IN VITRO PRODUCTION OF CAPRINE EMBRYOS THROUGH INTRACYTOPLASMIC SPERM INJECTION (ICSI) TECHNIQUE

KONG SOW CHAN

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UNIVERSITY OF MALAYA
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INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
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ABSTRACT

The present study evaluates the effect of PMSG-induced superovulation on the quantity and quality of oocytes retrieved via laparoscopic oocyte pick-up (LOPU) technique, the effect of different dosage of PMSG-induced, oocyte quality, IVM duration and chemical activation treatment following intracytoplasmic sperm injection (ICSI) on the *in vitro* developmental competence of goat embryos. A total of 84 goats from Jamnapari and Boer crossbred ranging from 6 to 42 months old were selected as oocyte donors and underwent oestrus synchronisation and superovulation. Oocyte retrieval (OR) was conducted through LOPU technique. Oocytes from different grades (A, B, C, D and E) and PMSG-induced dosages (1200 and 1500 IU) were cultured separately in microdroplets of IVM medium in the CO_2 incubator (5%) at 38.5^{o}C for duration 22 to 25 hours or 26 to 29 hours. Matured oocytes were fertilised *in vitro* through ICSI technique with different chemical activation treatments. ICSI-fertilised oocytes were then cultured *in vitro* in the CO_2 incubator (5%) at 38.5^{o}C to evaluate the further embryo developmental competence. No significant differences were observed in the quantity and quality of oocytes between two goat breeds used (4.92±0.32 oocyte retrieved per ovary) through LOPU technique from PMSG-superovulated donor goats. A significant difference ($P<0.05$) in the number of oocytes per ovary was retrieved from light (6.52±0.75) versus heavy body weights (3.38±0.44) goats. Repeatedly performing LOPU on the same donor goat up to 2 times did not significantly affect the quantity and quality of oocytes retrieved. Higher quantity ($P<0.05$) of oocytes per ovary were retrieved from the excellent (6.64±0.81) compared to satisfactory (3.44±0.58) ovarian response. There was a significant ($P<0.01$) positive correlation between the body weight and age in the Boer crossbred. A significantly higher ($P<0.05$) in the number of oocytes retrieved per ovary was obtained from PMSG-superovulated dosage at 1200 IU (6.21±0.66) compared to 1500 IU (4.48±0.33).

Higher maturation rates ($P<0.05$) were obtained from the PMSG dosage at 1200 IU (65.67±3.86%) compared to the 1500 IU (48.11±4.43%). Higher developmental rates at 4- and 8-cell ($P<0.05$) were obtained from PMSG-superovulated dosage at 1200 IU (63.04±7.72% and 58.60±7.65%, respectively) compared to 1500 IU (36.51±7.14% and 28.89±6.64%, respectively). As for IVM duration, higher maturation rates ($P<0.05$) were obtained at 22 to 25 hours (71.59±3.44%) compared to the 26 to 29 hours (38.74±4.30%). Maturation rates for Grades A and B oocytes were significantly higher ($P<0.05$) in IVM duration at 22 to 25 hours (90.56±4.18% and 91.32±3.23%, respectively) compared to 26 to 29 hours (59.03±8.27% and 51.33±8.94%, respectively). Oocytes from Grades A and B (75.42±5.01% and 73.14±5.32%, respectively) were significantly higher ($P<0.05$) compared with Grades D and E (9.20±5.20% and 5.56±4.04%, respectively) in both IVM durations. Higher cleavage and morula rates ($P<0.05$) were obtained in IVM duration at 22 to 25 hours (70.06±5.18% and 24.55±5.90%, respectively) compared to 26 to 29 hours (35.71±11.17% and 2.38±2.38%, respectively). Cleavage rates for Grade A oocytes were significantly higher ($P<0.05$) in IVM duration at 22 to 25 hours (78.92±7.36%) compared to 26 to 29 hours (38.89±20.03%). A higher ($P<0.05$) cleavage (73.2%) and morula rates (29.9%) were obtained from post-ICSI chemically activation with Ca^{2+} ionophore and 6-DMAP treatment than Ca^{2+} ionophore treatment, ICSI control and sham injection. It is suggested that PMSG dosage at 1200 IU and IVM duration at 22 to 24 hours for LOPU-derived oocytes should be taken into consideration when ICSI is conducted to produce *in vitro* developmental competent goat embryos. In conclusion, the results from the present study indicate that donor goats are satisfactorily able to be superovulated through PMSG+hCG treatment for LOPU and produce significantly higher rates of developmental competence oocytes for ICSI technique. However, further refinement studies are needed in the future to elucidate the various factors affecting the developmental competence of goat oocytes after superovulation, IVM and ICSI techniques.

ABSTRAK

Kajian ini menilai kesan superovulasi terinduksi PMSG ke atas perolehan kuantiti and kualiti oosit melalui teknik laparoskopi kutipan-oosit (LOPU), kesan dos terinduksi PMSG yang berlainan, kualiti oosit, tempoh IVM dan pelakuan aktivasi kimia selepas penyuntikan sperma intrasitoplasmik (ICSI) ke atas keupayaan perkembangan *in vitro* embrio kambing. Sejumlah 84 ekor kambing daripada baka Jamnapari dan kacukan silang Boer yang berjulat umur daripada 6 hingga 42 bulan dipilih sebagai penderma oosit dan melalui pensinkronian estus dan superovulasi. Perolehan oosit (OR) dijalankan melalui teknik LOPU. Oosit daripada gred yang berlainan (A, B, C, D dan E) dan dos terinduksi PMSG (1200 dan 1500 IU) dikultur berasingan dalam mikrotitisan medium IVM dalam inkubator CO_2 (5%) pada 38.5^{o}C bagi tempoh 22 hingga 25 jam atau 26 hingga 29 jam. Oosit yang matang disenyawakan secara *in vitro* melalui teknik ICSI dengan pelakuan aktivasi kimia yang berlainan. Oosit tersenyawa secara ICSI dikultur secara *in vitro* dalam inkubator CO_2 (5%) pada 38.5^{o}C untuk menilai keupayaan perkembangan embrio. Tiada perbezaan signifikan diperhatian pada kuantiti dan kualiti oosit yang diperolehi antara dua jenis baka kambing yang digunakan (4.92±0.32 oosit diperolehi per ovari) melalui teknik LOPU daripada kambing penderman tersuperovulasi-PMSG. Perbezaan signifikan ($P<0.05$) dalam bilangan oosit per ovari diperolehi daripada kambing berberat badan ringan (6.52±0.75) berbanding dengan kambing berberat badan berat (3.38±0.44). Prestasi LOPU dilakukan secara diulang ke atas kambing penderma yang sama sehingga 2 kali tidak meninggalkan kesan signifikan ke atas kuantiti dan kualiti oosit yang diperolehi. Lebih tinggi kuantiti ($P<0.05$) oosit per ovari diperolehi daripada respon ovari cemerlang (6.64±0.81) berbanding dengan yang memuaskan (3.44±0.58). Terdapat korelasi positif yang signifikan ($P<0.01$) antara berat badan dengan umur dalam kambing kacukan silang Boer. Signifikan tinggi ($P<0.05$) dalam

bilangan oosit diperolehi per ovari daripada dos tersuperovulasi PMSG pada 1200 IU (6.21±0.66) berbanding dengan 1500 IU (4.48±0.33). Kadar pematangan lebih tinggi ($P<0.05$) diperolehi daripada dos PMSG pada 1200 IU (65.67±3.86%) berbanding dengan 1500 IU (48.11±4.43%). Kadar perkembangan lebih tinggi pada 4- dan 8-sel ($P<0.05$) diperolehi daripada dos tersuperovulasi PMSG pada 1200 IU (63.04±7.72% dan 58.60±7.65%, masing-masing) berbanding dengan 1500 IU (36.51±7.14% dan 28.89±6.64%, masing-masing). Bagi tempoh IVM, kadar pematangan lebih tinggi ($P<0.05$) diperolehi pada 22 hingga 25 jam (71.59±3.44%) berbanding dengan 26 hingga 29 jam (38.74±4.30%). Kadar pematangan bagi oosit Gred A dan B lebih tinggi secara signifikan ($P<0.05$) dalam tempoh IVM pada 22 hingga 25 jam (90.56±4.18% dan 91.32±3.23%, masing-masing) berbanding dengan 26 hingga 29 jam (59.03±8.27% dan 51.33±8.94%, masing-masing). Oosit daripada Gred A dan B (75.42±5.01% dan 73.14±5.32%, masing-masing) adalah lebih tinggi secara signifikan ($P<0.05$) berbanding dengan Gred D dan E (9.20±5.20% dan 5.56±4.04%, masing-masing) dalam kedua-dua tempoh IVM. Kadar pembelahan dan morula yang diperolehi adalah tinggi ($P<0.05$) dalam tempoh IVM pada 22 hingga 25 jam (70.06±5.18% dan 24.55±5.90%, masing-masing) berbanding dengan 26 hingga 29 jam (35.71±11.17% dan 2.38±2.38%, masing-masing). Kadar pembelahan bagi oosit Gred A adalah lebih tinggi signifikan ($P<0.05$) dalam tempoh IVM pada 22 hingga 25 jam (78.92±7.36%) berbanding dengan 26 hingga 29 jam (38.89±20.03%). Kadar peratusan lebih tinggi ($P<0.05$) bagi pembelahan (73.2%) dan morula (29.9%) yang diperolehi daripada pelakuan aktivasi secara kimia pasca-ICSI dengan Ca^{2+} ionophone dan 6-DMAP berbanding dengan pelakuan Ca^{2+} ionophore, kawalan ICSI dan suntikan *sham*. Adalah dicadangkan bahawa dos PMSG pada 1200 IU dan tempoh IVM pada 22 hingga 24 jam bagi oosit diperolehi melalui LOPU seharusnya diambil kira semasa ICSI dijalankan untuk menghasilkan embrio kambing berkeupayaan berkembang secara *in vitro*. Kesimpulannya,

keputusan daripada kajian ini menunjukkan kambing penderma dapat disuperovulasi dengan memuaskan melalui pelakuan PMSG+hCG bagi LOPU dan menghasilkan kadar yang tinggi secara signifikan bagi oosit berkeupayaan berkembang bagi teknik ICSI. Walau bagaimanupun, kajian terperinci seterusnya adalah diperlukan pada masa hadapan untuk menjelaskan pelbagai faktor yang mempengaruhi keupayaan perkembangan bagi oosit kambing selepas teknik superovulasi, IVM dan ICSI.

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate	:	Kong Sow Chan	IC No: 830107-08-5060
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Name of Degree	:	Master of Science	
Title of Dissertation	:	*In Vitro* Production of Caprine Embryos through Intracytoplasmic Sperm Injection (ICSI) Technique	
Field of Study	:	Reproductive Biotechnology	

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%	percentage
µg	microgram
µl	microlitre
µM	micromolar
µm	micrometer
6-DMAP	6-dimethylaminopurine
ABEL	Animal Biotechnology-Embryo Laboratory
AI	artificial insemination
ANOVA	analysis of variance
ART	assisted reproductive technology
BCB	brilliant cresyl blue
BELE®	breed early lactate early
BME	basal media eagle
Ca^{2+}	calcium
CC	cumulus cell
CFO	cumulus-free oocyte
cFSH	caprine FSH
CG	cortical granule
CHX	cycloheximide
CIDR	controlled internal drug release
CL	corpus luteum
cm	centimeter
CO_2	carbon dioxide
COCs	cumulus-oocyte complexes
DMRT	Duncan Multiple Range Test
DMSO	dimethysulfoxyde anhydrous
DNA	deoxyribonucleic acid
DTT	dithiothreitol
e.g.	for example
eCG	equine chorionic gonadotrophin
EGA	embryonic genome activation
EGF	epidermal growth factor
EMiL	Embryo Micromanipulation Laboratory
ESS	oestrus sheep serum
ET	embryo transfer
et al.	*et alii* (and others)
FBS	foetal bovine serum
FCS	foetal calf serum
FF	follicular fluid
FGA	fluorogestone acetate
FSH	follicle stimulating hormone
G	gauge
g	gram
g	gravity, acceleration due to
G1-G2	Gardner's sequential media
G6PD	glucose-6-phosphate dehydrogenase
GC	granulose cell

GJC	gap junctional communication
GnRH	gonadotrophin-releasing hormone
GnSAF	gonadotrophin surge attenuating factor
GOEC	goat oviduct epithelial cells
GSH	glutathione
GVBD	germinal vesicle breakdown
hCG	human chorionic gonadotrophin
HEPES	N-(2-hydroxyethyl) piperizine-N'-(2-ethanesulphonic acid)
hMG	human menopausal gonadotrophin
hrs	hours
i.m.	intramuscular
ICSI	intracytoplasmic sperm injection
ID	inner diameter
IGFs	insulin-like growth factors
IPPP	Institute of Research Management and Monitoring
IPS	Institute of Graduate Studies
ISB	Institute of Biological Sciences
IU	international unit
IVC	*in vitro* culture
IVEP	*in vitro* embryo production
IVF	*in vitro* fertilisation
IVM	*in vitro* maturation
IVP	*in vitro* production
kg	kilogram
KSOM	potassium simplex optimisation medium
LH	luteinizing hormone
LOPU	laparoscopic oocyte pick-up
m	meter
M	molar
MAP	medroxyprogesterone acetate
MAPK	mitogen-activated protein kinase
MEM	minimum essential medium
mg	milligram
MI	metaphase I
MII	metaphase II
ml	millilitre
mM	millimolar
mm	millimeter
MOET	multiple ovulation-embryo transfer
mOsm/kg	milliosmol per kilogram
MPF	maturation-promoting factor
MPGF	male pronucleus growth factor
mSOF	modified synthetic oviductal fluid
MTOC	microtubule organising center
n	number
NaTuRe	Nuclear Transfer and Reprogramming Laboratory
NT	nuclear transfer
O_2	oxygen
^oC	degree Celsius
OD	outer diameter

oFSH	ovine FSH
OGS	oestrus goat serum
OR	oocyte retrieval
OSS	oestrus sheep serum
PB	polar body
PBS	phosphate-buffered saline
pFSH	porcine FSH
pg	pictogram
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
pl	pico liter
PMSG	pregnant mare serum gonadotrophin
PN	pronucleus
PVP	polyvinylpyrrolidone
PZD	partial zona dissection
RNA	ribonucleic acid
RO	reverse osmosis
rpm	rotation per minute
s.c.	subcutaneous
SCNT	somatic cell nuclear transfer
SEM	standard error of the mean
SOAF	sperm-associated oocyte-activating factor
SOF	synthetic oviductal fluid
SPSS	statistical package for social science
SS	steer serum
SUZI	subzonal sperm insertion
TCM-199	tissue culture medium-199
TUGA	transvaginal ultrasound-guided aspiration
UV	ultraviolet
v/v	volume:volume ratio
vs.	versus
w/v	weight:volume ratio
WID	well-in-drop
ZD	zona drilling
ZP	zona pellucida

Chapter 1

1.0 INTRODUCTION

Chapter 1

1.0 INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is a powerful technique in the field of assisted reproductive technology (ART) and provides exciting opportunities for studying the basic mechanisms of fertilisation and early embryo development. Nevertheless, its application in agriculture and conservation biology has been greatly hampered by the low success rate reported for this technique in respect of economically important species. Specifically, the rates of blastocyst formation and live newborn are greatly reduced when zygotes are generated by ICSI. The first live kid born to one of four recipients receiving six ICSI-derived two-cell embryos each was reported by Wang *et al.* (2003). Blastocyst yield from ICSI oocytes of prepubertal goats was 13% and 35%, respectively (Jimenez-Macedo *et al.*, 2006, 2007). In adult goats, Keskintepe *et al.* (1997) and Wang *et al.* (2003) obtained 18% and 35% of blastocysts, respectively. Therefore, ICSI remains a low efficiency technology in comparison with alternatives such as *in vitro* fertilisation (IVF) and its application is less widespread.

In mammals, ICSI was first studied by injecting human or hamster sperm into hamster oocytes (Uehara and Yanagimachi, 1976). The ICSI technique allows direct injection of a single sperm into the cytoplasm of a metaphase II (MII) oocyte to produce embryo *in vitro*. The ICSI procedure can bypass the process of sperm-egg binding, fusion and many upstream events associated with classical sperm-egg interactions, including plasma membrane and cortical region interactions. The ICSI

technique has provided an opportunity for investigation of fundamental aspects of fertilisation such as mechanisms of gamete interaction, sperm induced oocyte activation and first cell cycle control, as well as evaluation of oocyte quality because it reduces the variation due to sperm penetration and allows potential fertilisation and embryo development of each one of the MII oocytes injected (Jimenez-Macedo *et al.*, 2006). Apart from its application in the therapy of male infertility in humans, ICSI technology could also be useful in the animal production includes the use of genetically important male gametes for the preservation domestic and wild animals or to solve fertilisation problems in IVF systems. This technique can be used to extend the sperm vector for transgenic animal production and to use freeze-dried sperm for which sperm motility is not required. Some research groups have pursued an alternative procedure to pronuclear injection for producing transgenic animals using sperm as vector to introduce genes (sperm-mediated gene transfer; SMGT) (Lavitrano *et al.*, 2006). Creating transgenic mammals is currently a very inefficient process. In addition to problems with transgene integration and unpredictable expression patterns of the inserted gene, embryo loss occurs at various developmental stages. Yamuchi *et al.* (2007) demonstrates that DNA damage occurs after both the microinjection of pronuclei and ICSI-mediated transgenesis, albeit through different mechanisms. Dairy goats are a useful system for producing human therapeutics in their milk by genetic modification. New or improved technologies in mammalian transgenesis such as the use of lentiviral vectors (Whitelaw, 2004) and the 'renaissance' of ICSI approaches have shown that generation of transgenic animals now can be made easier (Moreira *et*

al., 2007).

In goats, superovulatory treatment typically consists of a combination of oestrous cycle control (usually involving application of progestagen implants) with an elevated dose of a gonadotrophin to induce the ovary to release more than the typical number of oocytes. The two most commonly used gonadotrophin treatments for superovulation in goats are follicle stimulating hormone (FSH) and equine chorionic gonadotrophin (eCG). FSH proved to be more efficacious than eCG (Armstrong *et al.*, 1983b; Nuti *et al.*, 1987; Mahmood *et al.*, 1991; Nowshari *et al.*, 1992), provided it contains an appropriate admixture of luteinising hormone (LH). PMSG has been used widely as a means of inducing superovulation in conjunction with embryo transfer technique for the purpose of genetic improvement and increasing large number of goat population. Not much work has been reported on the different dose response of PMSG on the number and quality of oocytes as well as embryo developmental rate through LOPU. To date, there is not a single one fulfills all expectations concerning predictability and reliability of the response from the numerous superovulation protocols in use. A host of environmental factors such as season, nutrition, health state, drug handling and insemination technique is known to contribute to the superovulatory variability. Therefore, vigorous research efforts are directed at the establishment of suitable superovulation regimes to augment the deployment of *in vitro* embryo production programmes and associated technologies based upon them in goats (Holtz, 2005).

The laparoscopic oocyte pick-up (LOPU) technique is very reliable and allows for the recovery of a predictable number of oocytes during each session. It is also less invasive than standard surgery (laparotomy) used for the recovery of *in vivo* zygotes and *in vivo* matured oocytes, thus allowing multiple recoveries from the same donor animal. Unlike oocytes collected from abattoir ovaries, LOPU-derived oocytes are recovered from animals of known health status. Moreover, LOPU in combination with IVP of embryos increased the number of offspring produced by genetically valuable does and enabled production of offspring from does unable to reproduce using AI or multiple ovulation-embryo transfer (MOET) such as prepubertal or aged goats. LOPU procedure can be repeated several times without ovarian damage or decrease in the donor fertility since it is less traumatic and results in fewer surgical adhesions than standard surgery (laparotomy) generally used to recovery of *in vivo* matured oocytes and embryos, therefore, extends the use of donor animal resources.

The mammalian oocyte acquires the ability to mature and accomplish its developmental competence during oogenesis and folliculogenesis, the proportion of developmentally competent oocytes increases with follicular size. Like other mammals, the primary oocytes of goat become arrested at the diplotene stage of meiosis at birth *in vivo*. However, they are capable of resuming meiosis spontaneously when removed from their follicles and cultured *in vitro* (Gilchrist and Thompson, 2007). The duration of *in vitro* maturation (IVM) employed in the IVP systems is somehow controversial due to the variations in protocol of oestrus synchronisation and superovulation, the reproductive status and breed of the goats as well as the

timing of oocyte retrieval. Nonetheless, the duration of IVM plays a critical role for subsequent development since an inappropriate timing of maturation results in abnormal chromatin (Dominko and First, 1997), oocyte aging (Hunter and Greve, 1997) and reduced development (Marston and Chang, 1964). A better understanding of these factors could ameliorate the selection of animals and oestrus synchronisation and superovulation from which oocytes are collected and thus improve the ICSI success rate. Therefore, the aim of the present study is an attempt to determine the appropriate timing for a complete IVM process.

To achieve normal fertilisation, sperm must pass through the egg vestments (the cumulus oophorus and zona pellucida) and fuse with oocyte plasma membrane. Male and female gametes may fail interact even when brought into close proximity during *in vitro* fertilisation (IVF). The success of IVF is dependent on both sperm maturation and capacitation for penetration of the oocyte and on oocyte quality to support embryo development. However, IVF not always provides good results because of male infertility or high fertilisation abnormalities such as polyspermy found using this technique. In order to minimise variability due to sperm capacitation and penetration, ICSI could be used as a technique for sperm and oocyte quality evaluation studies. The efficiency of ICSI depends on the techniques involved in micromanipulation, the treatments for permeabilisation of the sperm membrane and oocyte activation. Oocyte activation can be induced by a variety of physical and chemical agents, including an electrical pulse, ionomycin, Ca^{2+} ionophore, 6-dimethylaminopurine (6-DMAP) and ethanol. Recent studies have shown that species

differences exist in the oocyte activation after ICSI; in that regard, goat oocytes need artificial activation following ICSI to improve fertilisation, whereas injection alone is sufficient to activate oocytes in mice and humans. In sheep, Sham injection was able to activate the oocyte up to 16-cell but oocytes treated by ICSI developed to blastocyst stage at a percentage lower than IVF (8% versus 18%, respectively). In goats, Keskintepe *et al.* (1997) concluded that breaking of the sperm tail was helpful in initiating the fertilisation process, while just oocyte treatment with Ca^{2+} ionophores after injection did not have any effect on the fertilisation process. An alternative to chemical activation is ICSI with a Piezo-driven needle in order to break a hole into the oolemma. As a side effect, good developmental rates were obtained both in cows and goats when the mechanical pulses induced activation of oocytes. The study in the different methods of conventional ICSI procedure (alone versus chemical stimulation) is therefore essential to determine the appropriate combination and duration chemical activation for optimal production ICSI-derived goat embryos.

The main objectives of the present study were to obtain a large number of immature oocyte from PMSG-stimulated donor goats via the LOPU technique for *in vitro* ICSI-derived embryo production with special emphasis on PMSG dosage, IVM duration, oocyte quality as well as chemical activation treatment. This study was conducted according to the following specific objectives:

a) To retrieve immature oocytes from donor goats after oestrus synchronisation and PMSG-induced superovulation.

b) To evaluate the effect of genotype, body weight and age of donor goats after superovulation on quantity and quality of oocyte retrieved.

c) To evaluate the effect of oocyte retrieval cycle and ovarian stimulation response after superovulation on quantity and quality of oocyte retrieved.

d) To produce developmental competence oocytes and cleaved embryos from the IVM and ICSI techniques.

e) To evaluate the effect of PMSG dosage on quantity, quality, IVM and *in vitro* developmental competence of *in vitro* produced goat embryos through ICSI technique.

f) To evaluate the effect of IVM duration of different oocyte qualities on the embryo developmental competence *in vitro* following ICSI.

g) To evaluate the effect of oocyte chemical activation treatments on embryo developmental competence *in vitro* after ICSI.

Chapter 2

2.0 REVIEW OF LITERATURE

Chapter 2

2.0 REVIEW OF LITERATURE

2.1 BACKGROUND

In vitro embryo production (IVEP) in small ruminants provides an excellent source of low-cost embryos for basic research on developmental biology and physiology and for commercial application of the emerging biotechnologies such as nuclear transfer and transgenesis (Baldassare *et al.*, 2002). In addition, the application of IVEP and inter-specific transfer has been proposed as a strategy for the rescue of some endangered species (Ptak *et al.*, 2002). IVEP has been considerably developed in recent years due to the high variability in ovulatory response, fertilisation failures and embryo degeneration processes that affect yields obtained with traditional multiple ovulation and embryo transfer (MOET) (Cognie *et al.*, 2003; Baldassarre and Karatzas, 2004). Moreover, recent developments in IVEP have increased our understanding and knowledge of gamete physiology, the mechanism of fertilisation and embryo physiology and metabolism.

IVEP in goats is a rapidly advancing field. It offers an alternative to superovulation as a source of embryos for transfer and manipulation purposes. The various steps involved with the *in vitro* production of goat where the oocytes of follicular origin need to undergo *in vitro* maturation (IVM) before being exposed to *in vitro* capacitated sperm to be *in vitro* fertilised (IVF). Thereafter, the putative embryos are cultured *in vitro* for 5 to 7 days (IVC) and upon reaching the blastocyst stage, transferred to recipients or cryopreserved for future use (Crozet *et al.*, 1995; Izquierdo *et al.*, 1999; Ongeri *et al.*, 2001; Baldassarre *et al.*, 2003; Cognie *et al.*, 2003; Koeman *et al.*, 2003). The first kids born after complete *in vitro* maturation, fertilisation and culture were reported by Keskintepe *et al.* (1994a) and Pereira *et al.* (1995). During the following years there were only haphazard reports on *in vitro*-derived offspring

(Poulin *et al.*, 1996; Traldi *et al.*, 1998; Cognie *et al.*, 2001). Only very recently Baldassarre *et al.* (2003) reported the birth of an appreciable number of 150 *in vitro*-derived kids.

Intracytoplasmic sperm injection (ICSI) has been introduced as an alternative to assisted reproduction technologies, especially in humans. A major application of this technique for animal production includes use of genetically important male gametes for procreating wild and domestic animals. Amongst other advantages the attraction of the technique lies in the avoidance of potential polyspermia (Palomo *et al.*, 1999; Bhatia *et al.*, 2002) and the intriguing perspective of being able to predetermine the sex of the offspring by using sperm sex-sorted by flow cytometry (Parrilla *et al.*, 2004). When it will be possible to utilise sperm as carriers for altered chromosomal material, ICSI could also become a useful way of generating transgenic animals (Perry *et al.*, 2001).

The first live kid born to one of four recipients receiving six ICSI-derived two-cell embryos each was reported by Wang *et al.* (2003). Blastocyst yield from ICSI oocytes of prepubertal goats was 13% and 35%, respectively (Jiménez-Macedo *et al.*, 2006, 2007). In adult goats, Keskintepe *et al.* (1997) and Wang *et al.* (2003) obtained 18% and 35% of blastocysts, respectively. At present, the efficiency of ICSI is low and not conclusive. Therefore, the ICSI procedures in goats were not defined adequately to ensure consistent and reproducible success. Nevertheless, these reports are essential for future development and refinement of ICSI procedures. Moreover, with the success rates reported by human fertility clinics, it is probably only a matter of further sustained research effort until practicable solutions are found.

Table 2.1: Timeline of selected significant findings of oestrus synchronisation, superovulation, IVM, ICSI and IVC in goat

Year	Author	Significant event/ finding
1983a	Armstrong *et al.*	Differences in the ovarian responses to PMSG and FSH may be attributed primarily to differences in the biological half-life of each preparation.

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Year	Author	Significant event/ finding
1983b	Amstrong *et al.*	High incidence of early luteal regression after PMSG superovulation was associated with low recovery of embryos from reproductive tracts of Angora goats flushed later than Day 5 after onset of oestrus.
1986	Greyling and Van Niekerk	Two injections of cloprostenol synthetic analogue at the rate of 62.5, 125 and 250 µg administered 14 days apart were effective in synchronising goat during breeding season.
1989	Tsunoda and Sugie	Higher numbers of oocytes were recovered with FSH-treated (9.4) than PMSG-treated (5.7) goats.
1990	Amoah and Gelaye	Superovulation with PMSG are prone to premature regression of the induced corpus luteum results in short cycles and have the potential risk of embryo expulsion.
1991	Younis *et al.*	Achieved better fertilisation rates in goats by combining the use of luteinising hormone (LH) in IVM medium.
1992	De Smedt *et al.*	Maturation rates from goat oocytes derived from 2 to 6 mm and 1 to 1.8 mm sized follicles obtained were 86% and 24%, respectively.
1994	Krisher *et al.*	Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and GnRH is an effective supplement used with FSH superovulation regimes in dairy goats which enhance early embryo collection for DNA microinjection studies.
1994	Mani *et al.*	Good superovulation was achieved in Angora goat with porcine FSH (22 mg) divided in 4 decreasing dosage injected twice daily commenced one day before sponge removal after 17 days of progestagen treatment.
1995	Crozet *et al.*	Maturation rates from goat COCs derived from follicles of 2 to 3 mm, 3.1 to 5 mm and larger than 5 mm in diameter obtained were 70%, 83% and 97%, respectively.
1995	Martino *et al.*	Maturation rates for prepubertal goat COCs in TCM-199+FBS (10%) in presence or absence of granulosa cells (GCs) for 27 hours obtained were 72% and 76.9%, respectively.
1995	Yadav *et al.*	The oviductal cell co-culture had a marked effect on cleavage and development of goat IVF embryos; buffalo oviductal cells can be used well for goat embryo development.

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Year	Author	Significant event/ finding
1996a	Baril *et al.*	The repetition of treatment with eCG to induce oestrus in goats increases eCG binding, lowered efficiency of the hormonal treatment to synchronise oestrus and the associated decrease in fertility when goats are inseminated at a predetermined time.
1996	Freitas *et al.*	During anoestrus, fluorogestone acetate (FGA) intravaginal sponges for 11 days in conjunction with PMSG (750 IU) and cloprostenol (50 µg) 48 hours before sponge removal resulted in 87.5 and 93.8% oestrus response and fertility, respectively, in Alpine goats.
1996	Gall *et al.*	Maturation rates for culturing goat COCs in TCM-199+FCS (10%) after 20 and 27 hours of IVM obtained were 22% and 96%, respectively from 2 to 6 mm sized follicles and 3.5% and 8%, respectively from 1 to 1.8 mm sized follicles.
1996	Keskintepe *et al.*	The first to report goat development to the blastocyst stage *in vitro* and obtained offspring following uterine transfer.
1996	Pawshe *et al.*	Maturation rates for culturing goat COCs using Ham's-12+OGS (10%), Ham's-12+FCS (10%), TCM-199+OGS (10%) and TCM-199+FCS (10%) obtained were 49%, 64.8%, 62.6% and 90%, respectively.
1996	Pintado *et al.*	Synchronisation regimen does not interfere with the superovulatory response and that high percentages of premature luteal regression can be associated with FSH-based superovulatory regimes; administration of prostaglandin $F_{2\alpha}$ does not increase the number of animals showing premature luteal regression.
1996	Sharma *et al.*	Greater numbers of matured oocytes for *in vitro* fertilisation, 30 to 32 hours would be a better time for maturation rather than maturing them only for 24 hours.
1997	Mellado and Valdes	Ear implantation periods for goats usually extend from 9 to 14 days and often combined with PMSG and/or $PGF_{2\alpha}$ at 2 days before the end of ear implantation.
1997a	Mogas *et al.*	A significantly positive effect of the addition of different sources of granulosa cells to a maturation medium was observed on the percentage of maturation and embryo cleavage that overcame the *in vitro* developmental block from 8- to 16-cell stage goat embryos.
1997	Yadav *et al.*	The optimal duration of *in vitro* maturation of oocytes is 30 hours for goats on the basis of sequential configuration of chromosomes.

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Year	Author	Significant event/ finding
1998	Malik *et al.*	Goat peritoneal fluid could be used as an alternative medium to the conventional TCM-199 for generating goat embryos by *in vitro* techniques.
1998	Muna *et al.*	Oestrus synchronisation in Sudanese Nubian goats was achieved with double dose of cloprostenol (125 µg) together with intravaginal sponges impregnated with progesterone inserted for 16 days.
1998	Pintado *et al.*	The use of PMSG antibodies for superovulating goats is an efficacious treatment which increases the number of viable embryos collected.
1998	Saharrea *et al.*	Administration of hCG 84 hours after the onset of oestrus prevents premature luteal regression in goats superovulated with PMSG.
1999	Izquierdo *et al.*	The best result in blastocyst formation has been 10% using TCM-199 with EGC without serum with IVM-IVF prepubertal goat oocytes.
2000	Crozet *et al.*	The culture of granulosa cell-enclosed oocytes isolated from early antral follicles for 9 days supported goat oocyte growth and the acquisition of GVBD competence to a certain extent.
2000	Graff *et al.*	No difference was detected between the two stimulatory agents for the number of follicles and quality of oocytes harvested from stimulated goats, indicating that these two commercial FSH products could be used successfully for ovarian stimulation of anestrous dairy goats.
2000	Greyling and Nest	Dose of progestagen played no role in the efficiency of synchronisation, regardless of the breed, the mean pregnancy rate achieved is acceptable following fixed-time AI in both breeds and at both doses progestagen.
2000	Samake *et al.*	Maturation rates for culturing goat oocytes in TCM-199+FBS (10%) for 24 hours obtained were 100% from oestrus synchronised and superovulated goats.
2001	Behl and Pandey	Epidermal growth factor (EGF) influences the steroidogenesis by goat granulosa cells *in vitro* and plays an important role in the follicular growth and maturation.
2001	Mayor *et al.*	No significant differences in maturation rates obtained with glutathione or cysteine supplementation at different concentrations for culturing goat COCs in TCM-199+FBS (10%) for 27 hours.
2001	Oliveira *et al.*	Oestrus synchronisation using CIDR for 9 days combined with eCG (100 IU) and cloprostenol at CIDR removal results a 100% oestrus in Saanen goats within 24 hours.

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Year	Author	Significant event/ finding
2001	Rho *et al.*	Maturation rates for culturing goat COCs in M-199+FCS (10%) after 20, 24 and 27 hours of IVM obtained were 30%, 55% and 73%, respectively.
2001	Riesenberg *et al.*	The ovulation rates were satisfactory, suggesting that a single injection of pFSH or hMG provides an adequate stimulus to induce a superovulatory reaction in goats.
2001	Teotia *et al.*	GC monolayers better support cytoplasmic maturation of growing caprine oocytes, which is evident by a better maturation rate, active fertilisation, an improved cleavage rate and subsequently a higher rate of morula formation.
2002	Baldassarre *et al.*	The use of LOPU allows for improved control over the stage of maturation or development of the goat oocytes and produced zygotes, a less invasive means of recovery, thereby allowing for repeated usage of the oocyte donor animals and the ability to source the oocytes from live animals of known health status.
2002	Greyling *et al.*	Folltropin (20 mg) as a superovulation agent proved to be very efficient when used on South African Boer and Indigenous feral goats.
2002	Izquierdo *et al.*	There were no significant differences in embryo development between oocytes obtained from prepubertal and adult goats, and the embryo development from prepubertal goat oocytes were similar in the different culture systems compared.
2002	Rodriguez-Gonzalez *et al.*	The brilliant cresyl blue (BCB) test is useful in selecting more competent prepubertal goat oocytes for *in vitro* embryo production.
2002	Velilla *et al.*	Maturation rates for culturing goat COCs in TCM-199+FBS (10%) and staining with Hoechst after 15, 20 and 27 hours obtained were 25.7%, 36.9% and 51%, respectively.
2003	Baldassarre *et al.*	LOPU is a reliable and effective technique for the recovery of goat oocytes for production of goat zygotes.
2003	Bormann *et al.*	Addition of vitamins (MEM vitamins to SOF maturation medium) during oocyte maturation is beneficial for the subsequent goat blastocyst development and viability.
2003	Koeman *et al.*	Higher yields of oocytes were obtained from gonadotrophin-primed, prepubertal goats than from adults, while *in vitro* development was similar through LOPU technique.

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Year	Author	Significant event/ finding
2003a	Rodriguez-Gonzalez *et al.*	Maturation rates for culturing prepubertal goat COCs in TCM-199+SS (10%) supplemented with cysteamine (100 µM) for 27 hours obtained were 89.5% from brilliant cresyl blue (BCB) positive, 72.1% from BCB negative and 67.3% from control.
2003	Selvaraju *et al.*	There was beneficial effect of insulin on folliculogenesis and steroidogenesis as it improves superovulatory response in goats.
2003	Tajik and Esfandabadi	Foetal bovine serum (FBS), oestrus sheep serum (ESS) or oestrus goat serum (EGS) at 10, 15 or 20% can be substituted for one another for *in vitro* maturation of caprine oocytes.
2003b	Urdaneta *et al.*	Maturation rates for culturing goat COCs in TCM-199+SS (10%) supplemented with 100, 200 and 400 µM concentrations of cycteamine for 25 hours from BCB positive and negative obtained were 67.9% and 57.8%, respectively, 87.6% and 71.1%, respectively, as well as 77.1% and 61.4%, respectively.
2003	Wang *et al.*	Cutting sperm tails using the oocyte-holding pipette coupled with the PiezoDrill is an efficient approach for goat ICSI in terms of oocyte survival, pronuclear development and initial cleavage; mTALP-mKSOM culture system was more suitable for *in vitro* development of ICSI-derived goat embryos than G1.3-G2.3.
2004a	Baldassarre *et al.*	LOPU in combination with *in vitro* embryo production techniques is an efficient method for the early propagation of valuable goats produced by somatic cell nuclear transfer.
2004	Faruk *et al.*	There was no significant difference between alfaprostol and luprostiol treatment group on superovulation rate as well as recovered, fertilised and transferable embryos; however, significant was existed in the percentages of fertilised embryos where alfaprostol had the significant higher percentages compared to luprostiol treated group.
2004a	Gonzalez-Bulnes *et al.*	GnRH antagonist reduces plasma FSH and LH levels with suppression of the growth of large dominant follicles and a 2 fold increase in number of smaller follicles in Spanish goats.
2004	Pierson *et al.*	LOPU may be repeated up to five times in goats at different intervals and in different seasons with little or no important change in overall response.
2004	Silva *et al.*	Goat primordial follicles activate spontaneously *in vitro* and that both FSH and EGF stimulate an increase in follicle size by promoting oocyte growth.

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Year	Author	Significant event/ finding
2004	Tanaka *et al.*	Nutritionally induced anovulation after CIDR-G treatment is associated with a reduction in the frequency of LH pulses, and that insulin and NEFA, rather than the glucose concentration in the circulation, may be associated with the metabolic suppression of LH pulses.
2005	Goel and Agrawal	In PMSG-treated goats, comparatively more unfertilised ova or retarded embryos were recovered than in FSH-treated goats; the superiority of FSH preparations over PMSG was reflected in terms of total and transferable embryo production.
2005	Jimenez-Macedo *et al.*	Prepubertal goat oocytes need chemical activation (ionomycin plus 6-DMAP) to be fertilised using fresh semen and conventional ICSI; ICSI oocytes produced higher percentages of embryos developing beyond the 8-cell stage when embryos were cultured in G1.3/G2.3.
2005	Nagar and Purohit	Maturation rates for culturing goat COCs in TCM-199 with supplementation of 0, 10, 20, 50 and 100 ng/ml EGF for 28 hours obtained were 34.1%, 55.6%, 64.5%, 52.4% and 49.2%, respectively.
2005	Zhou and Zhang	The growth factors can individually or synergically regulate the survival and growth of goat oocytes; alternative addition of growth factors to culture medium could improve culture system for preantral follicle oocytes.
2006	Faruk *et al.*	The superovulation response was better with PMSG at the dose rate of 900 IU compared to 700 and 800 IU, respectively in black bengal goat.
2006	Han *et al.*	The well-in-drop (WID) system supported oocyte maturation and embryo development to a level similar to the conventional group system; goat oocytes acquired competence for development up to the 8- to 16- cell stage in follicles larger than 2 mm, but did not gain the ability to form morula/blastocyst until follicles larger than 3 mm in diameter; cumulus expansion increased with follicle size and decreased with increasing incidence of granulose cell (GCs) apoptosis.
2006	Jimenez-Macedo *et al.*	Sperm treatment with ionomycin plus heparin using the conventional ICSI protocol improved fertilisation rates in comparison to IVF; oocytes smaller than 125 µm were unable to develop up to blastocyst stage.

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Year	Author	Significant event/ finding
2006	Kharche *et al.*	Maturation rates for culturing goat COCs in TCM-199 medium with 0, 10, 15 and 20% OGS for 24 to 27 hours obtained were 28.6%, 61.9%, 72.7% and 78.6%, respectively.
2006	Lehloenya *et al.*	The pre-treatment with a GnRHa to the pFSH superovulation protocol outside their natural breeding season seemed to be detrimental to embryo production and quality in Boer goats, and is not warranted.
2006	Khanum *et al.*	Estrumate (125 µg/ml) treatment given in the form of two intramuscular injections (0.5 ml each) at 10 days interval is an effective method for the induction and synchronisation of the oestrous cycle with high fertility rate in Dwarf goats.
2006	Zhou and Zhang	The growth rate of caprine oocytes cultured in microdrops was significantly ($p<0.05$) higher than that in agar gel clots, whereas the viability of oocytes in microdrops was considerably ($p<0.05$) lower than that in agar gel clots.
2007	Anguita *et al.*	The size of oocytes from prepubertal goats is related to their capability to undergo meiotic maturation, *in vitro* fertilisation and to reach the blastocyst stage.
2007	Baldassarre *et al.*	LOPU-IVEP can be used successfully to extend the reproductive life of valuable goats that have acquired difficulties becoming pregnant by artificial insemination after multiple kiddings.
2007	Gibbons *et al.*	Retrieval of oocytes can be maximised without affecting oocyte quality, by repeating 'oneshot' FSH/eCG regimes and LOPUs at intervals as short as 4 days in goats.
2007	Jimenez-Macedo *et al.*	ICSI and embryo biopsy do not have negative effect on embryo quality and development, however, oocyte size has a positive relationship on blastocyst yield and quality in prepubertal goats.
2007	Katska-Ksiazkiewicz *et al.*	Selection of cumulus-oocyte complexes (COCs) based on the visual assessment of morphological features is the most important criterion in obtaining competent goat oocytes prior to IVM.
2007	Rahman *et al.*	Better maturation, fertilisation and embryo development rates were obtained with LOPU-derived goat oocytes than with the abattoir source; morula stage embryos were obtained using ICSI technique from *in vitro* matured heterogeneous goat oocytes without any artificial activation and first reported in Malaysia.

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Year	Author	Significant event/ finding
2007	Rodriguez-Dorta *et al.*	Co-culture of *in vitro* produced goat embryos with goat oviduct epithelial cells (GOEC) improves pregnancy and embryo survival rates, also leading to the birth of healthy offspring.
2007	Sayre	PGE_2 alone reduced hatching rate and $PGF_{2\alpha}$ alone had no effect on the development of goat embryos; high concentrations of PGE_2 with $PGF_{2\alpha}$ improved the hatching rates.
2007	Wang *et al.*	Slicing and puncture of the ovaries are alternative techniques for oocyte recovery; TCM-199 media supplemented with EGF or FCS in the presence of FSH are suitable for *in vitro* maturation and subsequent embryonic development of Boer goat oocytes.
2008	Abdullah *et al.*	LOPU 60 or 72 hours after FSH/hCG optimised yields of good quality oocytes for IVM and embryo production in goats.
2008	Anguita *et al.*	Development to the blastocyst stage of prepubertal goat oocytes depended upon the denuded oocytes having a minimum diameter of 125 µm.
2008	Chao *et al.*	A combination of a small dose (40 mg) of progesterone and 500 IU eCG was effective for oestrus synchronisation in Korean native goats; high embryo loss after synchronisation treatment and mating was caused by a functionally poor CL bringing low doses of progesterone.
2008	Fernandez-Moro *et al.*	Goats with oestrus synchronised by progestagen showed a higher number of pre-ovulatory sized follicles but a decreased oestradiol secretion when compared with does with oestrus synchronised by using prostaglandin analogues.
2008	Holtz *et al.*	Ovsynch synchronisation scheme (combining GnRH analog and prostaglandin administration) may be an useful alternative to the sponge-eCG treatment which be handy to synchronise does which have developed eCG antibodies.
2009	Anguita *et al.*	Oocyte developmental competence in prepubertal goats is influenced by oocyte diameter and COC morphology.
2009	Kharche *et al.*	The use of fatty acid free albumin resulted in a significantly higher ($P<0.05$) cleavage rate compared to unmodified albumin, and the supplementation of 20% oestrus goat serum in the fertilisation medium significantly ($P<0.05$) increased the cleavage rate of *in vitro* matured goat oocytes compared to defatted albumin.

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Year	Author	Significant event/ finding
2009	Rahman *et al.*	Better maturation, fertilisation and embryo development rates were obtained with LOPU-derived goat oocytes than with the abattoir source.
2010	Heidari *et al.*	A GnRH antagonist treatment can improve the superovulatory response by 64% and embryo recovery rate by 90% in goat.
2010a	Lehloenya and Greyling	Age and parity of the donor Boer goat had an effect on the ovarian activity following superovulation.
2010b	Lehloenya and Greyling	The inclusion of $PGF_{2\alpha}$ treatment in the superovulation protocol for Boer goats had no beneficial effect while the Day 0 protocol engaged in this trial resulted in a lower superovulation response.
2010	Lv *et al.*	Oocyte selection and β-mercaptoethanol supplementation can positively influence progression to metaphase II of oocytes harvested from ovaries of prepubertal goats, whereas high concentrations of oestradiol are inhibitory to *in vitro* maturation.

2.2 OESTRUS SYNCHRONISATION AND SUPEROVULATION

Oestrus synchronisation in goats focuses on the manipulation of either the luteal or the follicular phase of the oestrous cycle. In goats, the opportunity for control is greater during the luteal phase which is of longer duration and more responsive to manipulation. Strategies can be employed to extend the luteal phase by supplying exogenous progesterone or to shorten this phase by prematurely regressing existing corpora lutea (Wildeus, 2000). Successful techniques must not only establish tight synchrony but also provide an acceptable level of fertility upon fixed-time breeding, artificial insemination (AI), laparoscopic oocyte pick-up (LOPU) for oocyte and embryo retrieval or embryo transfer (ET). The latter is commonly accomplished through co-treatments using gonadotrophin. After these conditions are met, oestrus synchronisation becomes the basis for successful assisted reproduction technique (ART) programmes.

Progesterone or one of its synthetic analogues is preferred for the control of the oestrous cycle in most situations. Classically the progesterone treatment extends over 18 days, a period that is long enough for corpora lutea to undergo timely regression in all animals no matter what stage of the cycle the animals were at the outset. Most commonly, progestagen-containing vaginal pessaries are employed. The most widespread method of oestrus synchronisation used in goats involves progesterone or progestagen treatment, such as polyurethane sponges impregnated with fluorogestone acetate (FGA), medroxyprogesterone acetate (MAP) or Y-shaped silicone-coated devices (controlled internal drug release, CIDR) impregnated with progesterone for 9 to 11 days (Evans and Maxwell, 1987; Ritar *et al.*, 1989; Baldassarre and Karatzas, 2004). As an alternative to vaginal pessaries, implants impregnated with the highly potent synthetic progestagen norgestomet may be inserted under the skin on the upper side of the ear (Bretzlaff and Madrid, 1985, 1989; Holtz and Sohnrey, 1992; Yuswiati and Holtz, 1996; Freitas *et al.*, 1997; Graff *et al.*, 1999; Mellado *et al.*, 2000; Oliveira *et al.*, 2001; Medan *et al.*, 2002) or on the underside of the tail (East and Rowe, 1989). No difference in efficacy was found between sponges and subcutaneous implants in a comparative study (Holtz and Sohnrey, 1992). However, CIDR or subcutaneous implants are preferable because sponges frequently cause discomfort and may adhere to the vaginal wall causing problems with removal in small or nulliparous goat (Holtz, 2005). This is followed by a luteolytic dose of prostaglandin or an analogue (cloprostenol) administered either at the onset (Runianes and Menchaca, 2003) or, more commonly, at the end or 24 to 48 hours before the end of the progestagen treatment.

Progestagen insertion is aimed to decrease the endogenous LH release (Goodman and Karsh, 1980), inhibiting the occurrence of the oestrus, the LH surge and the ovulation until sponge withdrawal. This may lead to inadequate follicular development with persistent large follicles having deficient stereidogenesis if LH is not suppressed (Johnson *et al.*, 1996;

Vinoles *et al.*, 1999; Flynn *et al.*, 2000). The presence of aged follicles in progestagen-treated females affects fertility if ovulating (Ungerfeld and Rubianes, 1999; Vinoles *et al.*, 2001). Kaneko *et al.* (1991) and Rhodes *et al.* (1995a) demonstrated that oestradiol-17β is produced by the dominant follicle mainly during their growing phase; and to be reduced or stopped when the dominant follicle attained its maximum diameter. The high plasma oestradiol levels maybe caused either by the length of the progestagen treatment, by the intrinsic characteristics of ovarian function in goats, or by an interaction of both factors (Fernandez-Moro *et al.*, 2008). The duration of pessary insertion may cause a decrease in the progestagen release by the sponge and, thus, a lack of suppression of LH secretion. Rubianes and Menchaca (2003) proposed that the use of a 'short priming protocol' in which goats are treated with progestagen (5 days) and eCG (200–300 IU) is administered at sponge or CIDR removal. The present largest follicles will regress at the time of CIDR insertion emerged 4 or more days before; a new follicular wave emerged after CIDR insertion and 5 days later; and a young large follicle was present at the time of CIDR withdrawal. The follicle continued to grow and it became the largest follicle at eh time of device withdrawal. As a consequence of new understanding on ovarian follicular dynamics in goats, i.e. wave-like pattern, dominance and control of follicle wave turnover, they demonstrated that short progestagen priming could ensure the presence of young large follicles available for ovulation in most goats. When eCG was administered at the time of CIDR withdrawal ovulation occurred around 60 hours later. The progestagen treatment for longer than 5 days results in sub-luteal concentrations of progesterone that promote excessive growth and persistence of the largest dominant follicle, thus, leading to lower fertility (Runianes and Menchaca, 2003). In addition, the use of progestagen sponges in 'long protocol' treatments (18–21 days) does not require the use of a luteolytic agent but it has been shown to result in lower fertility rates in goats (Corteel *et al.*, 1988).

Ovarian superovulation in domestic animals may thus be used to increase the number of developmentally competent oocytes for *in vivo* or *in vitro* embryo production (Malhi *et al.*, 2008). In goats, superovulatory treatment typically consists of a combination of oestrous cycle control (usually involving application of progestagen implants) with an elevated dose of a gonadotrophin to induce the ovary to release more than the typical number of oocytes. The use of regimes with 'one shot' of follicle stimulating hormone (FSH) plus equine chorionic gonadotrophin (eCG) is given as a single treatment administered 36 hours prior to LOPU has shown to be as effective as traditional regimes with FSH multi-doses in goats (Baldassarre *et al.*, 1996, 2002). However, the high variability in the number of follicles and oocytes obtained in response to superovulation is a major limiting factor in goat IVEP programmes. This variability due to both extrinsic factors such as source, purity and batch of gonadotrophins and protocol of hormonal administration (Wollen *et al.*, 1985; Cognie, 1999); and intrinsic factors such as breed, age and reproductive status (Baril *et al.*, 1993) still remains despite refinements in the use of new gonadotrophin preparations and animal management systems (Cognie, 1999). Therefore, it is important to moderate the hormonal stimulation protocols based on physiological considerations in order to optimise the yield of high quality of oocytes (Sirard *et al.*, 2006).

2.2.1 Factors Affecting the Superovulatory Response of Goat

Factors causing such high viability are the major constraint for superovulatory response in goats being classified either as extrinsic (depending on the protocol treatment used for ovarian stimulation) such as season, nutrition and hormone preparations; or intrinsic factors (related to the physiological status of the animal) such as age, genetic differences and ovarian status at the time of treatment.

2.2.1.1 Age

The effect of donor age on the ovarian response to superovulation is an aspect almost always ignored in most farm animal species. However, age could contribute greatly to the variation in ovarian response to superovulation between animals within the same group as animals of different ages have been shown to have different physiological needs and respond differently (Jainudeen *et al.*, 2000). There is limited information in goats relating to the effect of age on the ovarian response to superovulation if compared to other ruminants.

Young goats recorded a longer time interval from CIDR removal to the onset of oestrus when evaluating the effect of age on oestrus response following superovulation (Lehloenya and Greyling, 2010a). Similar results have been reported in cattle (Drion *et al.*, 2001a) and goats (Baril *et al.*, 2000). The young goats may have responded slower and were less sensitive for the first time treated with exogenous hormones due to high sensitivity to the negative effects of steroids. It could imply that the hormonal threshold level is higher and a greater stimulus is needed to elicit an ovarian response (Baril *et al.*, 2000). Poorer superovulatory responses have been recorded in aged less than three years female goats (Mahmood *et al.*, 1991) and sheep (Torres *et al.*, 1987; Dingwall *et al.*, 1993; Lopes *et al.*, 2006). Several factors may contribute to this tendency such as the ovaries of young goats may not be mature enough and developing to be sensitised by exogenous gonadotrophin treatment, poor recruitment or stimulation of small follicles that could be attributed to the suppressive effect of a dominant follicle and eventually suppresses the total follicular recruitment. Thus, young goats maybe more sensitive to this inhibitory effect of the steroids and inhibin compared to adult goats (Driancourt, 2001; Senger, 2003).

Several studies have reported lower embryo developmental competence from oocytes of prepubertal than adult females, where the former results in a smaller number of viable embryos compared to those from adults (Palma *et al.*, 1993; Crozet *et al.*, 1995; Revel *et al.*,

1995; Khatir *et al.*, 1996; Keskintepe *et al.*, 1998; Cognie *et al.*, 2003). It has been reported that cytoplasmic deficiencies of prepubertal female oocytes resulted in low or incomplete developmental competence. These poor results are due to the cytopalsmic, ultrastructural, metabolic and nuclear abnormalities in prepubertal oocytes (Armstrong, 2001). Furthermore, cytoplasmic maturation of prepubertal oocyte being perturbed or incomplete has been reported from the studies of parthenogenic activation (Damiani *et al.*, 1996), nuclear (Mermillod *et al.*, 1998) and cytoplasmic transfer (Salamone *et al.*, 2001; Catt, 2002). High levels of abnormal fertilisation have been reported in prepubertal female oocytes (O'Brien *et al.*, 1996; 1997) such as failure of sperm head decondensation (Villamediana *et al.*, 2001), lack of sperm aster formation (Damiani *et al.*, 1996), asynchronous development of pronuclei or extrusion of maternal chromatin (Damiani *et al.*, 1996) and the incomplete or improper oocyte activation (Salamone *et al.*, 2001). Izquierdo *et al.* (2002) obtained 10% of blastocyst by using oocytes from abattoir goats at 2 months of age; and Koeman *et al.* (2003) obtained 8% of blastocysts with oocytes collected through LOPU-hormone stimulated goats from aged 2 to 5 months old. The low developmental ability that characterises oocytes from prepubertal goats (Izquierdo *et al.*, 1995) has been attributed to the presence of an abnormal protein profile (Levesque and Sirard, 1994), incomplete or delayed *in vitro* maturation (Damiani *et al.*, 1995), lack of sensitivity of the oocyte to its hormonal microenvironment (Levasseur, 1979), low male pronucleus formation (Mogas *et al.*, 1997b), a high rate of haploid (Villamediana *et al.*, 2001), polyspermic zygotes (Mogas *et al.*, 1997b), abnormal distribution of cortical granules (Velilla *et al.*, 2004) and mitochondrial morphology (Velilla *et al.*, 2006).

Several studies have reported that old age females generally become less reproductively competent and largely due to decreasing uterine health and oocyte viability (Carnevale *et al.*, 1993, 1997; Morris and Allen, 2002; Morel *et al.*, 2005). Oocyte viability is reported to reduce with old age and this may result from the ovulation of smaller pre-

ovulatory follicles which have not undergone final meiotic division and/or maturation prior to ovulation (Carnevale *et al.*, 1999). Santoro *et al.* (2003) speculated that this might be due to an impairment of final stages of antral follicle growth which in turn may be due to a number of factors including altered secretion of insulin-like growth factors (IGFs); a reduced response to circulating gonadotrophins; a decrease in gonadotrophin production or an increased sensitivity to feedback mechanisms such as inhibin. Morel *et al.* (2010) reported that a slower final growth rate of the pre-ovulatory follicle or a longer time interval from the achievement of maximum pre-ovulatory follicle diameter to ovulation in old animals related to reduction in gonadotrophin secretion and/or altered secretion of IGFs.

It has been reported that age of the animal was not considered to be a determinant factor in the superovulatory response (Hasler, 1992) and had no significant effect on the quantity and quality of oocytes harvested (Katska and Smorg, 1984; Wani *et al.*, 1999). Donaldson (1984a) also observed that there was no age effect on the total number of embryos recovered but a cut-off point beyond nine years of age was suggested, after which there was a decline in the response in terms of the percentage of transferable embryos recovered. The decline in superovulatory response maybe due to a reduction in the number of follicles is capable responding to gonadotrophin treatment in older animals (Lerner *et al.*, 1986). Oocytes have been harvested from the ovaries of prepubertal animals for *in vitro* maturation and *in vitro* fertilisation successfully indicating that age is not a constraint and the oocytes can be obtained from even prepubertal animals at a rate equivalent or higher than in pubertal animals (Salykbaev *et al.*, 1986; Armstrong *et al.*, 1994a). Despite this, oocyte from prepubertal animals compared to those from adults animals are capable of full development and result in the production of embryos are morphologically indistinguishable (O'Brien *et al.*, 1997) contain similar number of cells (O'Brien *et al.*, 1996; Koeman *et al.*, 2003), similar inner cell mass: total cell number ratio (Majerus *et al.*, 2000), glucose and pyruvate uptake

(Steeves *et al.*, 1999), protein profiles (Khatir *et al.*, 1998) and triglyceride content (Majerus *et al.*, 2000).

According to Eppig and Schroeder (1989) and Duby *et al.* (1996), follicles from prepubertal animals can be stimulated to grow and more oocytes could be retrieved with exogenous gonadotrophins. It has been reported that exogenous FSH treatments improve the developmental competence of oocytes from prepubertal and adult animals appears to be similar (Lu *et al.*, 1991; Pugh *et al.*, 1991; Koeman *et al.*, 2003). Baldassarre and Karatzas (2004) shown that the follicular response is maximised in prepubertal goats aged more than 3 months old following gonadotrophin stimulation. This is in agreement with previous studies in lambs (Earl *et al.*, 1995; Ledda *et al.*, 1999a; Ptak *et al.*, 1999a), acceptable development to term is obtained following the transfer to recipients of *in vitro* produced embryos from oocytes collected from prepubertal goats (Baldassarre *et al.*, 2004a). According to Wani *et al.* (1999), slicing and puncture techniques have been employed successfully instead of aspiration for the retrieval of oocytes from small size ovaries in animals aged below one year old. On the other hand, Lerner *et al.* (1986) demonstrated that the increased number of oocytes and embryos could be obtained with increasing doses of FSH following gonadotrophin stimulation in older animals, leading to recruit and maintain of a greater proportion of secondary or small antral follicles. It has been reported that LOPU followed by IVEP is an efficient method for the reproductive rescue of valuable female goats that have acquired reduced fertility primarily as a result of increased age (Baldassarre *et al.*, 2007).

In vitro embryo production (IVEP) systems now allow oocytes from very young animals to undergo fertilisation and form embryos capable of development to normal offspring, albeit at somewhat reduced efficiencies compared to oocytes from adult animals. They also can overcome infertility associated with advanced age of animals and women. However, the interactions between external and internal cues that drive young mammals to

puberty are complicated and still not fully understood (Ebling, 2005). Further research aims at better understanding of the mechanisms underlying developmental deficiencies and abnormalities of oocytes from young to aged animals. Such research will be crucial to the development of new oocyte-based technologies for improvement of IVEP in goats.

2.2.1.2 Breed

Differences in responsiveness to gonadotrophins among various breeds of cattle (Critser *et al.*, 1979; Holness *et al.*, 1980; Donaldson, 1984b), sheep (Torres *et al.*, 1987; Vivanco *et al.*, 1994) and mice (Spearow, 1988) have been reported. These studies demonstrated that animals with a genetic tendency toward high ovulation rates have a greater superovulatory response (Bindon *et al.*, 1986; Synder, 1986). Most of the differences in superovulatory response were related to the different prolificacy of the breeds used in multiple ovulation and embryo transfer (MOET) (Cahill and Dufour, 1979), with highly prolific breeds are more sensitive to gonadotrophin stimulation (Bindon *et al.*, 1971; Smith, 1976; Piper *et al.*, 1982) and hence respond better than less prolific breeds (Kelly *et al.*, 1983; Bindon *et al.*, 1986). Picazo *et al.* (1996) reported that these differences were also found when comparing non-prolific breeds, where an interaction between the type of gonadotrophin used and the breed. It has been demonstrated that Boer goat tended to demonstrate a high oestrus response, a shorter time interval from CIDR removal to onset of oestrus as well as exhibiting a shorter duration of the induced oestrus period among other breeds of goat. These characteristics may be ascribed to the Boer goat higher sensitivity to exogenous hormones and high fertility especially during the natural cycle when the ovaries are very sensitive to hormonal stimulation (Lehloenya *et al.*, 2005). Differences in superovulatory responses between breeds may be related either to a differential kinetic behaviour of the exogenous gonadotrophin or to a differential follicular dynamics and function in response to the hormone (Ammoun *et al.*,

2006). It has been reported that administration of a single dose oFSH (1.32 mg) provokes a significant increase in the plasma FSH in all the animals, in absorption and elimination of the hormone between breeds (McNeilly, 1985; Fry *et al.*, 1987), however, differences may be caused either by the FSH preparation used or by the route of administration or by the high inherent variability of FSH elimination (Demoustier *et al.*, 1988; Prakash *et al.*, 1999). Furthermore, a high individual variability within breeds was also found and the differences might be related to a higher expression or a higher sensitivity of FSH receptors in the ovary (Driancourt *et al.*, 1986; Abdennebi *et al.*, 1999; Dufour *et al.*, 2000). Thus, due to the variation in superovulatory responses derived from genetic factors (Bondurant, 1986), further studies are required to determine the causes for individual variability and, from a practical point of view, for the selection of high responsive individuals within each breed to establish a reliable method of superovulation as the first step toward the establishment of reliable least variable *in vitro* embryo production programmes.

2.2.1.3 Nutrition

Nutrition is considered to be an important factor affecting reproductive function in domestic ruminants influencing the onset of ovarian cyclicity in post-partum sheep (Tchamitchian *et al.*, 1973; Restall and Starr, 1977) and goats (Walkden-Brown *et al.*, 1994). Reproductive performance is commonly correlated with body weight changes. For instance, the mean body weight at puberty varied depending on the dietary energy level in the Boer goat (Greyling, 1988) and severe body weight loss is usually accompanied by anoestrus (Richards *et al.*, 1989) in a variety of species. However, specific research on the effect of body weight on the superovulatory response of goat is limited. Most of the available information in domestic animals regarding the effect of nutrition on the embryo recovery rate and quality was from superovulated cattle and sheep.

Dietary intake can influence ovarian activity via effects at various levels of the hypothalamus-pituitary-ovarian axis. Changes in the plane of nutrition can affect follicular growth (Gutierrez *et al.*, 1997; Gong *et al.*, 2002; Diskin *et al.*, 2003; Mihm and Bleach, 2003) by inducing changes in plasma metabolites and metabolic hormones such as insulin and insulin-like growth factor-I (IGF-I) (Armstrong *et al.*, 2001; Ferguson *et al.*, 2003) and/or in hormones and growth factors in follicular fluid (Landau *et al.*, 2000). Dietary changes cause an immediate and rapid change in a range of metabolic humoral agents including glucose and insulin. Changes in insulin are closely related to changes in IGF-I and IGF-II concentrations. These ligands are present in a range of tissues including follicular fluid and are likely to have endocrine, paracrine and autocrine actions in response to changes in nutrition (Monget and Martin, 1997). A detrimental effect of high live weight gain and dietary intake could have been expected on embryo production according to results *in vivo* in sheep (McEvoy *et al.*, 1993; Creed *et al.*, 1994; McEvoy *et al.*, 1995) and in cattle (Mantovani *et al.*, 1993; Negrao *et al.*, 1997; Yaakub *et al.*, 1999), or *in vitro* in cattle with impaired embryo development associated with high plasma concentrations of both insulin and IGF-I (Armstrong *et al.*, 2001). Thus, diet can profoundly alter endocrine signalling pathways. However, the effect that these changes ultimately have on fertility is unclear. The relationship between the developing oocyte and the follicular environment *in vivo* is a sensitive balance of hormonal and receptor communication. Although structural abnormalities have been reported in oocytes from superovulated compared with non-stimulated heifers (Assey *et al.*, 1994), there are few reports on the effects of nutrition on oocyte quality in cattle, sheep or goat; or on oocyte morphology in non-stimulated or superovulated goat.

Dietary intake can also affect oocyte morphology (O'Callaghan *et al.*, 2000), oocyte developmental capacity and embryo production. Overfeeding has been shown to be detrimental to oocyte quality, increased embryo mortality or delay embryo development *in*

vivo (Mantovani *et al.*, 1993; McEvoy *et al.*, 1995; Negrao *et al.*, 1997) and *in vitro* (Papadopoulos *et al.*, 2001). Conversely, overfeeding can also lead to reduced pregnancy rates (Parr *et al.*, 1987) and a decrease in the rate of development *in vitro* and the viability of embryos collected on day 2 after fertilisation (Creed *et al.*, 1994). Undernutrition has been shown to delay embryo development during the first 2 weeks after fertilisation (Parr *et al.*, 1987; Abecia *et al.*, 1997) and increase embryo mortality in the first 2 weeks of pregnancy (Rhind *et al.*, 1989a; Abecia *et al.*, 1995), whereas dietary restriction could have a positive effect on oocyte quality (Lozano *et al.*, 2003) and blastocyst production *in vitro* (McEvoy *et al.*, 1997; Nolan *et al.*, 1998b; Armstrong *et al.*, 2001).

The relationship between nutrition and reproduction in ruminants is complex and; responses are often variable and inconsistent. In sheep, low dietary intake can reduce ovulation rate (Smith, 1991) and dietary supplements containing high energy and protein can increase ovulation rate in ewes with poor body condition that are not supplemented with exogenous gonadotrophins (Downing *et al.*, 1995). Gong *et al.* (2002) demonstrated that feeding heifers with 200% maintenance requirements for a short period significantly increases the number of small follicles, confirming that increased dietary intake can enhance the recruitment of ovarian follicles in cattle (Gutierrez *et al.*, 1997). In addition, they showed that increased dietary intake can also enhance the response to a standard superovulatory protocol with FSH. The enhanced superovulatory response apparently resulted from a dietary induced increase in small follicle populations, as both the number of large follicles before ovulation and the number of CL were significantly correlated with the number of small follicles at the start of FSH stimulation (Gong *et al.*, 2002). Thus, it is likely that short-term energy supply is directly involved in follicle growth because cattle on restricted dietary intake have altered follicular growth characteristics (Murphy *et al.*, 1991; Rhodes *et al.*, 1995b). There is a strong negative relationship between dietary intake and systemic progesterone in

sheep (Rhind *et al.*, 1989b; McEvoy *et al.*, 1995). McEvoy *et al.* (1995) reported that a higher proportion of ova from ewes on a low diet were considered viable compared with those produced in ewes on a high diet. In cattle, restricting the energy intake before the animals were killed enhanced the subsequent *in vitro* development of the oocytes (McEvoy *et al.*, 1997; Nolan *et al.*, 1998a,b; Armstrong *et al.*, 2001). Adamiak *et al.* (2005) demonstrated that feeding level on oocyte quality is dependent on the body condition of the animal, with the high level of feeding being beneficial to oocytes from animals of low body condition but detrimental to oocytes from animals of moderately high body condition.

These findings indicate that some of the effects of nutrition on reproduction may influence the oocyte. It is not known whether the effects of diet on embryo development are solely confined to the period of embryo development or whether diet can also affect oocyte development before fertilisation which is subsequently manifested in poor embryo development. The way nutrition influences embryo production remains to be fully characterised. It may affect oocyte development before fertilisation, early embryo development or uterine environment. This is why nutritional management of donor goat in programmes of superovulation and LOPU need to be adapted to optimise the production of oocytes and embryos (Scaramuzzi and Murray, 1994).

2.2.1.4 Exogenous gonadotrophin used

Roover *et al.* (2005) demonstrated that optimisation of oocyte production has been an important goal throughout LOPU research during the past decade due to the constant need for more oocytes of good quality. Effects of exogenous hormones on stimulation of goat follicles have been studied (Lambert *et al.*, 1986; Amoah and Gelaye, 1990). The two most commonly used gonadotrophin treatments for superovulation in goats are follicle stimulating hormone (FSH) and equine chorionic gonadotrophin (eCG). FSH is much more expensive and due to

its shorter half-life requires multiple doses, whereas eCG is inexpensive, available in the local market and requires less veterinary input because it requires a single injection (Monniaux *et al.*, 1983).

FSH proved to be more efficacious than eCG (Armstrong *et al.*, 1983b; Nuti *et al.*, 1987; Mahmood *et al.*, 1991; Nowshari *et al.*, 1992), provided it contains an appropriate admixture of luteinising hormone (LH). The use of eCG with or without a follow-up with eCG antibodies (Pintado *et al.*, 1998), in many cases, did not deliver the anticipated response (Saharrea *et al.*, 1998; Cognie, 1999). The action of eCG is more FSH-like than LH-like. The LH component in the preparation causes luteinisation of premature follicles, desensitization of LH receptors, premature ovulation and drastically decreases the superovulalory response (Herrler *et al.*, 1991; Boland *et al.*, 1991; Breuel *et al.*, 1991). A decreased FSH/LH ratio in eCG results in fewer ovulations and tends to promote the formation of ovarian cysts (Murphy *et al.*, 1984; Boland *et al.*, 1991). Lower superovulatory responses with eCG may be associated with its relatively long circulating half-life, resulting in excessive follicular development and failure of ovulation, as eCG remains active in the blood for 5 to 7 days after administration (Siddiqui *et al.*, 2002). FSH has surpassed eCG as the method of choice for superovulation because in general the results, i.e. ovulation response and; number of total and transferable embryos were reported better in FSH- than eCG-treated goats (Amstrong *et al.*, 1983a,b; Tsunoda and Sugie, 1989; Goel and Agrawal, 1990; Mahmood *et al.*, 1991; Pampoukidou *et al.*, 1992; Pendelton *et al.*, 1992).

Baldassarre and Karatzas (2004) reported that repeated use of eCG has been reported to result in poor fertility in goats due to the presence of anti-eCG antibodies developed as an immune response to previous treatments. The presence of such antibodies has been clearly linked to a delay in the occurrence of oestrus, LH peak and ovulation in the synchronized animals, which may explain the lower fertility (Chemineau *et al.*, 1999; Roy *et al.*, 1999;

Drion *et al.*, 2001b). The repeated superovulation treatment of goats with porcine FSH (pFSH) is associated in the goat with the appearance of anti-FSH antibodies and a decrease in the ovulation rate after third treatment (Ryan *et al.*, 1991), therefore, leads to an immunologically induced decline in superovulatory response (Beckers *et al.*, 1990; Remy *et al.*, 1991; Baril *et al.*, 1993). This is a problem not encountered in cows, presumably due to a greater homology of porcine and bovine as compared to caprine FSH (Remy *et al.*, 1991). However, it has been reported that superovulatory response in goats is maintained by means of treating several times with caprine (cFSH) or ovine (oFSH) FSH (Baril *et al.*, 1993). Superovulation with either eCG or FSH causes severe alterations of the normal reproductive physiology of the donor animal (Greve *et al.*, 1995). During ovarian superovulation, plasma progesterone, oestradiol, inhibin and gonadotrophin surge attenuating factor (GnSAF) concentrations are increased, and there is a concomitant reduction in endogenous pulsatile secretion of LH. The effects on the reproductive system of these various endocrine changes should not be ignored, as they may contribute to the great variation encountered in standard superovulatory protocols (Sendag *et al.*, 2008).

Efforts have been made to neutralise the long action of eCG in the circulation and thus reduce post-oestrus ovarian follicular stimulation by using antiserum against it (Gonzalez *et al.*, 1994). Treatment with eCG antibodies have been reported to improve ovulation rates (Dhondt *et al.*, 1978; Dieleman and Bevers, 1987) and embryo quality (Kummer *et al.*, 1980; Saumande *et al.*, 1984). The mechanism responsible for inducing such effects is not known. According to Dieleman *et al.* (1993), the use of anti-eCG requires a critical timing and minor compromise that adversely affect the yield of embryos. In addition the individual animal variation in response to the anti-eCG often obscures the potential benefit of using an anti-eCG injection (Dieleman *et al.*, 1993). Several attempts have been made to devise less labour-intensive treatment regimes without compromising oocyte or

embryo yield. Batt *et al.* (1993), Baldassarre *et al.* (2002, 2003, 2007) and Gibbons *et al.* (2007) almost equalled the oocytes or embryo yield obtained with the traditional multiple injection regimen by applying a 'one shot'-treatment regimen consisting of a single dose of FSH combined with a moderate dose of eCG (e.g. 60 to 80 mg FSH and 300 IU eCG). The simplicity of this treatment is appealing by increasing the percentage of large follicles in relation to small follicles prior to oocyte recoveries in goats (Gibbon *et al.*, 2007). On the other hand, ovulation can be synchronised more precisely by administering GnRH around the time of oestrus. This improves the success of oocyte or embryos retrieval at a controlled stage of development for specific applications (Pierson *et al.*, 2003). It has been reported terminal follicular growth is impaired in goat by administration of GnRH before superovulation, and this treatment-beneficial effect on the ovulatory response is negated by an increase in the proportion of unfertilised ova and degenerated embryos (Cognie *et al.*, 2003).

2.2.1.5 Route of superovulatory gonadotrophin administration

A route of administering a superovulation treatment might also lead to variation in superovulation response as it determines the rate of gonadotrophin absorption (Dobbs *et al.*, 1994). There is very little information on goats regarding the route of the superovulatory gonadotrophin treatment administration (Mahmood *et al.*, 1991; Pendleton *et al.*, 1992; Selvaraju *et al.*, 2003; Goel and Agrawal, 2005). It has been observed that the absorption of FSH is faster following an i.m. injection compared to the subcutaneous (s.c.) administration of FSH in human. However, the s.c. administration of FSH has been shown to lead to a higher number of developing follicles when compared to the i.m. route of administration as it is generally accepted that the uptake of gonadotrophin is slower following s.c. administration. Thus the route of gonadotrophin administration could be crucial in timing of the

superovulatory treatment, positioning of the time of ovulation and determining the efficiency of superovulation (Dobbs *et al.*, 1994).

The superovulatory treatment (FSH) is generally administered intramuscularly (i.m.) and observed a high variation in ovulation rate in most of the studies conducted in goats (Armstrong *et al.*, 1983a; Selgrath *et al.*, 1990; Mahmood *et al.*, 1991). Therefore, the s.c. route of gonadotrophin administration prove to lead to more predictable results than i.m. route. Several studies have demonstrated that the s.c. route of gonadotrophin administration lead to a lower number of unfertilised ova and thus enhances fertility (Senthil Kumar *et al.*, 2003; Lehloenya and Greyling, 2009). Lehloenya and Greyling (2009) showed that the higher number of unfertilised ova in the i.m. route of hormone-treated goats may have been caused by factors related to a poor fertilisation rate, e.g. uterus environment, faster and higher oestrogen levels. The occurrence of a low fertilisation following superovulation is often caused by poor synchronisation (hormonal imbalance) and the timing of ovulation. This has been proven by an increase in the fertilisation rate as indicated by higher number of embryos recovered following superovulation with pFSH and when ovulation was synchronised using LHRH in goats (Akinlosotu and Wilder, 1993). Although the s.c. route of pFSH administration can induce follicles to grow and ovulate, however, the recruited oocytes do not attain complete maturation. The prolonged period of s.c. stimulation and lower FSH levels could contribute to this phenomenon. Therefore, the oocytes could be fertilised, but cannot sustain the viability of the embryo. These oocytes that do not attain complete maturation are generally recruited from the follicles less than 2 to 3 mm in size. Furthermore, it has been found that viable embryos are only associated with the recruitment of follicles of 4 to 6 mm size in diameter in goats (Gonzalez-Bulnes *et al.*, 2003a, 2004a). Lehloenya and Greyling (2009) demonstrated the s.c. route resulted in lower variances which may indicate a more predictable response in the efficiency of superovulation in Boer goat when using pFSH; and

could also be an acceptable technique of embryo flushing used although no significant differences were found between s.c. and i.m. routes of gonadotrophin administration regarding the number of transferable embryos.

2.2.1.6 Effect of ovarian follicular status

The major factor leading to the variable ovulation rate and oocyte or embryo output currently seems to be the follicular status, i.e. the number of small and responsive follicles and the absence of a dominant follicle, which can directly be affected by the presence of GnRH of the donor at the onset of superovulatory treatment (Rubianes and Menchaca, 2003; Gonzalez-Bulnes *et al.*, 2004a). The presence of a dominant follicle at the onset of superovulation has been reported to inhibit the growth of other ovarian follicles and decrease the ovarian response in small ruminants (Guilbault *et al.*, 1991; Rubianes *et al.*, 1995; Kafi and McGowan, 1997; Lopez-Sebastian *et al.*, 1999; Gonzalez-Bulnes *et al.*, 2002, 2004a; Rubianes and Menchaca, 2003). Several studies have reported that the superovulatory response in sheep and goats is closely related to the number of gonadotrophin responsive follicles stimulated and the absence of a dominant follicle on the ovary at the onset of superovulation (Brebion *et al.*, 1990; Baril *et al.*, 1996b; Heidari *et al.*, 2010). It has been previously shown that the use of a GnRH antagonist increases the number of the small gonadotrophin-responsive follicles (Gonzalez-Bulnes *et al.*, 2003b). This phenomenon is generally the result of the blockage of the GnRH receptors by the application of an antagonist which then causes a rapid decrease in the circulating LH and FSH levels, thus preventing the LH surge (Dobson *et al.*, 1997; Campbell *et al.*, 1998; Bellmann *et al.*, 2002; Doody *et al.*, 2003; Rubians and Menchaca, 2003; Gonzalez-Bulnes *et al.*, 2004a). On the other hand, it has been observed that once the numbers of small follicles increase and develop, the response to superovulation is greater (Gonzalez-Bulnes *et al.*, 2004a). Low LH concentration can be

achieved by administrating an exogenous gonadotrophin-releasing hormone antagonist (GnRHa) or agonist prior to the superovulatory treatment (Dobson *et al.*, 1997; Campbell *et al.*, 1998; Gonzalez-Bulnes *et al.*, 2004a; Stout and Colenbrander, 2004). GnRH antagonist pre-treatment thus regressed the dominant follicles at the start of the superovulation treatment as reported in the ewe (Brebion *et al.*, 1990) and human (Doody *et al.*, 2003; Fanchin *et al.*, 2003).

It has been previously reported that the long-term use of a GnRH antagonist led to an adverse effect on terminal follicular growth, fertilisation rate and embryo viability in the superovulated goats (Cognie *et al.*, 2003). Generally, the GnRH antagonist inhibits follicular growth and causing atresia in some of the follicles. It has also been suggested that the deteriorating follicles regrow; and producing a large number of anovulatory follicles and unfertilized ova following superovulation (Cognie *et al.*, 2003; Lehloenya *et al.*, 2006; Heidari *et al.*, 2010). Earlier reports indicated that the GnRH receptors are not limited to hypothalamic tissue and can also be found in other body tissues such as the ovary, placenta, oviduct and embryo (Kawamura *et al.*, 2004; Senqupta and Sridaran, 2008). It is hypothesized that the GnRH antagonist blocks the GnRH receptors present in the reproductive tissues and prohibiting natural GnRH binding, thus, bringing the signaling cascade involved in oocyte maturation to a halt (Heidari *et al.*, 2010). Cognie *et al.* (2003) proposed that the shortening of the GnRH antagonist pre-treatment period may solve this problem.

Persistent follicles are a problem especially in eCG-stimulated goats (Armstrong *et al.*, 1983a; Dutta *et al.*, 1993). During oestrus synchronisation, follicular dominance is prolonged due to dominant follicles growing to larger sizes and persisting in the ovary for longer periods of time as compared with typical dominant follicles, if progesterone is lacking (Sirois and Fortune, 1990; Revah and Butler, 1996; Flynn *et al.*, 2000). It was demonstrated in ewes

that persistent follicles can produce variable concentrations of androstendione, testosterone, oestradiol and progesterone (Meinecke-Tillmann *et al.*, 1983) and luteinise (Hermann, 1992). The long stimulatory action of eCG due to its slow metabolic clearance seems to be responsible for this condition (Ryan *et al.*, 1991). If these large persistent follicles ovulate, fertility may diminish (Mihm *et al.*, 1994; Smith and Stevenson, 1995; Austin *et al.*, 1999; Vinoles *et al.*, 2001). Furthermore, numerous studies on goat superovulation make reference to premature luteal regression (Armstrong *et al.*, 1983a,b; Tervit *et al.*, 1986; Pendleton *et al.*, 1992) and hormonal profiles during regression have been reported (Battye *et al.*, 1988; Borque *et al.*, 1993). A higher incidence of premature corpus luteum regression in eCG- versus FSH-superovulated goats was recognised (Armstrong *et al.*, 1983a; Pendleton *et al.*, 1992; Rosnina *et al.*, 1992; Riesenberg *et al.*, 2001). This could possibly be ascribed to hormonal imbalances due to a deficiency in circulating LH (Armstrong *et al.*, 1983a; Pendleton *et al.*, 1992; Saharrea *et al.*, 1998). Pintado *et al.* (1996) reported that exogenous administration of synthetic prostaglandins does not increase the incidence of early regression and luteal failure in superovulated goats and it does not interfere with the superovularoty response.

The number of recovered and viable oocytes or embryos could be associated with the number of follicles of 4 to 6 mm in size (Gonzalez-Bulnes *et al.*, 2003a). The lower recovery rate obtained with a higher number of 2 mm follicles seem to indicate that the smallest follicles grew until pre-ovulatory stages but were not able to release a viable oocyte. Studies performed on ewes and cows state that survival of these follicles are associated with disturbances in the ovulation process (Rubianes *et al.*, 1997), presence of anovulatory luteinised follicles (Monniaux *et al.*, 1983) or deficiencies in the release of a fertilisable oocyte (D'Occhio *et al.*, 1997; Stock *et al.*, 1996). These studies are emphasised that viability rate was increased in follicles with a diameter of 5 to 6 mm which could indicate the presence

of changes in oocyte developmental competence during the latter stages of maturation in the smaller follicles (Telfer *et al.*, 1999). In this way, the acquisition of meiotic competence of goat oocytes is correlated with follicular size as the oocytes are fully competent to mature in follicles larger than 3 mm (De Smedt *et al.*, 1994).

The observation of the ovarian follicular status at the onset of superovulatory treatment by transrectal ultrasonography made in heifers (Guilbault *et al.*, 1991) and ewes (Gonzalez-Bulnes *et al.*, 2000) has allowed the identification of some of the variability in superovulatory response, taking in consideration ovarian factors such as the number and size of follicles present in the ovaries at the onset of FSH treatment. However, the ultrasonographic screening of the ovarian follicular population has not yet been employed in superovulated goats until recently (Riesenberg *et al.*, 2001). Transrectal ultrasonography can be used as a practical criteria for selection of donor goats which could avoid the treatment of poor responding females and provides a basis for the study of suitable treatments to make ovarian follicular populations uniform (Bungartz and Niemann, 1994; Gonzalez-Bulnes *et al.*, 2003b).

To date, there is not a single one fulfills all expectations concerning predictability and reliability of the response from the numerous superovulation protocols in use. The variability in number of ovulations and yield of viable oocytes or embryos remains the main drawback. A host of environmental factors such as season, nutrition, health state, drug handling and insemination technique is known to contribute to that variability. Therefore, vigorous research efforts are directed at the establishment of suitable superovulation regimes to augment the deployment of *in vitro* embryo production programmes and associated technologies based upon them in goats (Holtz, 2005).

2.3 RETRIEVAL OF PRE-OVULATED OOCYTES

Production of embryos either by *in vivo* or *in vitro* is a well-established practice in small ruminants for spreading or conserving desirable genes of valuable individuals (Cognie *et al.*, 2004; Gonzalez-Bulnes *et al.*, 2004b). *In vitro* embryo production (IVEP) has been considerably developed in recent years due to the high variability in ovulatory response, fertilisation failures and embryo degeneration processes that affect yields obtained with traditional multiple ovulation and embryo transfer (MOET). *In vitro* production (IVP) of goat embryos is a rapidly advancing field and it offers an alternative to superovulation as a source of embryo for transfer and manipulation purposes. Application of *in vitro* techniques (*in vitro* maturation, fertilisation and culture) to species such as the goat would facilitate the production of large numbers of embryos from a single genetically valuable animal. The success of IVEP programme is largely dependent on the continuous supply of competent oocyte. To date, goat oocytes have been harvested principally from ovariectomised (Younis *et al.*, 1991; Keskintepe *et al.*, 1994b) or slaughtered goats (Crozet *et al.*, 1995; Keskintepe *et al.*, 1996; Pawshe *et al.*, 1996; Mogas *et al.*, 1997a; Izquierdo *et al.*, 1999, 2002). Laparoscopic oocyte pick-up (LOPU) has provided an efficient and relatively non-invasive method for the collection of oocytes from small ruminants, such as goats (Graff *et al.*, 1999; Baldassarre *et al.*, 2002), sheep (Baldassarre *et al.*, 1994, 1996) and cattle (Armstrong *et al.*, 1992, 1994b; Baldassarre, 1998) in which other techniques may not be feasible (transvaginal ultrasound-guided aspiration, TUGA) or desirable (slaughterhouse, laparotomy).

2.3.1 Laparoscopic Oocyte Pick-Up (LOPU)

In vitro production of embryos using immature oocytes recovered by laparoscopy has the potential to overcome some of the problems associated with standard MOET techniques (Tervit, 1996; Baldassarre *et al.*, 2002; Cognie *et al.*, 2003). Currently, most IVP protocols

combine with oestrous synchronisation and stimulation of follicular development by short gonadotrophin treatments in order to recover high number of oocytes through LOPU technique (Alberio *et al.*, 2002; Baldassarre *et al.*, 2002, 2003). Graff *et al.* (1995) showed that LOPU in goats enabled more of the available follicles to be aspirated, resulting in more oocytes recovered compared with TUGA recovery. The procedure was first described by Snyder and Dukelow (1974) in sheep but the potential of the technique was not fully realised until IVEP technologies were developed 20 years later in both sheep (Baldassarre *et al.*, 1994; Tervit, 1996; Kuhholzer *et al.*, 1997) and goats (Baldassarre *et al.*, 1994, 2002; Graff *et al.*, 1995; Pierson *et al.*, 2004). Briefly, the donor goat is restrained on a standard laparoscopy table under general anaesthesia and follicles are aspirated under laparoscopic observation using a 20G needle mounted in a plastic pipette connected to a collection tube and a vacuum line. The LOPU procedure only takes between 10 to 20 minutes per goat depending the number of follicles to be aspirated, to reduce animal stress (Kuhholzer *et al.*, 1997). The technique is less invasive but still entails full anaesthesia and requires sophisticated equipment and considerable technical skill (Baril *et al.*, 1993; Flores-Foxworth, 1997).

LOPU technique is an extremely powerful tool for production and propagation of genetically valuable animals by given its repeatability and reliability as a source of immature oocytes, which always results in more than 5 oocytes aspirated per donor while individual variation in the response to gonadotrophin treatment remains (Baldassarre and Karatzas, 2004). It has been also reported that LOPU is an efficient technique for the retrieval of high quantity and quality oocytes from prepuberal, pubertal or ageing animals; where MOET is not possible (Koeman *et al.*, 2003; Baldassarre *et al.*, 2007). Baldassarre *et al.* (2002) have showed that follicular response is maximised in prepubertal goats at more than 3 months of age following transfer to recipients of *in vitro* produced embryos from oocytes collected from prepubertal goats. Recovery of oocytes by LOPU from gonadotrophin-stimulated prepubertal

goats resulted in a significantly higher yield from 60 to 90 days old goats than from 90 to 150 days old goats (Baldassarre *et al.*, 2002). Furthermore, it has been demonstrated that LOPU followed by IVEP is an efficient method for the 'reproductive rescue' of valuable female goats that have acquired reduced fertility primarily as a result of increased age (Baldassarre *et al.*, 2007).

LOPU procedure can be repeated several times without ovarian damage or decrease in the donor fertility since it is less traumatic and results in fewer surgical adhesions than standard surgery (laparotomy) generally used to recovery of *in vivo* matured oocytes and embryos (Stangl *et al.*, 1999; Alberio *et al.*, 2002; Baldasssarre *et al.*, 2003; Baldassarre and Karatzas, 2004), therefore, extends the use of donor animal resources (Pierson *et al.*, 2004). Repeated LOPU followed by IVP has the potential to produce more offspring than traditional MOET because it is less variable in results (Baldassarre and Karatzas, 2004) and may avoid poor results caused by poor ovulation rates, early corpus luteum (CL) regression and poor fertilisation (Cognie *et al.*, 2003; Baldassarre and Karatzas, 2004). It has been demonstrated that hormonal treatment and LOPU can be repeated up to 5 times in the same donor goats with minimal surgical adhesions and no important change in overall response, in which many donors subjected to more than five LOPU procedures become pregnant and kid after insemination or natural mating (Pierson *et al.*, 2004). Stangl *et al.* (1999) also reported that LOPU had no detrimental effects on the fertility of donor animals even when repeated up to 20 times.

Graff *et al.* (1999) have proposed TUGA as a preferred system for the recovery of oocytes from valuable animals because LOPU had a greater risk for post-surgical adhesion formation. However, the rate of recovery of oocytes was significantly lower than those obtained by LOPU. TUGA technique is commonly used in large domestic livestock, particularly in cattle and not much practiced in small ruminant such as goats. Snel-Oliveira *et*

al. (2002) found that repeated TUGA in prepubertal calves caused histological lesions in the ovaries, and could alter the normal ovarian function and possibly affect future fertility. In contrast, Pierson *et al.* (2004) indicated that no adhesions that could adversely affect or prevent further LOPU were reported although occasional adhesions of the omentum to the abdominal wall were observed after LOPU. Furthermore, Baldassarre *et al.* (2003) indicated that the utilisation of appropriate aspiration techniques and grasping instruments may minimise trauma to the reproductive tract resulting in few if any surgical sequels. Thus, LOPU may preferable over TUGA for the propagation of valuable animals and endangered species.

With respect to time interval between gonadotrophin stimulation and LOPU, it has been demonstrated that the interval can be reduced to periods as short as 4 days without affecting follicular development and oocyte quality. The reiteration of the protocols had no effect on the number of follicles present, as described when longer intervals were applied either with (Alberio *et al.*, 2002; Baldassarre *et al.*, 2003) or without hormonal treatment (Kuhholzer *et al.*, 1997) in the successive LOPU. LOPU can be also repeated at intervals as short as a week after multidose hormonal treatments (Tervit *et al.*, 1992, 1993; Alberio *et al.*, 2002) without diminishing good quality oocytes harvest (Tervit *et al.*, 1992; Stangl *et al.*, 1999; Alberio *et al.*, 2002). Other studies also demonstrated that the ovary enables to recover from LOPU in less than 5 weeks because LOPU is less traumatic because only the theca is perforated during aspiration to the ovary, whereas during TUGA, the needle usually reaches the follicle following perforation of the ovarian stroma (Pierson *et al.*, 2004). In IVP programmes where large numbers of animals are available, it may be possible to use a longer interval; however, a period as short as 5 to 7 weeks between procedures may be advantageous in situation where donor animal resources are limited. Shorter intervals allow for improved management of animal resources and lower production costs by allowing one to do more with

a given number of animals. It is possible to generate more offspring from genetically valuable animals using LOPU technique since it can be repeated many times in the same donor within a short time period.

2.3.2 Laparotomy and Ovariectomy

The number of high quality oocytes harvested from an ovary is an important consideration for the *in vitro* production of embryos. *In vivo* matured oocytes are obtained either by surgical or laparoscopic methods (Baldassare *et al.*, 1994). These methods are expensive and the number of oocytes recovered per ovary is very small (Pawshe *et al.*, 1994a). Ovaries of slaughtered animals are the cheapest and most abundant source of primary oocytes for the large scale production of embryos through IVP procedures (Agrawal *et al.*, 1995).

Several techniques have been used for the collection of oocytes from the ovaries goats (Mogas *et al.*, 1992; Pawshe *et al.*, 1994b), sheep (Wahid *et al.*, 1992a,b) and cattle (Iwasaki *et al.*, 1987; Katska, 1984; Katska and Smorg, 1984). The most common techniques used are the slicing of the ovary (Wahid *et al.*, 1992a,b), aspiration of visible ovarian follicles (Slavik *et al.*, 1992; Watson *et al.*, 1994) or follicular dissection (Fukui *et al.*, 1988). It has been reported that ovary slicing is a simple and more efficient technique than follicle aspiration (Martino *et al.*, 1994; Pawshe *et al.*, 1994b), however, the production of more debris will interfere the number of oocytes retrieved. Puncture of ovarian surface by an 18G hypodermic needle is also a simple and efficient method of recovering a high number of morphologically normal goat oocytes; and thus can be used as an alternative to the aspiration or slicing method for (Mogas *et al.*, 1992; Pawshe *et al.*, 1994b). The lower number of oocytes recovered by the aspiration method may be attributed to the presence of some follicles embedded deeply within the cortex, which are released by puncture or slicing of the ovary; and some of the

oocytes may even be lost during aspiration of follicles which is not possible when using the slicing or puncture method.

Oocyte aspiration from ovarian follicles is performed by simple aspiration of follicular contents using a syringe and a needle of 18 to 22G (Keskintepe *et al.*, 1996, 1998; Kuhholzer *et al.*, 1997; Yadav *et al.*, 1998; Ptak *et al.*, 1999b; Stangl *et al.*, 1999; Berlinguer *et al.*, 2004; Morton *et al.*, 2005) or an aspiration pump set to various pressures (Smith *et al.*, 1994; Baldassarre *et al.*, 1996; Anel *et al.*, 1997; Alberio *et al.*, 2002; Velilla *et al.*, 2002). Previous studies have demonstrated that the increase in vacuum pressure causes an increase in velocity of the moving fluid which may lead to stripping away of cumulus cell and deforming the vitelline membrane (Fry *et al.*, 1997); and the total number of oocytes recovered but reduces the number of viable oocytes (Tervit *et al.*, 1995; Rodriguez *et al.*, 2000, 2006). Furthermore, Ward *et al.* (2000) observed that an increase in vacuum pressure collapsed the follicle around the needle, preventing the flow of fluid or trapping the oocyte, thereby, preventing the increase in oocyte recovery rate which is normally associated with an increase in aspiration pressure. The majority of studies have observed that oocyte developmental competence is affected by vacuum pressure during oocyte aspiration (Smith *et al.*, 1994; Bols *et al.*, 1996; Ward *et al.*, 2000; Morton *et al.*, 2008). However, Tervit *et al.* (1995) reported the rates of oocyte development similar to that of the blastocyst stage after oocytes were aspirated with vacuum pressure of 15, 25 or 50 mmHg. Aspiration pressure affects oocyte developmental competence through mechanical forces exerted on the oocyte during aspiration, contact with tubing and the length of time between removal from the follicle. Factors such as tubing length and diameter, needle length and gauge all affect the flow rate and there can be considerable difference in flow rates of the oocytes through the two aspiration systems using the same vacuum pressure. Although, the effect of vacuum pressure during oocyte harvesting on oocyte retrieval and *in vitro* development of goat

oocytes has not been reported as it was in sheep (Morton *et al.*, 2008), it is highly probable that this is the case also in goats.

The presence of a CL also adversely affected the number of oocytes recovered. The cause of a low number of oocytes per ovary with a CL may be attributed to the fact that a CL inhibits the growth of follicles and increases their atresia (Hafez, 1993). In adult goat ovaries, conventionally, oocytes are recovered by follicle aspiration selecting follicles bigger than 3 mm diameter. From prepubertal goat ovaries, slicing the ovary allows collection of more oocytes per ovary than by follicle aspiration but the morphological quality is lower (Martino *et al.*, 1994). Wani *et al.* (1999) reported that large ovaries are expected to yield a higher number of oocytes and the cause may be attributed to the low number of visible follicles present in small ovaries, whereas the large sized ovaries have a good pool of visible follicles for aspiration. In Malaysia, the abattoir source is extremely limited due to the low slaughtering activities as consequences of shortage of breeding stock; and normally older or culled goats are chosen to slaughter (Rajikin, 1995; Anna, 2007), thus, LOPU could be an alternative method of oocyte retrieval for the IVEP programmes in goats.

2.4 *IN VITRO* MATURATION (IVM)

IVM has particular significance being the platform technology for the abundant supply of mature and good quality oocytes; as it is one of the essential steps in the IVP process of goat. Goat oocytes have been studied for different aspects of maturation (Pawshe *et al.*, 1996; Mogas *et al.*, 1997a; Yadav *et al.*, 1997; Kharche *et al.*, 2005). In most studies, the basic medium was supplemented with hormones and different concentrations of serum. The maturation medium and the selection of protein supplements and hormones for IVM play an important role in subsequent IVF or ICSI and *in vitro* development (Pawshe *et al.*, 1996). Embryo development is influenced by events occurring during oocyte maturation. For

successful IVM, oocytes must undergo synchronically nuclear and cytoplasmic maturation. In all mammalian species, nuclear maturation can be achieved when the oocytes are removed from the antral follicles and are cultured *in vitro* (Thibault *et al.*, 1987). Cytoplasm of the oocyte may play a crucial role in assembling the correct metabolic environment for production of sufficient energy for cellular functions during maturation, cleavage and blastocyst formation. The reduced development of *in vitro* derived zygotes in goats suggests that the conditions of IVM do not support cytoplasmic maturation. So it is very important that the improvement of the *in vitro* maturation system for oocytes be aimed at defining *in vitro* conditions more similar to the *in vivo* environment.

2.4.1 The Physiology of Oocyte Maturation

Oogenesis is characterised by a unique process of cell division occurring only in gametes, called meiosis; whose goal is the production of haploid cells highly specialised for fertilisation. Mammalian oocytes are arrested at the diplotene stage of the first meiotic prophase, also known as germinal vesicle (GV) stage during their growth (Voronica and Wessel, 2003). Meiotic competence is defined as the ability of an oocyte to undergo germinal vesicle breakdown (GVBD), progresses to metaphase I (MI), extrude the first polar body and reach metaphase II (MII). Meiotic competence is sequentially acquired during the final phase of oocyte growth. During subsequent follicle growth, the oocytes acquired the ability to progress to MI in follicles between 1.0 to 1.8 mm in diameter; and to achieve nuclear maturation up to MII in follicles more than 2 mm in diameter. Meiotic competence of oocytes is acquired in antral follicles between 0.5 to 3 mm in diameter in goats (De Smedt *et al.*, 1994).

The oocyte undergoes significant changes while enclosed within the follicle, especially when it becomes dominant and approaches ovulation. These changes have been

termed as 'oocyte capacitation' or 'prematuration' and play a key role in the oocyte acquisition of developmental competence (Hyttel *et al.*, 1997; Dieleman *et al.*, 2002). The final phase of oocyte maturation within the ovulatory follicle is initiated by the pre-ovulatory surge of luteinising hormone (LH), which triggers resumption of meiosis and its progression to MII, at which stage it is ovulated. It is well known that removal of the oocyte from the follicle and placement in culture results in spontaneous resumption of meiosis (Pincus and Enzmann, 1935). Fully grown of goat oocytes are able to resume meiosis *in vivo* after the LH surge or spontaneously after their release from the follicle and subsequent *in vitro* culture (Chang, 1951; Edwards, 1965; Gilchrist and Thompson, 2007). Oocytes recovered from large antral follicles develop to blastocysts at significantly higher rates than those from smaller follicles (Pavlok *et al.*, 1992; Lonergan *et al.*, 1994; Crozet *et al.*, 1995), suggesting that the stage of follicular development at which the oocyte is removed is important in determining its ability to develop into a transferable embryo (Hyttel *et al.*, 1997; Dieleman *et al.*, 2002). Furthermore, Machatkova *et al.* (2000) who utilised the growth phase of the first follicular wave to obtain oocytes of high developmental competence. In addition, a higher proportion of *in vivo* matured oocytes reach the blastocyst stage when compared to their *in vitro* matured counterparts (Greve *et al.*, 1987; Marquant-Le *et al.*, 1989; Van de leemput *et al.*, 1999; Dieleman *et al.*, 2002; Rizos *et al.*, 2002a), which is almost certainly related to the quality of the oocytes at the outset of IVM (Cognie *et al.*, 2003). Acquisition of oocyte developmental competence occurs continuously throughout folliculogenesis, and the influence of follicle size and follicle atresia on developmental competence (Mermillod *et al.*, 1999).

Oocyte competence is acquired during the growth phase when the synthesis and storage of proteins, ribosomal as well as heterogeneous ribonucleic acid (RNA) take place (Crozet *et al.*, 1981); and implies its ability to complete not only nuclear maturation but also cytoplasmic changes needed to maintain embryo development. These cytoplasmic changes

include protein and RNA storage; development of calcium regulatory mechanisms; changes in the activity of maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK); and redistribution of cellular organelles. Naito *et al.* (1992) suggested that MPF plays an important part in the regulation of cytoplasmic maturation. Oocytes in meiotic arrest (GV) display relatively low activity of MPF. MPF activity then appears just before GVBD and increases until MI and is followed by a sudden decrease in oocytes releasing the first polar body (Hashimoto and Kishimoto, 1988; Choi *et al.*, 1991; Dekel, 1996; Zernicka-Goetz *et al.*, 1997); and increases again at MII as well as remains high until fertilisation in goat (Dedieu *et al.*, 1996; Eppig, 1996). The above studies show that the final steps of oocyte capacitation and maturation are critical for subsequent embryonic development but the relative importance of each of these steps is still unknown.

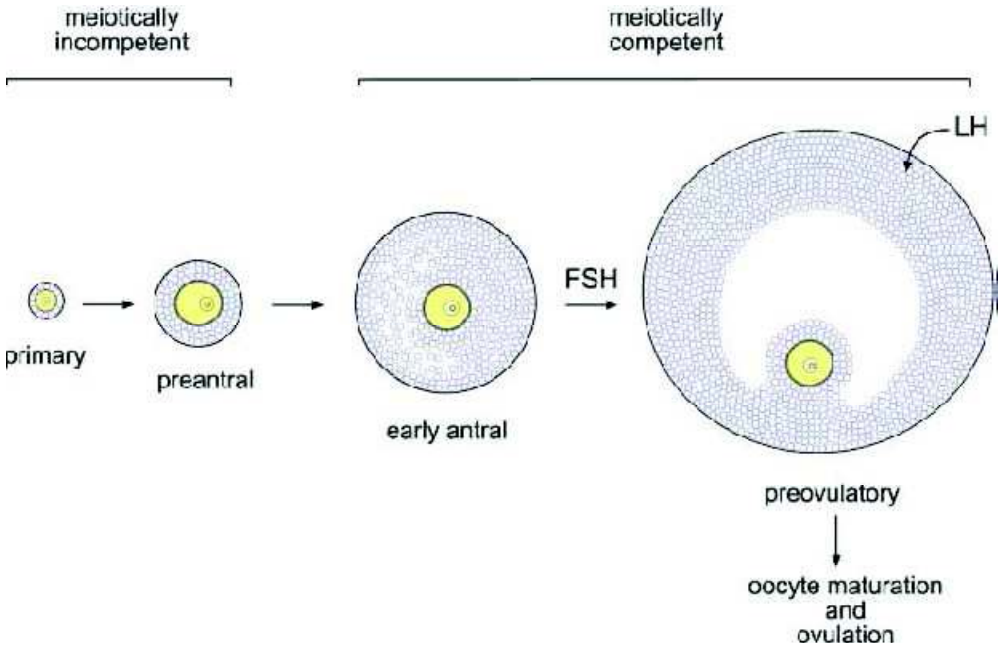

Figure 2.1: Development of the mammalian ovarian follicle. Follicles consisting of somatic cells (blue) surrounding an oocyte (yellow), grow within the ovary. Oocytes within the smallest (primary and preantral) follicles are meiotically incompetent and will not mature spontaneously if removed from their follicles. At the early antral stage, the oocytes acquire meiotic competence and are able to mature if isolated from their follicles. At this stage, follicles are recruited for further growth by FSH. The follicle enlarges and develops LH receptors on the outermost, mural granulosa cells. A surge of LH stimulates the oocyte to resume meiosis, as well as ovulation of the mature oocyte. Adapted from Mehlmann (2005).

2.4.2 Nuclear and Cytoplasmic Maturation

Oocyte maturation is defined as reinitiation of the first meiotic division, progression to MII and the accompanying cytoplasmic processes occurring within the oocyte that are essential for fertilisation and that support early embryo development (Ferreira *et al.*, 2009). It is a long process which oocytes acquire their intrinsic ability to support the subsequent stages of development and ultimately reaching activation of the embryonic genome. This process involves complex and distinct events of nuclear and cytoplasmic maturation. Nuclear maturation mainly involves chromosomal segregation, whereas cytoplasmic maturation involves organelle reorganisation and storage of messenger RNA (mRNA), proteins and transcription factors that act in the overall maturation process, fertilisation and early embryogenesis (Sirard, 2001). The transcripts and proteins stored in the cytoplasm of the oocyte are of fundamental importance for the maturation process and for ensuring the progression of early embryo development to the 8-cell stage, when the embryonic genome is activated and the synthesis of new proteins becomes necessary. This phase is denoted as embryonic genome activation (EGA) and the expression of certain genes during this period will determine the success of embryogenesis in the pre-implantation stage (Meirelles *et al.*, 2004).

Nuclear maturation refers to the ability of the oocyte nucleus to progress from the GV stage to MII stage of meiosis. Sui *et al.* (2005) found that the configurations of GV chromatin in goat differ from those of other species in that the chromatin did not condense into a perinucleolar ring. Configuration of GV chromatin has been found that associated with the developmental competence of oocytes. They classified GV chromatin of goat oocytes based on the size of nucleoli and the degree of chromatin condensation; as GV1: large nucleoli and diffuse chromatin; GV2: medium-sized nucleoli and condensed net-like or clumped chromatin; GV3: small nucleoli and net-like or clumped chromatin and; GV4: no nucleolus

but clumped chromatin. A large variation exists in the GV stage (among 1 to 4) of oocytes retrieved from a range of antral follicles both at the time of collection and after defined period of IVM (Grupen *et al.*, 1997a; Nagai *et al.*, 1997; Funanhashi *et al.*, 1997). This variation appears to be a result of difference in GV status in oocytes obtained from different follicle sizes within the aspiration range (McGaughey *et al.*, 1979; Grupen *et al.*, 1997a). Continued asynchronous meiotic progression throughout IVM gives rise to a population of aged oocytes which have different developmental abilities following embryo IVP (McGaughey and Polge, 1972; Motlik and Fulka, 1976; Funahashi and Day, 1993; Ocampo *et al.*, 1993; Christmann *et al.*, 1994; Grupen *et al.*, 1997a).

Cytoplasmic maturation can be described as processes modifying the oocyte cytoplasm that are essential for fertilisation and pre-implantation embryonic developmental competence. Mechanisms that have been implicated in cytoplasmic maturation include the occurrence of stage-specific processes, such as the synthesis of specific proteins (Schultz and Wasarman, 1977), the ability to release cortical granules (Abbott *et al.*, 1998), to release calcium from intracellular stores (Carroll *et al.*, 1996), to relocalise mitochondria (Van Blerkom and Runner, 1984) and to decondense sperm head (Usui and Yanagimachi, 1976; Thibault, 1977), which significantly contribute to success fertilisation. Cytoplasmic maturation is acquired in a step-wise manner (Eppig and Schroeder, 1989), requiring complete antral development (Ceconi *et al.*, 1996) and maintenance of functional gap junctions between somatic and germ cells (Buccione *et al.*, 1990; Ceconi *et al.*, 1996; Eppig *et al.*, 1996). The cumulus cells play an important role in cytoplasmic maturation, with increased number of cumulus cell layers and cumulus-oocyte complex (COC) compactness pre-IVM correlated with improved developmental outcome (Shioya *et al.*, 1988; Abeydeera, 2002).

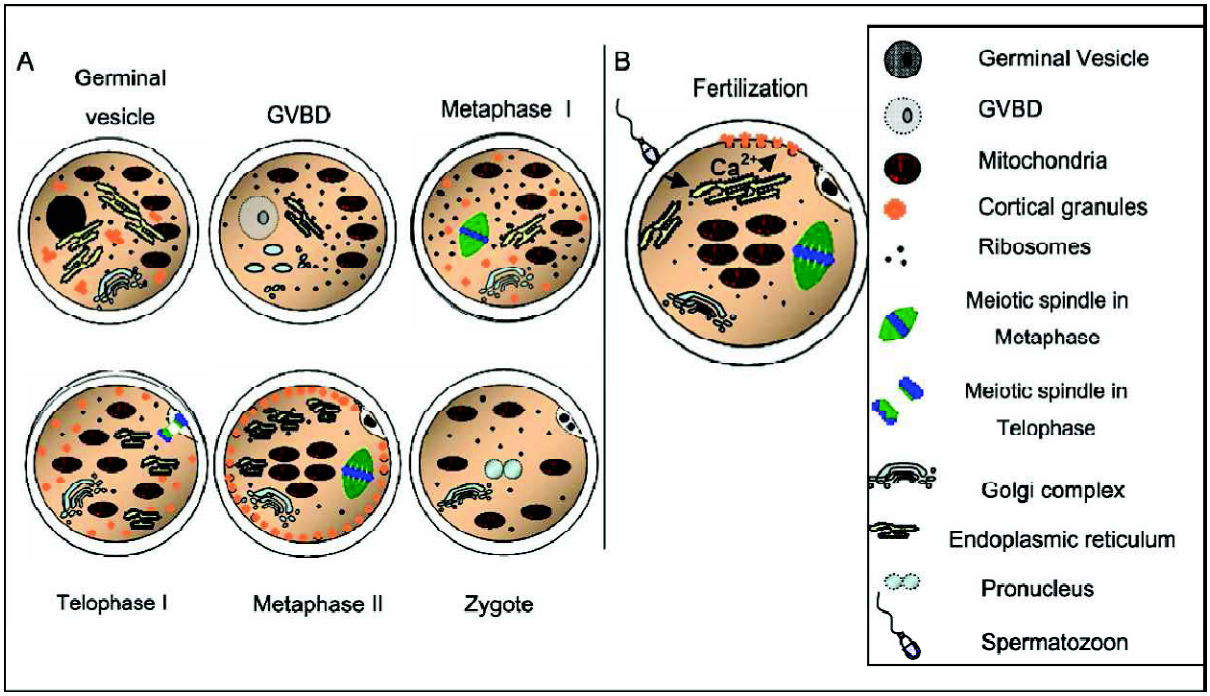

Figure 2.2: Schematic overview of the distribution of cytoplasmic organelles during maturation, fertilisation and zygote formation. A. Nuclear maturation progression and cytoplasmic organelle movement from the immature stage of germinal vesicle to the mature stage of metaphase II and zygote formation. B. Organelle distribution and the mechanism of cortical granule content release, secondary to intracellular calcium (Ca^{2+}) release after the entry of the sperm into the oocyte during fertilisation. Adapted from Ferreira *et al.* (2009)

2.4.3 Factors Affecting IVM of Goat Oocytes

The IVM oocytes has been reported to be affected by several factors such as donor age (Izquierdo *et al.*, 2002), follicle size (Pavlok *et al.*, 1992; Blondin and Sirard, 1995), oocyte diameter (Hyttel *et al.*, 1997), developmental stage of oocyte (Hagemann *et al.*, 1999), composition of media (Lonergan *et al.*, 1997), hormones (Zuelke and Brackett, 1990) and serum (Avery *et al.*, 1998). The rate of oocyte maturation has implications for subsequent embryo development (Dominko and First, 1992; Rajikin *et al.*, 1994; van der Westerlaken *et al.*, 1994; Rajikin, 1995; Teotia *et al.*, 2001). Inappropriate timing of maturation could lead to the formation of abnormal chromatin (Dominko and First, 1997), oocyte aging (Hunter, 1989; Hunter and Greve, 1997) and impaired development (Marston and Chang, 1964).

2.4.3.1 Donor age

Meiotic competence is acquired when oocytes have accumulated cell cycle regulating molecules in sufficient amounts to enable resumption of meiosis. It is closely correlated to oocyte size, which in turn is correlated with follicle size. Since follicles of prepubertal animals are usually smaller than those of adults, and thus it is difficult to separate donor age factor from the effects on oocyte competence, which related to follicle size. Cytoplasmic factors play roles in cell cycle regulation including regulation of meiosis (Balakier and Czolowska, 1977), and hence can contribute to failure of meiotic maturation in oocytes that have otherwise reached meiotic competence. Cytoplasmic deficiencies also are expressed as failure of sperm penetration and decondensation, inability to form male pronuclei (MPN), failure of block to polyspermy, early cleavage failure, failure to reach or survive the transition from maternal to embryonic genomic expression, and developmental failure leading to embryonic losses at later pre-implantation and post-implantation stages of development (Armstrong, 2001).

Several studies have demonstrated that the developmental capacity of embryos from prepubertal goats after IVM and IVF is similar to those from adult goats but were unable to proceed in development resulting in low blastocyst rate (Martino *et al.*, 1994, 1995; Izquierdo *et al*, 1999). Other studies also demonstrated the inferior developmental capacity of the oocytes from prepubertal animals not only after superovulation treatment (Wright *et al.*, 1976; Pinkert *et al.*, 1989; French *et al.*, 1991), but also after IVM (Kajihara *et al.*, 1991; Levesque and Sirard, 1992; Torner *et al.*, 1992; Palma *et al.*, 1993; Thonon *et al.*, 1993). This may be attributed to the presence of an abnormal protein profile (Levesque and Sirard, 1994), incomplete or delayed IVM (Damiani *et al.*, 1995; Kuhholzer *et al.*, 2001; Salamone *et al.*, 2001), lack of sensitivity of the oocyte to its hormonal microenvironment (Levasseur, 1979), high rates of polyspermy (Palomo *et al.*, 1999); failure of sperm head decondensation and

MPN formation (Mogas *et al.*, 1997b); low blastocyst production rate (Izquierdo *et al.*, 2002) and high percentage of haploid embryos (Villamediana *et al.*, 2001) in oocytes derived from prepubertal goats (Izquierdo *et al.*, 1995). On the other hand, it has reported no differences in the rates of cleavage and blastocyst development between oocytes obtained from prepubertal and adult goats (Mogas *et al.*, 1997a; Koeman *et al.*, 2000).

It has been reported that the differences between prepubertal and adult oocytes in terms of ultrastructure including abnormal chromatin and microtubule configurations (Damiani *et al.*, 1996; de Paz *et al.*, 2001), reduced levels and activity of histone H1 kinase (indicative of MPF activity) and MAPK (Damiani *et al.*, 1998; Salamone *et al.*, 2001), reduced amino acid uptake, metabolism glucose, glutamine and pyruvate (Gandolfi *et al.*, 1998; Steeves and Gardner, 1999), different patterns of mRNA and proteins (Levesque and Sirard, 1994; Khatir *et al.*, 1996, 1998; Gandolfi *et al.*, 1998), and aberrant calcium oscillation post-fertilisation (Damiani *et al.*, 1996, 1998). Therefore, the kinetics of nuclear maturation between prepubertal and adult goat oocytes is needs to be further studied.

2.4.3.2 Follicle size and oocyte diameter

It has been found that a direct positive relationship between follicle diameter, oocyte diameter, meiotic competence and embryo development in goats (Crozet *et al.*, 1995, 2000). It has been demonstrated that oocytes from follicles more 6 mm in diameter produce blastocysts *in vitro* at substantially greater rates than those from 2 to 6 mm follicles (Lonergan *et al.*, 1994), and follicles more 2 mm yield oocytes capable of fertilisation but lack of the ability to cleave beyond the 8-cell stage (Pavlok *et al.*, 1992). In most species, oocytes from follicles less than 3 mm in diameter are not fully meiotically competent, resulting in low rates of MII and have extremely limited ability to support embryo development following IVM (Abeydeera, 2002). Crozet *et al.* (1995) reported that the diameter of follicles has been positively related to

oocyte diameter and to oocyte developmental competence in adult goats, which follicles larger than 5 mm contain oocytes which are more competent to develop up to blastocyst stage. Most of the prepubertal goat ovaries contain a great number of small follicles between 2.5 to 3 mm in diameter (Martino *et al.*, 1994).

Oocyte diameter is a determinant factor in acquiring meiotic competence (Hyttel *et al.*, 1997); and can generally be used as an indicator of oocyte growth, as there is an intensive synthesis of RNA during this phase that causes an increase in size (Crozet *et al.*, 1981; Lazzari *et al.*, 1994; Lonergan *et al.*, 1994). In this regard, smaller oocytes tend to follow an abnormal path of meiotic maturation, resulting in disturbances in the maturation process (Lechniak *et al.*, 2002). In several species, the oocyte ability to resume and complete meiosis *in vitro* has been attributed to the oocyte diameter (Motlik and Fulka, 1986; Durinzi *et al.*, 1995; Fair *et al.*, 1995; Ledda *et al.*, 1999b; Otoi *et al.*, 2000), and the ability to undergo successful fertilisation and embryo development (Moor and Trounson, 1977; Otoi *et al.*, 1997; Lucas *et al.*, 2003). It has been demonstrated that oocytes smaller than 95 µm diameter are unable to resume meiosis *in vitro*, acquiring meiotic competence between 95 to 100 µm diameter. Oocytes of 95 to 104 µm diameter are capable of fertilisation *in vitro*, but have a limited ability to develop to the morula stage. With increasing growth, their ability to develop to the blastocyst stage *in vitro* gradually increases until they reach optimum rates at a maximum diameter of 135 µm (Arlotto *et al.*, 1995; Fair *et al.*, 1995; Harada *et al.*, 1997). In both adult (De Smedt *et al.*, 1994) and prepubertal (Martino *et al.*, 1995) goat oocytes, meiotic oocyte competence has been classified in terms of the oocyte diameter as incompetent oocytes (less than 110 µm), partially competent oocytes (110 to 125 µm) and competent oocytes (125 to 135 µm).

2.4.3.3 Oocyte quality

Oocyte is a complex cell with many organelles, each of which must be in the appropriate state for the maturation of cell (Trimarchi and Keefe, 2006). Any dysfunction or dislocation of oocyte components such as meiotic spindle, cortical granules or mitochondria can decrease the oocyte viability and has a crucial impact on embryo development and quality (Sun *et al.*, 2001b; Coticchio *et al.*, 2004; Combelles and Racowsky, 2005). Oocyte quality or developmental competence is acquired during folliculogenesis as the oocyte grows and during the period of oocyte maturation. Sirard *et al.* (2006) pointed out the influence of the oocytes quality on the resumption of meiosis, cleavage of zygote, embryo development to blastocyst stage, the uterine implantation and healthy offspring birth. Cytoplasm changes which accompany the oocyte growth include mRNA transcription and protein synthesis (Sirard *et al.*, 1989; Kastrop *et al.*, 1991). These processes are necessary for the meiotic maturation of oocyte, activation of the zygotic genome, and blastocyst formation (Barnes and First, 1991; De Sousa *et al.*, 1998).

ARTs involving ovarian stimulation or collection of immature oocytes followed by IVM can perturb this process and reduced oocyte developmental competence. There are a variety of other processes occurring within the cytoplasm of the oocyte that are required for complete developmental competence following fertilisation although nuclear maturation may be completed successfully. Successful completion of these events is independent of nuclear maturation and is collectively referred to as cytoplasmic maturation (Sun and Nagai, 2003; Krisher, 2004). An oocyte that has not completed cytoplasmic maturation is of poor quality, and thus unable to successfully complete normal developmental processes (Vassena *et al.*, 2003). However, the cellular mechanisms that impart oocyte developmental competence are entirely unclear. Any effort to use ARTs in the IVEP programmes will be inefficient at best until the mechanisms involved in oocyte quality are elucidated.

For IVEP, prospective detection of competent oocytes is critical. Quality of goat oocytes are selected using vague criteria such as cumulus investment surrounding the oocytes and uniformity of the cytoplasm (Shioya *et al.*, 1988; Sirard *et al.*, 1988; Mochizuki *et al.*, 1991; Madison *et al.*, 1992; Lonergan *et al.*, 1994; Chaubal *et al.*, 2006), follicle diameter (Crozet *et al.*, 1995; Romaguera *et al.*, 2010), oocyte diameter (Jiménez-Macedo *et al.*, 2006; Anguita *et al.*, 2007) and the brilliant cresyl blue (BCB) test (Rodríguez-González *et al.*, 2002). These shortcomings are the impetus to evaluate the developmental competence of individual oocytes and to establish a more objective indicator for oocyte selection to improve the outcome of IVEP.

Antral follicle contains two anatomically and functionally different types of somatic granulosa cells, which is mural granulosa cells that line the inner layer of the follicle wall; and the cumulus cells which enclose the oocyte (Wassarman, 1988). The oocyte is most sensitive to changes in the cumulus cells since they are in close contact with the oocyte (cumulus-oocyte complex, COC) (Buccioine *et al.*, 1990; Driancourt and Thuel, 1998). Factors secreted by the granulosa cells are deposited in the follicular fluid (FF) surrounding the COC and mediate oocyte growth and survival; and play an important role in follicle growth. Several studies showed that gap junctional communication (GJC) from the major anchorage between the oocyte and cumulus cells during all stages of follicle development, which the cumulus cells transmit the signals to the oocyte (Coskun and Lin, 1994). The corona radiata layer of cumulus cells extends cytoplasmic processes through zona pellucida to form gap junctions with the oocyte surface (Anderson and Albertini, 1976; Gilula *et al.*, 1978). These interactions enable the follicular cells to supply the maturing oocyte with nucleotides, amino acids and phospholipids, while maintaining ionic balance and mRNA stability (Gilula *et al.*, 1978; Heller and Schultz, 1980). Cumulus cells are known to supply nutrient (Eppig, 1982; Haghighat and Van Winkle, 1990; Laurincik *et al.*, 1992), and/or

messenger molecules for the development of the oocyte (Lawrence *et al.*, 1978; Thibault *et al.*, 1987; Buccione *et al.*, 1990); and to mediate the hormonal support emerged by the action of FSH, which induces meiotic resumption (Zuelke and Brackett, 1990; Fagbohun and Downs, 1991). Visual assessment of morphology features particularly on the cellular vestments and cytoplasm uniformity surrounding an oocyte is the most important criteria for selection goat oocytes before maturation (Hashimoto *et al.*, 1998; Sun *et al.*, 2001a; Tanghe *et al.*, 2002), and the degree of expansion of cumulus cells can be used as a morphological indicator oocyte quality following IVM for successful fertilisation and embryo development (Ball *et al.*, 1983; Chen *et al.*, 1993; Qian *et al.*, 2003; Han *et al.*, 2006). It is reported that the oocyte quality is better when the oocyte is surrounded by more layers of cell (Blondin and Sirard, 1995; Zeuner *et al.*, 2003; Warriach and Chohan, 2004; Yuan *et al.*, 2005, Rahman *et al*, 2007a). Han *et al.* (2006) showed that developmental competence of goat oocytes with the same COC morphology, follicle size and the same grade of cumulus expansion may differ significantly, and thus suggests that the developmental potential of an oocyte is determined by multifactorial interactions.

Table 2.2: Criteria for grading goat COC

Grade	Characteristics
A	COC with more than 5 complete layers of cumulus oophorus and corona radiata (cumulus-corona) cells and finely granulated homogeneous ooplasm.
B	COC with 3 to 5 complete layers of cumulus-corona cells and finely granulated homogeneous ooplasm.
C	COC with 1 to 2 complete layers of cumulus-corona cells or COC with 3 to 5 partially invested cumulus-corona cell layers and finely granulated homogeneous ooplasm.
D	Cumulus-free oocytes (CFO) or oocyte with incomplete investment of cumulus-corona cells (1 to 2 layers) and finely granulated homogeneous ooplasm.
E	Degenerating oocyte or oocyte with abnormal size, shape and heterogeneous ooplasm or apoptotic oocytes in jelly-like cumulus-corona cells investment.

Adapted from Rahman *et al.* (2007a)

Crozet *et al.* (1995) concluding that follicles larger than 5 mm contain oocytes which are more competent to develop up to blastocyst stage, in which, oocytes could have enough time to be submitted to pre-maturation processes and to acquire a complete developmental

competence. Anguita *et al.* (2007) observed a higher blastocyst rate in oocytes larger than 125 to 135 µm prior to IVM, whereas oocyte smaller than 125 µm were only able to develop to morula. It has been demonstrated that BCB test is a useful tool and can be used for the selection of more developmental competent goat oocytes (Rodríguez-González *et al.*, 2002, 2003; Urdaneta *et al.*, 2003b; Jimenez-Macedo *et al.*, 2006; Katska-Ksiazkiewicz *et al.*, 2007). The intracellular activity of glucose-6-phosphate dehydrogenase (G6PD), an enzyme synthesized in the first-half of the S-phase during the oocyte growth phase can determine through BCB stain (Wassarman, 1988). G6PD activity plays a critical role in cell growth by providing NADPH for redox regulation (Tian *et al.*, 1998). BCB is a blue compound reduced to a colourless one by G6PD activity. Oocytes that have finished their growth phase will show decreased G6PD activity indicating complete oocyte maturation whereby will exhibit blue colouration because BCB will remain unreduced state in the ooplasm (Rodríguez-González *et al.*, 2002). Increased G6PD enzymatic activity has been related to deleterious effects on maturation and fertilisation of oocytes (Ericson *et al.*, 1993).

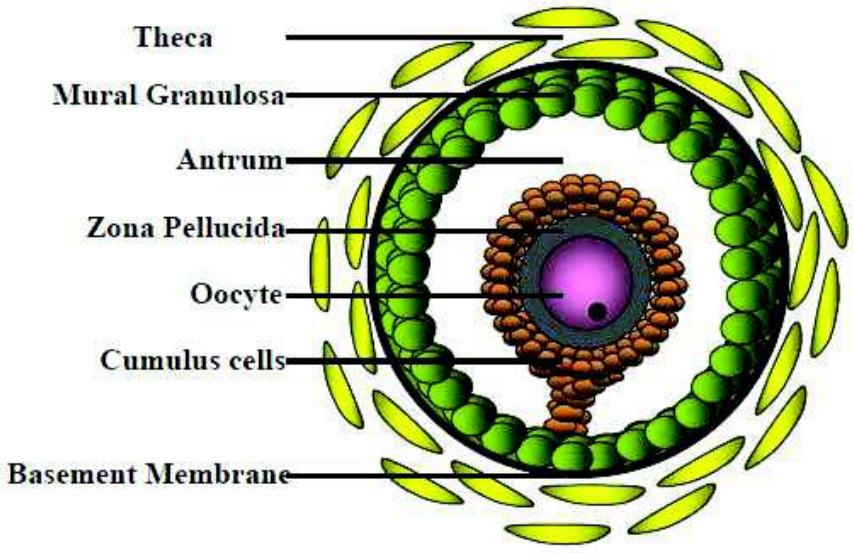

Figure 2.3: Schematic representation of the antral follicle components. Adapted from Wassarman (1988).

2.4.3.4 IVM duration

The duration of IVM may play a critical role for subsequent development since an inappropriate timing of maturation results in abnormal chromatin (Dominko and First, 1997), oocyte aging (Hunter, 1989; Hunter and Greve, 1997) and reduced development (Marston and Chang, 1964). It has been demonstrated that the time necessary for goat oocytes to reach metaphase II is about 27 hours, independent of the physiology of the animals (Martino *et al.*, 1994; Song and Iritani, 1987; Le Gal *et al.*, 1992; Rho *et al.*, 2001). Furthermore, peak MII of goat oocytes was reported at 27 hours when follicles were stimulated by gonadotrophin treatment (De Smedt *et al.*, 1992). On the other hand, Younis *et al.* (1991) reported that the 24 hours of IVM duration is the best time for goat oocyte maturation *in vitro*. Sharma *et al.* (1996) and Yadav *et al.* (1997) found that the optimal duration of IVM for goat abattoir-oocytes was 32 and 30 hours, respectively, in a study on the basis of sequential configuration of chromosomes.

The ability for goat oocytes to achieve meiotic maturation is acquired in follicles larger than 2 mm in diameter (De Smedt *et al.*, 1992; Crozet *et al.*, 1995; Gall *et al.*, 1996). Oocytes that are derived from smaller follicles may require longer maturation time (Qian *et al.*, 2001), which may promote full meiotic competence and confer to a larger number of oocytes the ability to resume meiosis and complete meiotic maturation (Crozet *et al.*, 2000). In general, oocyte have already grown beyond the same point for synthesizing some specific RNAs and protein before being isolated from the follicles, to acquire full competence to support the development of oocyte fertilised *in vitro*. Sharma *et al.* (1996) demonstrated that M1 arrest in some goat oocytes observed up to 36 hours may be related to the different developmental stages of oocytes before mature *in vitro* (Yadav *et al.*, 1997; Crozet *et al.*, 2000). A synchronous oocyte nuclear maturation and steroidogenesis in the majority of the follicles is present when there is a normal periovulatory peripheral pattern of progesterone

and LH in plasma, whilst asynchronous oocyte nuclear maturation (premature activation or meiotic arrest), is concurrently with abnormal follicular fluid endocrinology (Callesen *et al.*, 1986). High concentrations of intrafollicular steroids (oestradiol and progesterone) are associated with a higher incidence of premature condensation of chromatin in goat oocytes (Kumar *et al.*, 1992). However, it has been reported that prolongation of metaphase II arrest before insemination leads to a gradual loss of successful fertilisation and embryo development (First *et al.*, 1988), and an inappropriate time point of insemination can also lead to impaired development (Marston and Chang, 1964).

The different oocyte maturation time-scale may be accounted for by difference in media, serum percentage, volume of the media used and the culture system (Baldassarre *et al.*, 1996; Samake *et al.*, 2000; Teotia *et al.*, 2001; Bormann *et al.*, 2003; Cognie *et al.*, 2003). It has been reported that there is an increase of oocytes with abnormal chromosome constitution probably due to a change in pH, osmolarity or accumulation of toxic materials in the media during later stages of maturation (Shea *et al.*, 1976; McGaughey, 1977; Deb and Goswami, 1990). Furthermore, variations in the timing of the oocyte maturation process *in vitro* could be probably due to oocyte quality, which likely to be affected by age of the donor goat (Izquierdo *et al.*, 2002; Koeman *et al.*, 2003).

2.4.3.5 IVM media

Embryo development is influenced by events occurring during oocyte maturation. For successful IVM, oocytes must undergo synchronically nuclear and cytoplasmic maturation. Immature goat oocytes are conventionally matured in buffered TCM199 supplemented with L-glutamine, pyruvate, hormones (FSH, LH and 17β-oestradiol) plus serum (10 to 20%) (Mogas *et al.*, 1997a).

Gonadotrophins are the primary regulators of nuclear maturation in mammalian oocyte *in vitro* (Moor and Trounson, 1977). Oocytes matured *in vitro* in the presence of gonadotrophins and oestradiol showed high maturation and fertilisation rates compared with those matured without hormones (Saeki *et al.*, 1990; Galli and Moor, 1991; Totey *et al.*, 1992; Ladda *et al.*, 1997). Gonadotrophins alter the metabolism of the cumulus cells and induce resumption of meiosis in the oocytes (Salustri and Siracusa, 1983) by interrupting the mode of inhibitory substances through the gap junctions (Ball *et al.*, 1983). Addition of LH for IVM enhanced the quality of oocytes which was reflected in an increased embryo yield after IVC. FSH was found to enhance early embryo development rather than meiotic maturation (Eyestone and Boer, 1993). Oestradiol improves the completion of maturational changes including the synthesis of presumed male pronucleus growth factor (MPGF) (Moor, 1978; Thibault *et al.*, 1975).

Maturation media for goat oocytes are generally supplemented oestrus goat serum (EGS) (Pawshe *et al.*, 1996; Tajik and Esfandabadi, 2003), oestrus sheep serum (ESS) (Tajik and Esfandabadi, 2003), foetal bovine serum (FBS) (Martin-Lunas *et al.*, 1996), calf serum (Crozet *et al.*, 1993); and combination of bovine serum albumin and EGS (Rajikin *et al.*, 1994) are used by several laboratories (Cognie *et al.*, 2003). Oestrus serum was found to be necessary for FSH-induced cumulus expansion in goat (Keskintepe *et al.*, 1994b; Mogas *et al.*, 1992, 1993; Pawshe *et al.*, 1994b,c). Tajik and Esfandabadi (2003) obtained high maturation rates of goat oocytes after 24 to 25 hours of culture in IVM supplemented with either 10% of EGS, ESS or FCS, and did not find differences among the serum.

Follicular fluid (FF) recovered from non-atretic (Cognie *et al.*, 1995) or gonadotrophin-stimulated follicles (more than 4 mm) (Cognie and Poulin, 2000) can be used as a supplement in maturation medium, which had beneficial effect on goat oocyte maturation (Cognie *et al.*, 2003). Follicular cells are reported to provide nutrients for the oocyte and

energy by the granulosa cells (GC). It has been shown that GC interactions with COC during IVM are involved in imparting developmental competence to the maturing oocytes (Crister *et al.*, 1986; Barboni and Mattiali, 1996). These cells initiate protein and/or polypeptide synthesis which render the cytoplasm competent to assume normal cooperation with the male genome (Thibault *et al.*, 1987). Tyagi *et al.* (1997) reported that GC monolayer delays maturation of goat oocytes for a few hours; but improves ooplasmic maturation (Teotia *et al.*, 2001) and increases the maturation rate. The secretions of GC may promote the synthesis of oocyte glutathione. Glutathione (GSH) has important roles to play in male pronuclear formation (Calvin *et al.*, 1986) and early development and the levels increase during maturation and decreases during fertilisation and embryo development (Yoshida *et al.*, 1993). GSH participates in various mechanisms such as amino acid transport, protein synthesis, reduction of disulphides and protection against oxidative damage (Urdaneta *et al.*, 2003b).

The supplementation cysteamine to the IVM media improve embryo development rates, increases intracytoplasmic GSH concentration and protects cells from culture oxidative stress. Cysteamine increased intracytoplasmic GSH and embryo development in prepubertal goat oocytes (Rodriguez-Gonzalez *et al.*, 2003b). Supplementation with 100 µM of cysteamine improved embryo yield in oocytes from adult (Cognie *et al.*, 2003) and prepubertal goats (Urdaneta *et al.*, 2003b).

Antibiotics are frequently added to embryo culture media to prevent bacterial and fungal contamination. Penicillin and streptomycin are most commonly used but gentamicin is an alternative (Zhou *et al.*, 2000). It has been demonstrated that streptomycin interfered with the maturation of immature goat oocytes but did not affect the subsequent development of mature goat oocytes. In media for somatic cell culture, streptomycin or streptomycin plus penicillin retard or depress protein and DNA synthesis (Amonn *et al.*, 1978; Moss *et al.*, 1984), and it is probable that this is the case also in cultures of immature goat oocytes.

Gentamicin is known to be stable over a wider pH range than either penicillin or streptomycin and maintains its biological activity in the presence of serum (Schafer *et al.*, 1972). Kang *et al.* (2004) suggested that gentamicin is the antibiotic of choice for preventing bacterial contamination of goat oocyte maturation media.

2.5 INTRACYTOPLASMIC SPERM INJECTION (ICSI)

The ICSI technique has been used successfully in humans in order to solve male infertility throughout the world since it was first introduced clinically in 1992 (Palermo *et al.* 1992), as a treatment of last resort for men with extremely severe oligo- and asthenozoospermia but also as a 'more efficient' treatment in less severe cases in which standard IVF could be envisaged (Tesarik, 1996). It is even possible to use epididymal and testicular sperm with similar results (Nagy *et al.* 1995; Shulman *et al.* 1999; Vernaeve *et al.* 2003). However, ICSI remains a low efficiency technology in comparison with alternatives such as *in vitro* fertilisation (IVF) and its application is less widespread in domestic animals (Younis *et al.*, 1989; Goto *et al.*, 1990; Keefer *et al.*, 1990; Meintjes *et al.*, 1996; Squires *et al.*, 1996; Dell *et al.*, 1997). Among the domestic animals, most of the ICSI studies are conducted in cattle followed by pig and sheep, however, there is paucity of information with regards to ICSI research attempts in goats; and the success are limited with only one live birth kid (Wang *et al.*, 2003).

2.5.1 Origin and Application of ICSI

Micromanipulation assisted fertilisation technology has significantly improved and it is now possible to circumvent the oocytes barriers to sperm penetration and fusion. Gordon and Talansky (1986) demonstrated that an artificial opening (zona drilling) introduced in the zona pellucida before insemination enhanced fertilisation by allowing the sperm greater access to

the oocyte. This concept led to the technique of partial zona dissection (PZD), and eventually the first live birth from assisted fertilisation in the mouse (Gordon and Talansky, 1986) and human (Malter and Cohen, 1989). However, PZD has not been widely used because it requires a relatively high number of progressively motile sperm and moreover, the incidence of polyspermy was high even few sperm were used. Therefore, the microinjection of sperm into the perivitelline space (subzonal sperm insertion, SUZI) was developed. This technique led to the birth of live offspring in the mouse and human (Ng *et al.*, 1988), but the incidence of monospermic fertilisation was not high as expected, demonstrated again the limitations of SUZI. To overcome these limitations, microinjection of single sperm into the ooplasm of metaphase II (MII) oocyte (intracytoplasmic sperm injection, ICSI) was developed. The ICSI technique can bypass the process of sperm penetration of cumulus cells, corolla oophorus, zona pellucida and oolemma during fertilisation; and it is effective for functional disorders such as disorders of capacitation, acrosome reaction and sperm-egg fusion (Van Steirtegehem *et al.*, 1993, 1996). This technique was reported with success for the first time in hamsters 30 years ago (Uehara and Yanagimachi 1976). Since Uehara and Yanagimachi (1977) obtained the first hamster born by ICSI, other live births have been obtained with this fertilisation technique, such as human (Palermo *et al.*, 1992), mice (Kimura and Yanagimachi, 1995), cattle (Goto *et al.*, 1990), sheep (Catt *et al.*, 1996), horse (Cochran *et al.*, 1998), pig (Martin, 2000) and goats (Wang *et al.*, 2003). In goats, the first study using ICSI as a fertilisation method with development until blastocyst stage was reported by Keskintepe *et al.* (1997). The first kid born by this method was reported by Wang *et al.* (2003) using a piezo-drill ICSI, Jimenez-Macedo *et al.* (2006) studied different protocols of ICSI to fertilise prepubertal goat oocytes and obtained ICSI-derived blastocyst from oocytes larger than 125 µm of prepubertal goats, and Rahman *et al.* (2009) obtained ICSI-derived morula from different oocyte source.

In laboratory or livestock species, ICSI is used as a reproductive option to solve different problems but the focus is not about low male fertility. As ICSI requires far fewer sperm to fertilise the same number of oocytes than conventional artificial insemination (AI) or IVF, it is potentially valuable in using very valuable semen samples most effectively. Furthermore, the use of sex-sorted semen is becoming more and more popular in the recent years because of their potential application in animal production, and ICSI could be a great help with this type of semen samples where very low numbers of sperm are available and they are mostly of low motility (Iritani, 1991; Rath *et al.*, 1999; Probst and Rath, 2003). This technique may be suitable for cryopreservad oocytes (Fujihira *et al.*, 2004; Rho *et al.*, 2004; Fabbri, 2006) as the problems of polyspermy caused early extrusion of cortical granules in the cryopreserved oocytes (Vincent *et al.*, 1990), and ICSI can bypasses these events.

The ICSI technique has not only applied to rescue infertile male strains but also could be used in livestock species in several areas such as biodiversity conservation, transgenic production (Kurome *et al.*, 2007; Hirabayashi, 2008; Hirabayashi *et al.*, 2008) or to solve fertilisation problems in IVF systems. Some research groups have pursued an alternative procedure to pronuclear injection for producing transgenic animals using sperm as vector to introduce genes (sperm-mediated gene transfer; SMGT) (Lavitrano *et al.*, 2006). The production of transgenic animals by ICSI-SMGT has been achieved in pigs (Lai *et al.*, 2001), mice (Perry *et al.*, 1999; Moreira *et al.*, 2004) and rats (Hirabayashi *et al.*, 2005). New or improved technologies in mammalian transgenesis such as the use of lentiviral vectors (Whitelaw, 2004) and the 'renaissance' of ICSI approaches have shown that generation of transgenic animals now can be made easier (Moreira *et al.*, 2007).

2.5.2 Difference between Normal Fertilisation and ICSI

Fertilisation requires accurate cytoplasmic events mediated by the centrosome, the cell's microtubule organising center (MTOC). The centrosome is a complex organelle composed of many different proteins (Joshi *et al.*, 1992; Stearn *et al.*, 1992; Salibury, 1995) such as γ-tubulin which is important in both microtubule nucleation and in defining the polarity of the assembled microtubules (Joshi *et al.*, 1992). Maternal γ-tubulin is drawn to the sperm centrosome shortly after sperm penetration to assist the formation and subsequent elongation of the sperm aster (Stearn *et al.*, 1992). As these sperm astral microtubules elongate throughout the cytoplasm, they come into contact with the female pronucleus which is then translocated towards the male pronucleus resulting in pronuclear migration and apposition (Wu *et al.*, 1996). Therefore, the centrosome serves a central role in the fertilisation process which microtubule nucleation is necessary to bring about the union of the paternal and maternal genomes in the activated zygote cytoplasm at first mitosis. This crucial event signals the completion of the fertilisation process and has been demonstrated in zygotes (Asch *et al.*, 1995; Simerly *et al.*, 1995; Wu *et al.*, 1996). Furthermore, defects in centrosome function during microtubule elongation may result in the failure of normal fertilisation, and therefore, they suggesting a link between centrosomal function and infertility (Asch *et al.*, 1995; Simerly *et al.*, 1997).

The similarities between IVF- and ICSI-derived oocytes in terms of their cytoskeletal dynamics suggest that an injected sperm behaves in much the same manner as a sperm which undergoes oolemma binding and fusion. However, DNA synthesis and pronuclear migration can be delayed by several hours after ICSI; conversely, after IVF, pronuclear migration has been completed within 12 hours post-insemination and DNA synthesis is detected in both pronuclei. Furthermore, sperm decondensation is altered after the injection of sperm

(Hewitson *et al.*, 1996; Sutovsky *et al.*, 1996) and it may prevent the import of maternal nuclear proteins during the process.

On the other hand, the sperm plasma membrane and contents of acrosome never enter the oocyte; and the sperm nucleus is incorporated into the oocyte through membrane fusion between two gametes during normal fertilisation. In contrast, the entire sperm plus a small amount of extracellular medium are deposited in an oocyte during ICSI. Obviously, ICSI oocyte must do extra work that normally fertilised oocyte do not need to do. This includes the 'elimination' of sperm plasma and acrosomal membranes, various macromolecules added onto the sperm plasma membrane during epididymal maturation and ejaculation, acrosomal contents with a spectrum of hydrolysing enzymes and glycoproteins, and medium components co-injected with sperm during ICSI, such as polyvinyl pyrrolidone which makes sperm within the injection pipette more manoeuvrable) (Yamagimachi, 2005). Therefore, it is quite remarkable that ICSI oocytes survive the operation and develop in to live offspring. It is also somewhat astonishing that the pattern of intracellular Ca^{2+} oscillations following ICSI is quite different from that after normal fertilisation, yet, ICSI oocytes, if not all, develop into apparently normal offspring.

2.5.3 Factors Affecting ICSI Produced Goat Oocytes

To date, ICSI has so far had only limited success in goat and only a single goat kid has been born after this technique was used. Therefore, some important factors affecting ICSI outcomes are described mostly based on other species ICSI and it is highly probable that this is the case also in goats.

2.5.3.1 Effect of goat oocyte

Many of the events that prepare the female gamete for fertilisation and make it capable of supporting the initiation of embryonic development take place during oocyte maturation (Gioia *et al.*, 2005). After sperm penetration, the sperm undergoes chromatin decondensation, nuclear swelling and pronuclear formation. During this sperm chromatin structure reorganisation and particularly during protamine-histone replacement, profound DNA epigenetic modifications take place. In the same mature ooplasm and at the same time, the maternal genome is also modified and prepared for integration with the paternal genome. The ability of the oocyte to carry out the correct genome remodelling after fertilisation therefore represents a solid opportunity to characterise oocyte maturation (Gioia *et al.*, 2005). In general, this characterisation takes place on *in vitro* matured oocytes since their production is less costly and time-consuming than that of *in vivo* matured oocytes, which are less readily available.

During meiotic arrest, the nuclear status and morphology of MII oocytes do not change. However, cytoplasmic changes occur when the arrest period is prolonged (Chian *et al.*, 1992). Kikuchi *et al.* (2000) observed an increased likelihood of parthenogenetic activation by aged oocytes that could be attributed in part to the gradual decrease of MPF activity in oocytes during prolonged culture. Bai *et al.* (2006) also performed an experimental design reconstructing oocytes with MII chromosome spindle and cytoplasm from aged and fresh oocytes by the use of nuclear transfer. They showed that in *in vitro* aged oocytes, loss of developmental potential after parthenogenetic activation and IVF was mainly due to cytoplasmic deficiencies rather than nuclear deficiencies. The age of oocyte can affect embryo viability after ICSI; delayed injection may lead to oocyte aging and failure of male pronucleus formation, thus reducing the fertilisation rate (Zheng *et al.*, 2004) or lower embryo development after somatic cell nuclear transfer (Cervera and Garcia-Ximenez, 2003).

In addition, duration of *in vitro* maturation process could affect fertilisation rates after ICSI (Garcia-Rosello *et al.*, 2006a) and further embryo development.

However, several ICSI studies suggested incomplete terminal differentiation of the oocyte as the main cause for the low capacity of *in vitro* matured oocytes developed after fertilisation (Probst and Rath, 2003; Garcia-Rosello *et al.*, 2006a). In order to solve these *in vitro* maturation problems, some strategies have been developed. Chemically defined media have been developed to contribute to an understanding of the basic molecular mechanism of embryonic development. It has been reported that embryos derived from oocytes matured in a defined medium based on TCM199 containing cysteine and EGF developed up to the blastocyst stage but at a lower efficacy than in a serum supplemented medium (Kishida *et al.*, 2004). Furhtermore, Kobayashi *et al.* (2006) demonstrated that the addition of cysteine or β-mercaptoethanol to a defined maturation medium can enhance blastocyst formation after ICSI.

It has been demonstrated in many species that a critical oocyte size is necessary for the resumption of meiosis and maturation. In a study of oocytes from goats aged 1 to 2 months (prepubertal phase) with IVM and IVF, Anguita *et al.* (2007) demonstrated that oocyte diameter was positively correlated with the percentage of oocytes that reached MII and with the percentage of fertilised oocytes that developed beyond the blastocyst stage. Oocyte diameter was also positively correlated with the intracellular amount of the p34 protein and with the degree of activity of maturation promoting factor. The highest percentage of blastocysts (12%) was obtained from oocytes with a diameter of more than 135 µm on the day of retrieval, which originated from follicles measuring 2 to 3 mm in diameter. Some studies have been undertaken to determine the relationship between follicle diameter and the competence of the oocyte to develop up to the blastocyst stage. Jimenez-Macedo *et al.* (2006) demonstrated a positive correlation between oocyte diameter and embryo development in goat ICSI. It has been concluded that the impaired development of embryos

from oocytes of prepubertal goats may be related to oocyte diameter more than to the physiologic condition of the animals. Goat oocytes acquire meiotic competence when their diameter is more than 136 µm both in adult (De Smedt *et al.*, 1994) and prepubertal (Martino *et al.*, 1994) females.

2.5.3.2 Effect of activation treatment of goat oocyte

The success of ICSI depends on the protocol used for each species. In some species, fertilisation and developmental rates obtained with ICSI have been low probably due to inappropriate oocyte activation or sperm capacitation that results in non-pronuclei formation. However, the efficiency of this procedure is still far from expectations in goat. In goat ICSI, oocyte activation plays a key role in the technique success. Mechanical sperm injection into the ooplasm is sufficient in certain species to activate the oocyte in rabbit (Keefer, 1989), hamster (Hoshi *et al.*, 1992), human (Tesarik and Sousa, 1995), mouse (Kuretake *et al.*, 1996) and sheep (Gomez *et al.*, 1998) for further embryonic development. However, it has been demonstrated that some species such as cattle (Keefer *et al.*, 1990; Rho *et al.*, 1998a), pigs (Probst and Rath, 2003) and goats (Jiménez-Macedo *et al.*, 2005) need the aid of chemical activation after ICSI to improve the embryo development. ICSI was combined with chemical activation such as ethanol (Hamano *et al.*, 1999), Ca^{2+} ionophore (Goto *et al.*, 1990; Probst and Rath, 2003), ionomycin alone (Fulka *et al.*, 1991; Rho *et al.*, 1998b); or combined with 6-dimethilaminopurine (6-DMAP) (Fulka *et al.*, 1991; Rho *et al.*, 1998b; Chung *et al.*, 2000), or electrical activation (Prochazka *et al.*, 1993; Lee *et al.*, 2003; Probst and Rath, 2003) in order to improve activation of oocytes. Piezo-drill ICSI was reported to increase both activation and cleavage rates in horses (Choi *et al.*, 2003; Galli *et al.*, 2003), goats (Wang *et al.*, 2003) and cows (Katayose *et al.*, 1999; Wei and Fukui, 2002; Galli *et al.*, 2003).

During fertilisation, sperm entry triggers a series of intracellular calcium oscillations critical to oocyte activation. MPF and MAPK are the most likely targets of calcium-stimulated events because inactivation of these kinases is a prerequisite to the resumption and completion of meiosis, subsequent pronuclear formation and DNA synthesis (Lorca *et al.*, 1993). However, the soluble sperm factors are the only oocyte activation factors except for the physical stimulation of the injection procedure in the case of ICSI (Horiuchi and Numabe, 1999). It is reported that the activation due to the injected sperm alone was sufficient to lower the MPF activity of oocytes after ICSI, however, it was not maintained. Following the elevation of Ca^{2+} concentration in ooplasm, which was induced only by the presence of the injected sperm, MPF activity of oocytes without activation after ICSI was temporarily raised but could not be maintained at low levels, and a shortage of cyclin B which promoting progression toward the first cleavage was apparently inhibited in the ooplasm by the temporary elevation (Fujinami *et al.*, 2004).

In all mammalian species, calcium plays an important role in the intracellular signaling responsible for the initiation and propagation of oocyte activation. It is known that Ca^{2+} ionophore is able to mimic fertilisation very closely by elevating oocyte intracellular Ca^{2+} levels (Jones *et al.*, 1995). The pattern of the increase in intracellular Ca^{2+} in oocytes is reported to differ among the various chemical treatments used to activate the oocytes and facilitate normal fertilisation (Miyazaki *et al.*, 1992; Fissore and Robl, 1993, 1994; Nakada and Mizuno, 1998). For example, reagents like the calcium ionophore A23187, ionomycin and ethanol induce only one increase in intracellular Ca^{2+} during a single treatment, which differs from the repeated spike-like increases in intracellular Ca^{2+} that occur characteristically during normal fertilisation, and was insufficient to completely inactivate the MPF due to reaccumulation of cyclin B (Liu and Yang, 1996), and brought the oocytes to arrest again at the MIII stage (Liu and Yang, 1996; Rho *et al.*, 1998b; Chung *et al.*, 2000). Therefore, these

triggers have been accompanied by other chemicals, such as cycloheximide (CHX) as a protein synthesis inhibitor (Galli *et al.*, 2003) or 6-dimethylaminopurine (6-DMAP) as a protein kinase inhibitor (Rho *et al.*, 1998b; Ock *et al.*, 2003; Oikawa *et al.*, 2005) that can directly or indirectly inactive the MPF without changing the intracellular calcium profiles. it is a histone kinase inhibitor and prevents the reaccumulation of MPF, and this yields an improvement in the efficiency of oocyte activation (Susko-Parrish *et al.*, 1994).

2.5.3.3 Effect of PVP

Polyvinylpyrrolidone (PVP) has been used successfully in ICSI to increase viscosity of sperm solution in order to facilitate the process of sperm immobilisation. PVP is also used as a vehicle facilitating smooth movement of the sperm inside the injection needle, and thus PVP is also injected intracytoplasmically. Sperm are first suspended in a medium containing PVP and a single sperm is chosen and injected into the oocyte together with a small amount of medium in ICSI procedure (Hlinka *et al.*, 1998).

Exposure of sperm to PVP has recently been found to cause submicroscopic changes in sperm structure and the sperm nucleus appeared to be damaged, both in terms of shape and in the texture of the chromatin which was frequently decondensed (Strehler *et al.*, 1998). The PVP-induced sperm nuclear damage may have been due to breakdown of sperm membranes (Strehler *et al.*, 1998). Furthermore, Dozortsev *et al.* (1995a,b) suggested that the presence of PVP in the oocyte caused some delay between sperm injection and the beginning of calcium oscillations after ICSI. Consequently, it is likely that exposure of sperm to PVP may suppress embryonic development. It probably cannot either diffuse out of the oocyte or be readily digested by lysosomal enzymes because PVP is a large polymer (molecular weight, 360,000) (Jean *et al.*, 2001). Thus, it is expected that PVP injected into the oocyte during ICSI will remain there for a prolonged interval. Feichtinger *et al.* (1995) suggested that chromosomal

abnormalities in ICSI-derived pregnancies could be related to the injection of PVP into the oocyte during the ICSI procedure.

Palermo *et al.* (1992) used 10% (w/v) to slow down the sperm for manipulation and injection of a single sperm that resulted in the first ICSI pregnancy. Since then majority of laboratories utilise PVP in ICSI procedure even though there is evidence suggesting that PVP negatively influences intracytoplasmic processes following ICSI, since there is the potential embryonic toxicity may include chromosomal abnormalities (Ashwood-Smith, 1971; Feichtinger *et al.*, 1995) and may interfere with sperm nuclear decondesation (Dozortsev *et al.*, 2005). Although it has been reported that results obtained from PVP-free procedures (Jean *et al.*, 1996; McDermott *et al.*, 1996; Butler and Masson, 1997) were comparable with those using PVP (Van Steirteghem *et al.*, 1995), the extensive application of the PVP-free system is prevented by the fact that the immobilisation of the motile sperm and the injection procedure itself have some difficulties without the presence of PVP during ICSI process. In addition, it has been demonstrated that PVP solution affected the acrosomal status of sperm and enhance pronuclear formation in ICSI (Kato and Nagao, 2009). There are no detailed reports regarding the specific effects of PVP on sperm function and embryonic development in goats, however, all ICSI research in goat used PVP to slow down the sperm for manipulation and injection. Therefore, PVP will still be the substances of choice in ICSI for modulating sperm motility unless its potential harmful effects are proven to be true and safer; as well as better alternative to PVP is found.

2.5.3.4 Effect of sperm immobilisation

Sperm immobilisation before ICSI is considered necessary for efficient fertilisation to occur (Catt and O'Neill, 1995; Fishel *et al.*, 1995; Gerris *et al.*, 1995; Svalander *et al.*, 1995; Van de Bergh *et al.*, 1995; Palermo *et al.*, 1996; Vanderzwalmen *et al.*, 1996). Reported methods

of sperm immobilisation include pipetting (Redgment *et al.*, 1994; Gearon *et al.*, 1995), squeezing; and piezo method (Yanagida *et al.*, 1998). The squeezing method has involved touching (Palermo *et al.*, 1993; Tesarik *et al.*, 1994; Sibler, 1995), rubbing (Atiee *et al.*, 1995; Vanderzwalmen *et al.*, 1996), stroking (Sakkas *et al.*, 1996) or pressing (Dozortsev *et al.*, 1994; Payne *et al.*, 1994; Sibler *et al.*, 1995).

Immobilisation motile sperm by gently touching its tail with the injection needle at the edge of the sperm-PVP droplet prior to ICSI, and thus leads to an increase in the fertilisation rate (Fishel *et al.*, 1995; Van de Bergh *et al.*, 1995; Vanderzwalmen *et al.*, 1996). The sperm flagellum is then crushed by sliding the injection needle on the mid-portion of the flagellum until a kink on the flagellum is seen, and care should be taken not to damage the mid-piece because it contains the male centrosome that contribute a major role in fertilisation. Plasma membrane disruption after immobilisation sperm is to facilitate the release of sperm-associated oocyte-activating factor (SOAF) once the sperm is inside the oocyte, which induces oocyte activation (Miyazaki and Igusa, 1981; Cuthbertson *et al.*, 1981; Dozortsev *et al*, 1994; Tesarik *et al*, 1994; Dozortsev *et al.*, 1997), thus proper immobilisation technique is necessary to ensure the leakage of SOAFs into the ooplasm. This was confirmed in a study by Homa and Swann (1994) who suggested that oocytes are activated at fertilisation by the diffusion of a protein from the sperm into the ooplasm, with subsequent calcium oscillations and hyperpolarization of the oocyte. The activation of the oocyte triggers a series of biochemical processes in the ooplasm and ooplasmic factors such as thiol-reducing agents (Perreaults *et al*, 1984; Homa and Swann, 1994; Van Blerkom *et al*, 1994) are involved directly in the process of sperm chromatin decondensation (Tesarik and Kopecny, 1989; Montag *et al.*, 1992), second body emission, pronuclear formation and exocytosis (Stice and Robl, 1990; Swann, 1990). Alternatively, immobilising the sperm before ICSI may prevent the interference of the sperm with the cytoskeleton and metaphase spindle of the oocyte.

2.5.3.5 Effect of goat sperm

ICSI can produce healthy offspring regardless of concentrations, morphology, and motility of sperm as long as the sperm nucleus has intact genetic integrity (Yanagimachi, 2005). It has been demonstrated that ICSI supports a sperm-associated product activates the Ca^{2+} releasing machinery of the oocyte from inside the oocyte cytoplasm, favouring an inside-out sequence of events to active Ca^{2+} release (Fissore *et al.*, 2002). These Ca^{2+} oscillations activate the oocyte and determine MPF inactivation, sperm head decondensation and, pronuclei formation with the initiation of embryo development. Therefore, it is suggested that signalling events which are important for oocyte activation could be triggered by sperm components in the absence of sperm-oocyte plasma membrane interactions, and supporting the idea that a sperm factor is responsible for the initiation of oocyte activation (Williams, 2002). However, differences in sperm treatments prior to ICSI that may result in different amounts of damage to the sperm plasma membrane have been shown to affect the timing of onset of calcium oscillations, oocyte activation and pronuclear formation after ICSI (Yanagida *et al.*, 1997; Kasai *et al.*, 1999; Garcia-Rosello *et al.*, 2006b; Morozumi *et al.*, 2006).

Under some circumstances, fertilisation fails to occur and the oocyte remains in the MII stage although the sperm is within the oocyte (Ma *et al.*, 1994; Schmiady *et al.*, 1986). The failure of fertilisation after ICSI may result from either the lack or deficiency of activating factors in the sperm or from the lack of ooplasmic factors triggering sperm chromatin decondensation (Van Blerkom *et al.*, 1994; Yanagida *et al.*, 1999). Decondensation of the sperm nucleus and oocyte activation is initiated by mutual interactions of the factors within the ooplasm and sperm sub-membrane components (Perry *et al.*, 1999). The prerequisite of this process is the permeabilisation of the sperm membrane following fertilisation (Kasai *et al.*, 1999). ICSI eliminates sperm membrane breakdown, which occurs during natural fertilisation as sperm penetrate the oolemma, however, the procedures that are

used for sperm capacitation before ICSI are not sufficient in comparison with complex process of capacitation during IVF, where sperm also capacitates passing through several natural barriers (cumulus cells, zona pellucida). The persistence of the acrosome or its substructures over the anterior part of the injected sperm might prevent the import of maternal nuclear proteins during sperm decondensation (Hewitson *et al.*, 2000) and cause the insufficient sperm head decondensation. It has been demonstrated that inappropriate capacitation of sperm before injection that does not allow the release of sperm factors responsible for oocyte activation (Stricker, 1999) and/or results in block of sperm head decondensation. Therefore, a number of treatments have been used for destabilisation of the sperm membrane prior to ICSI, such as freezing and thawing (Catt and Rhodes, 1995; Perreault *et al.*, 1988), crushing the sperm tail with a micropipette (Keskintepe *et al.*, 1997) or laser shot (Montag *et al.*, 2000), and removal of the acrosome and tail by sonication (Goto, 1993; Keefer, 1989).

In order to increase sperm membrane permeabilisation, acrosome reaction and sperm head decondensation following ICSI, sperm have been treated by various chemicals such as heparin (Keefer, *et al.*, 1990; Chen and Seidel, 1997; Wei and Fukui, 1999), caffeine (Gato, 1990; Iwasaki and Li, 1994; Wei and Fukui, 1999) and Ca^{2+} ionophore (Chen and Seidel, 1999; Wei and Fukui, 1999). Furthermore, sperm also have often been pre-treated by various methods including treatment with Triton X-100 (an anionic detergent) that induces membrane damage and dissolves nuclear proteins (Lee and Yang, 2004; Tian *et al.* 2006), using dithiothreitol (DTT) to reduce disulfide bonds (Perreault *et al.*, 1988; Rho *et al.*, 1998a,b; Suttner *et al.*, 2000) or progesterone (Katayama *et al.*, 2002). It has been reported in goat (Jimenez-Macedo *et al.*, 2006, 2007) that sperm must be capacitated to complete nuclear decondensation and pronuclear development following ICSI. In goats, a stronger sperm treatment using heparin plus ionomycin improved *in vitro* fertilisation and embryo

development results, both in adult (Wang *et al.*, 2002) and prepubertal goats (Urdaneta *et al.*, 2004). However, it has demonstrated that species-specific differences in the stability of sperm nuclei exist probably due to the different disulfide bonding in the sperm nuclei (Perreault *et al.*, 1988). These treatments focussed on to the strategy that removal of sperm membranes may improve male nuclear formation and make the sperm-borne oocyte activating factor more easily available to the cytoplasm of the oocyte, could be useful for embryo development of sperm-injected oocytes (Qian *et al.*, 1996; Rho *et al.*, 1998a; Wei and Fukui, 1999; Suttner *et al.*, 2000).

2.5.3.6 Procedural effect of the ICSI technique

Although ICSI is now considered routine, it remains a very demanding technique to master, due partly to its inherent technical difficulty and partly to the heterogeneity of the cases. It is generally agreed that the ICSI procedure is subject to a learning curve (Shen *et al.*, 2003) and that one common technical failure is not depositing the sperm within the oocyte cytoplasm. In this situation, the oocyte membrane may not have been broken during attempts to aspirate the ooplasm into the ICSI needle. Thus, the sperm is deposited next to the membrane so that when the oolemma returns to its original position, the sperm is pushed out into the perivitelline space, or is trapped inside a sac formed by the membrane (Esfandiari *et al.*, 2005). The sperm may also adhere to the tip of the injection needle or remain within the injection needle and be inadvertently pulled out upon withdrawal of the needle from the cytoplasm. The degeneration of oocytes after ICSI is often a result of a fault in the ICSI technique, e.g. an injection pipette that is too large or not sharp enough. Aspiration of the ooplasm is always used to make sure that the oocyte membrane is broken during injection. However, if the ooplasm is aspirated too much, degeneration of the oocyte frequently results. It has been speculated that ICSI might lead to irregular chromosome segregation (Macas *et*

al., 1996; Rosenbusch and Sterzik, 1996). It could be postulated that the cytoskeletal architecture (and in particular the second metaphase spindle) could be disturbed during the ICSI procedure, either by the injection needle itself, or by substances such as culture medium and PVP injected into the oocyte. Injury to the cytoskeleton after ICSI might lead to minor disturbances in the complex machinery need for normal karyokinesis, and that cytokinesis that would lead to abnormal chromosomal division, either immediately or during later divisioins. However, technical features of the injection procedure do not appear to influence the rate of chromosomal abnormalities in embryos (Dumoulin *et al.*, 2000).

It has previously been reported that orientation of the first polar body (PB) of the MII oocyte at the 6 o'clock position during ICSI produces a significantly higher proportion of embryos (Stoddart and Fleming, 1999). Proper orientation of the polar body and needle position are also important since improper positioning can damage or disrupt the metaphase plate during needle entry. In addition, disturbances in the nuclear spindle may dispose oocytes to aneuploidy or maturation arrest. Thus, perturbation of the cytoskeletal integrity of the oocyte may critically influence the fate of the embryo. During ICSI, the location of the first polar body is commonly used as an indication of the spindle position, with the assumption that they are located in close proximity. To avoid damage to the spindle, oocytes are injected at the 3 o'clock position with the first polar body at the 6 or 12 o'clock position (Javed *et al.*, 2010).

2.6 *IN VITRO* EMBRYO CULTURE

The improvement of *in vitro* culture systems are important for production of embryos with high developmental competence that are used in agricultural and biomedical research and animal biotechnology (Hansen and Block, 2004). Embryo biotechnology is also important for accelerating genetic improvement of livestock and for emerging technologies such as somatic

cell nuclear transfer (SCNT) or cloning (Edwards *et al.*, 2003; Faber *et al.*, 2003, 2004). The early embryogenesis is a complex process characterised by the use of maternal proteins and transcripts to supporting the development of the embryo until its genome activation (embryonic genome activation) (EGA) leading to the synthesis of new transcripts and proteins at the right amount and stage of development (Memili and First, 1999). Since EGA sets the stage for later development, aberrations during early embryogenesis can have deleterious effects on the developmental potential and/or the survival of the embryo/foetus (Niemann andWrenzycki, 2000; Khosla *et al.*, 2001; Lazzari *et al.*, 2002; Fleming *et al.*, 2004; Wrenzycki *et al.*, 2005). In fact, embryo mortality still remains as one of the biggest causes of infertility in livestock (Hansen, 2002).

While the intrinsic quality of the oocyte constitutes the primary determinant of blastocyst rate, the post-fertilisation culture environment has the biggest influence on blastocyst quality (Hendriksen *et al.*, 2000; Rizos *et al.*, 2002a; Lonergan *et al.*, 2003; Krisher, 2004). Embryos are susceptible to a wide range of stressors *in vitro* (Lane, 2001; Lane and Gardner, 2005) including inappropriate media formulations, media supplementation, problems in the culture system, technical issues, or lack of appropriate quality control and quality assurance (Gardner, 2004). These stress factors can trigger response mechanisms designed to preserve the homeostatic balance in the embryo. Short-term responses observed include changes in morphology (Pollard and Leibo, 1994; Abe *et al.*,1999; Boni *et al.*,1999; Crosier *et al.*, 2000, 2001; Abe and Hoshi, 2003), cell proliferation and apoptosis (Knijn *et al.*, 2002; Gjørret *et al.*, 2003), metabolism (Khurana and Niemann, 2000; Thompson, 2000; Leese, 2002; Houghton and Leese, 2004), transcriptome (Wrenzycki *et al.*, 1999, 2001, 2005; Rizos *et al.*, 2002b; Fabian *et al.*, 2005; Corcoran *et al.*, 2006; Sagirkaya *et al.*, 2006), and proteome (Katz-Jaffe *et al.*, 2005).

In most mammalian species, the requirements for normal embryo development are not yet defined. The inadequacy of culture conditions leads either to a developmental block or to a loss of viability even though morphological aspects seem to be preserved. Despite the use of various culture media and methods, development of goat embryos is frequently arrested at the 8- to 16-cell stage (Bavister, 1988). In an ideal scheme for *in vitro* embryo development, the culture medium supplements, additives and environment together would offer a milieu in which a matured and fertilised oocyte could cleave and develop up to the morula/blastocyst stage (Bavister, 1988; Thompson, 1996). Embryonic development *in vitro* is affected by several factors such as co-culture with somatic cells (Smith *et al.*, 1992), supplementation with antioxidants (Kitagawa *et al.*, 2004), growth factors (Grupen *et al.*, 1997b) and oxygen tension (Karja *et al.*, 2004; Booth *et al.*, 2005). Moreover, another important factor is embryo density during culture (Lane and Gardner, 1992) which influences the interaction of embryonic factors in the microenvironment. The embryonic factors enhance the development of embryos through the autocrine-paracrine action.

2.6.1 *In Vitro* Culture Media

Different strategies have been undertaken to formulate exclusive media for *in vitro* embryo production (Tervit *et al.*, 1972; Summers and Biggers, 2003). The success in culturing pre-implantation mammalian embryos has been gained throughout the improved knowledge of their nutritional requirements by empirical alterations of culture conditions (Barnett and Bavister, 1996). The composition of the basal culture medium in which embryos are cultured is an important factor in their developmental processes. The choice of media for temporary maintenance of embryos ranges from commercial complex media designed for cell culture such as TCMI99 and Ham's FI0, to very simple formulation, such as the media Chatot-Ziomek-Bavister (CZB) (Chatot *et al.*, 1989) and Synthetic Oviductal Fluid (SOF) (Tervit *et*

al., 1972). It seems that the effect of culture medium, the culture conditions and the use of various cells for co-culture are highly related and the success of the process depends on the combination used (Fukui *et al.*, 1991; Bavister, 1995). Accumulation of embryo toxic metabolites during static culture systems might explain the low quality of *in vitro* produced bovine embryos. Renewal of culture media at 72 hours post-insemination has been suggested in order to meet the dynamic nature of early embryo metabolism (Steeves and Gardner,1999; Leese *et al.*,1998, Leese, 2002, 2003; Houghton and Leese, 2004).

Many different types of semi-defined and defined media including SOF (Tervit *et al.*, 1972; Krisher *et al.*, 1999), CR1aa and CR2 (Rosenkrans and First, 1991), CZB (Ellington *et al.*, 1990), KSOM (Erbach *et al.*, 1994), G1.2 and G2.2 (Gardner, 1994), BECM (Dobrinsky *et al.*, 1996; Lim *et al.*, 1999), G1 (Krisher *et al.*, 1999) and IVD101 (Abe and Hoshi, 2003) have been designed to simplify *in vitro* culture and allow better control of the culture conditions (Marquant-Leguienne and Humblot, 1998). These media may be supplemented with amino acids, which are important regulators of early embryonic development (Kim *et al.*, 1993b; Rosenkrans and First, 1994; Liu and Foote, 1995; Steeves and Gardner, 1999; Rezaei and Chian, 2005), growth factors to improve the development to the blastocyst stage (Lonergan *et al.*, 1996; Palma *et al.*, 1997; Palasz *et al.*, 2000) and implantation rate (Block and Hansen, 2007), gluthathione, superoxide dismutase or taurine, cysteamine and β-mercaptoethanol as antioxidants; chelators such as EDTA (Olson and Seidel, 2000b), or desferrioxamine (Harvey *et al.*, 2007), vitamins (Olson and Seidel, 2000a), and other molecules such as coenzyme Q10, (Stojkovic *et al.*, 1999), sodium citrate and myo-inositol (Holm *et al.*, 1999), hyaluronan (Stojkovic *et al.*, 2002; Palasz *et al.*, 2006), and insulin-transferrin-selenium (ITS) (Palasz *et al.*, 2000).

In vivo, the developing embryo migrates from the oviduct to the uterine lumen where the fluid composition and gas atmosphere are likely different (Fukui *et al.*, 1996; Gandhi *et*

al., 2000; Thompson, 2000; Macklon *et al.*, 2002; Gardner, 2004; Houghton and Leese, 2004; Biggers *et al.*, 2005, 2006). The increased knowledge of early embryo development and maternal reproductive tract micro-environment suggest the use of two media during embryo culture. Sequential media have been developed to respond to the scalable requirement of developing embryos (Bavister, 1995; Gardner and Lane, 1997; Pool, 2004). The design of sequential media resides on the switch from pyruvate metabolite (the preferred nutrient of the early cleavage stage when low levels of oxygen are consumed) to glucose during the post-compaction period, which meets the increased energy demand for blastulation, differentiation, and growth (Houghton and Leese, 2004; Lopes *et al.*, 2007; Harvey, 2007; Donnay *et al.*, 1999; Thompson and Peterson, 2000). Moreover, the amino acids are adjusted at a restricted subset in earlier embryonic stages and a full array of twenty amino acids after genomic activation (Steeves and Gardner, 1999; Lane *et al.*, 2003). By minimizing the amount of byproduct accumulation (i.e. ammonium), sequential media such as G1.2/G2.2 provide a quantitative and qualitative embryo production comparable to those obtained from co-culture systems (Lane *et al.*, 2003). The removal of ammonium accumulation and serum in sequential media reduces the risk of large offspring syndrome (Van Wagtendonkde Leeuw *et al.*, 2000). Other researchers (Ongeri *et al.*, 2001; Borman *et al.*, 2003; Koeman *et al.*, 2003; Wang *et al.*, 2003; Herrick *et al.*, 2004; Jimenez-Macedo *et al.*, 2005) had also employed G1-G2 sequential culture medium (Gardner and Lane, 1997) successfully culture IVF or ICSI-derived goat embryo to blastocyst stage.

Co-culture generally provides high developmental rates and good quality blastocysts and high pregnancy initiation rates (Massip *et al.*, 1996; Rizos *et al.*, 2001). Izquierdo *et al.* (1999) suggested that development with oviductal cells was superior to that in cumulus cells or in medium alone; thus, the effect was tissue-specific. It also appears that the beneficial effect of the co-culture does not depend on the species from which the cells are taken, it have

also reported that there is a very low species specificity for oviduct epithelial cells since bovine oviduct epithelial cells also provided a good possibility for development of goat embryos (Buggin-Daubie *et al.*, 1992; Betteridge, 1995). Several investigators have reported the superiority of co-culture with oviductal ephitelial cells when compared to culture without cells in embryos from the goat and found that cumulus cells did not support embryonic development as well as oviductal cells (Sakkas *et al.*, 1989; Prichard *et al.*, 1990; Buggin-Daubie *et al.*, 1992; Shamsuddin *et al.*, 1993; Rorie *et al.*, 1994). In goat, Keskintepe *et al.* (1994b) obtained a higher number of embryos reaching the morula stage with cumulus cells than with oviductal ephitelial cells but these authors used an atmosphere with 90% of N_2 for embryo co-culture. According to Bavister (1988), the means by which oviductal tissue supports early development *in vitro* are not well known but it seems likely that these cells produce material beneficial to the young embryos, remove substances with negative effects from the media and/or reduce the oxygen concentration, or both.

In addition, co-culture systems were supplemented with both defined (hormones) and undefined components (serum). Serum has been commonly used as a component of co-culture media and addition of serum into the culture medium improves the kinetics of embryo development (Lazzari *et al.*, 2002; Lequarre *et al.*, 2003; Rizos *et al.*, 2003), cell number and the number of blastocysts reaching the blastocyst stage (Holm *et al.*, 2002; Lazzari *et al.*, 2002). However, serum contains many kinds of components such as proteins, amino acids, carbohydrates, trace elements, hormones, growth factors, cell attachment and spreading factors, and some, as yet, undefined factors (Takagi *et al.*, 1991). Since the biological activity of serum differs remarkably from batch to batch (Ellington *et al.*, 1990; Bavister, 1995), improved culture media without serum should be developed in order to obtain a more controlled system.

2.6.2 Culture System

Several factors of culture are known to perturb the development, morphology and gene expression of embryos. These factors are associated with culture media (composition and physicochemical properties) and/or the culture environment (such as oxygen concentration and light exposure) (Ho *et al.*, 1994; Harvey *et al.*, 2004; Fischer-Brown *et al.*, 2005a,b; Gyu-Jin *et al.*, 2007; Takenaka *et al.*, 2007). Besides the differences in basal media, oxygen concentration and supplementation with undefined additives (serum or albumins), other known or unknown variables related to the culture system are susceptible to perturb the embryo development and its gene expression profile. These factors include physical nature of embryo culture, temperature, embryo density, use of oil, degree of humidification and surface area of gas exchange which is likely to be different among laboratories. For example, the lack of a stabilised incubator environment as a result of constraints of incubator size and/or number of technicians working with incubators, the degree of technical experience while preparing embryos for culture and exposure to light, room temperature and quality of atmosphere are subjected to vary between laboratories. Moreover, unknown elements in embryo culture, such as volatile organics and other contaminants that the majority of laboratories do not have the resources to monitor could also influence the embryo development and gene expression pattern. Additionally, embryos themselves have varying abilities to develop in any one particular culture system, depending on their genetic background (Hansen, 2007; Thompson *et al.*, 2007), and the sperm used (Palma and Sinowatz, 2004; Fischer-Brown *et al.*, 2005a,b). Oxygen concentration during embryo culture is known to influence development, but much of the evidence on long term effects of oxidative stress is inconclusive (Leese *et al.*, 1998). It has been demonstrated that some of the beneficial effects of somatic cells for embryo culture are due to the reduction of oxygen tension surrounding the embryos or the production of antioxidants (Fukui, *et al.*, 1991; Poulin

et al., 1994; Van Steenbrugge *et al.*, 1996). The deleterious effect of high oxygen tension on early embryonal development has also been investigated in various species such as goat (Batt *et al.*, 1991), sheep (Wright *et al.*, 1976; Thompson *et al.*, 1990) and cattle (Nakao and Nakatsuji, 1990; Thompson *et al.*, 1990; Trounson, 1992; Nagao *et al.*, 1994). Some researchers (Nagao *et al.*, 1994; Poulin *et al.*, 1994; Cognie *et al.*, 1995; Fujitani *et al.*, 1996) have found that reducing the oxygen concentration to 5% enhance blastocyst development of embryos without somatic cell support.

Chapter 3

3.0 MATERIALS AND METHODS

Chapter 3

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3.1 INTRODUCTION

The main objective in this study was to produce viable goat embryos through intracytoplasmic sperm injection (ICSI) technique. Surgery for oocyte retrieval (laparoscopic oocyte pick-up, LOPU) was conducted once a week using either one or two donor goats per surgery session at the Nuclear Transfer and Reprogramming Laboratory (NaTuRe), Institute of Research Management and Monitoring (IPPP), University of Malaya. All the media and reagents were prepared in the Embryo Micromanipulation Laboratory (EMiL), Institute of Biological Sciences, Faculty of Science, University of Malaya. All the experiments involving *in vitro* maturation (IVM), intracytoplasmic sperm injection (ICSI) and *in vitro* culture (IVC) were conducted in the NaTuRe laboratory. This study was conducted from May 2009 to April 2010.

3.2 EXPERIMENTAL ANIMALS

A total of 84 does were selected as oocyte donors which were underwent oestrus synchronisation and superovulation for a total 42 surgery session. In the present study, the experimental does comprising of Boer crossbred and Jamnapari breed; and the age ranging from 6 to 42 months at the time of LOPU. The experimental goats were sourced from the ISB Mini Livestock Farm, University of Malaya located

approximately 1 km from the laboratories. They were fed a diet consisting of good quality Napier grass and commercial pellets (goat/sheep pellet, KMM Berhad, Malaysia) twice daily and given water *ad libitum*. All experimental does used were maintained under good conditions and with welfare guidelines. Furthermore, frozen Jermasia buck semen was obtained from sperm preservation laboratory, ISB Mini Livestock Farm, University of Malaya. The semen was supplied in 0.5 ml French straws and stored in the laboratory where ICSI was done in the liquid nitrogen tank at -196^{o}C.

3.3 MATERIALS

Materials used in the present study included various equipment, chemical, reagents and media, labwares and disposables as well as study samples. These are briefly described in the following sections:

3.3.1 Equipment

Each of the equipment used in the present study with model number, manufacturer's and supplier's name are listed in Appendix Table 1.1. The commonly used equipment included autoclave, centrifuge, CO_2 incubator, dissecting microscope, fluorescent microscope, flushing and aspiration system, inverted microscope with micromanipulation, laminar flow cabinet, laparoscopic system, liquid nitrogen tank, microforge, micropipette dispenser, micropipette grinder, micropipette puller, osmometer, oven, pH meter, stage warmer, stereomicroscope, surgical set, ultrapure

purification water system and water bath.

3.3.1.1 The ICSI microscope and micromanipulation system

An inverted microscope with 4X, 10X, 20X and 40X objectives, a heated stage set at 38.5^{o}C, a high quality camera and a monitor was used in the present study. The microscope also fitted with the Hoffman modulation contrast system (preferred to Nomarski system) and Narishige hydraulic micromanipulators was used to perform ICSI. The micromanipulation system was comprised of 1) a set of left and right arms of the micromanipulators that were either electronically or mechanically controlled by their respective joysticks, 2) a set of joysticks that allow control of coarse and fine three-dimensional (X-Y-Z) positioning and also precise linear displacement simultaneously. The joysticks were either hydraulically driven based on de Fonbrune's system or electrically driven system, and 3) air-filled injection and holding units were comprised of microtool holder connected to air-tight syringe (3 ml) by Teflon plastic tubing. The micromanipulators that control the injection and holding systems were positioned ergonomically to prevent back pains due to prolong work on the micromanipulation system. For instance, the injection was done from the right micromanipulator but the joystick controller was placed on the left side of the microscope. Likewise, the holding needle was placed on the left micromanipulator but its movements were controlled by the joystick controller on the right side of the microscope.

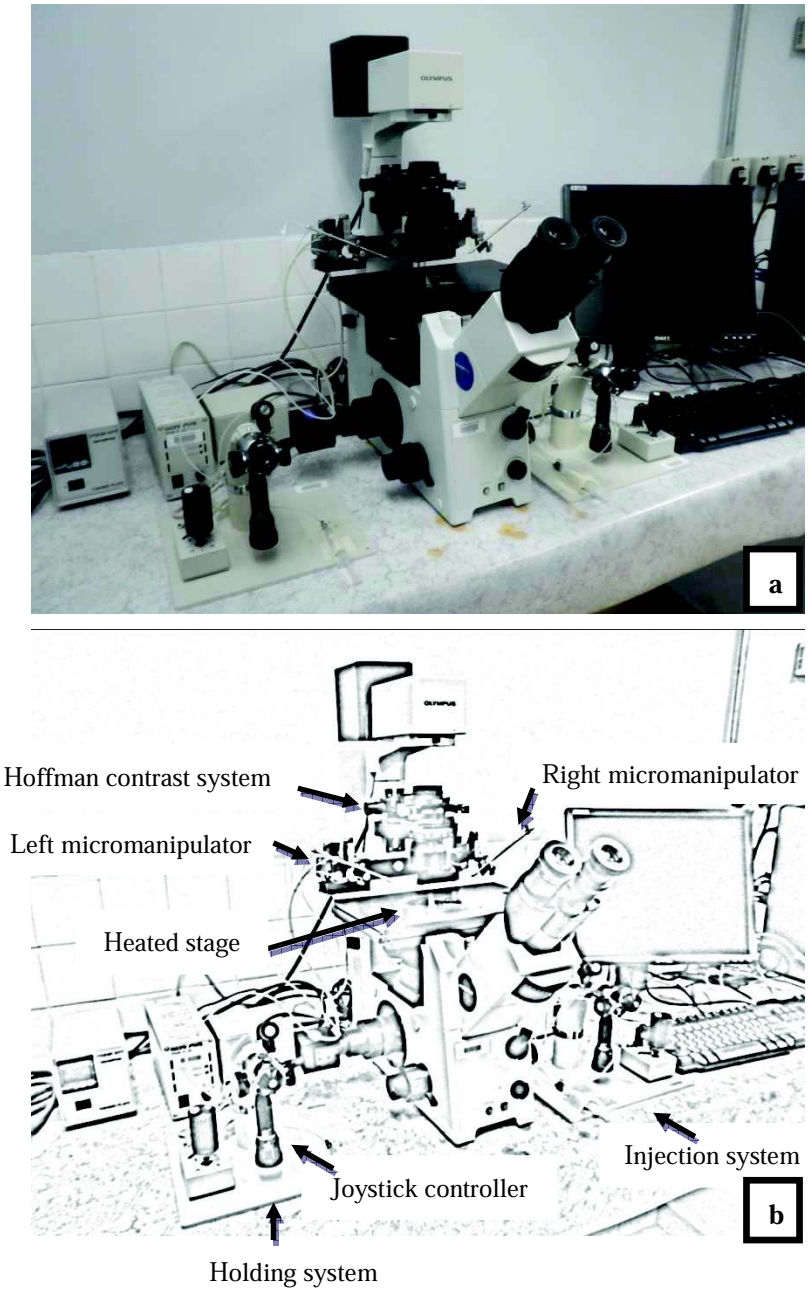

Figure 3.1: Set-up for intracytoplasmic sperm injection. (a) Original photograph; (b) Labelled photograph. The inverted microscope equipped with Hoffman modulation contrast optics, a heated stage and two identical sets of micromanipulators and injectors. The sets on the left side used for manipulating the holding needle, the set on the right side for manipulating the injetion needle.

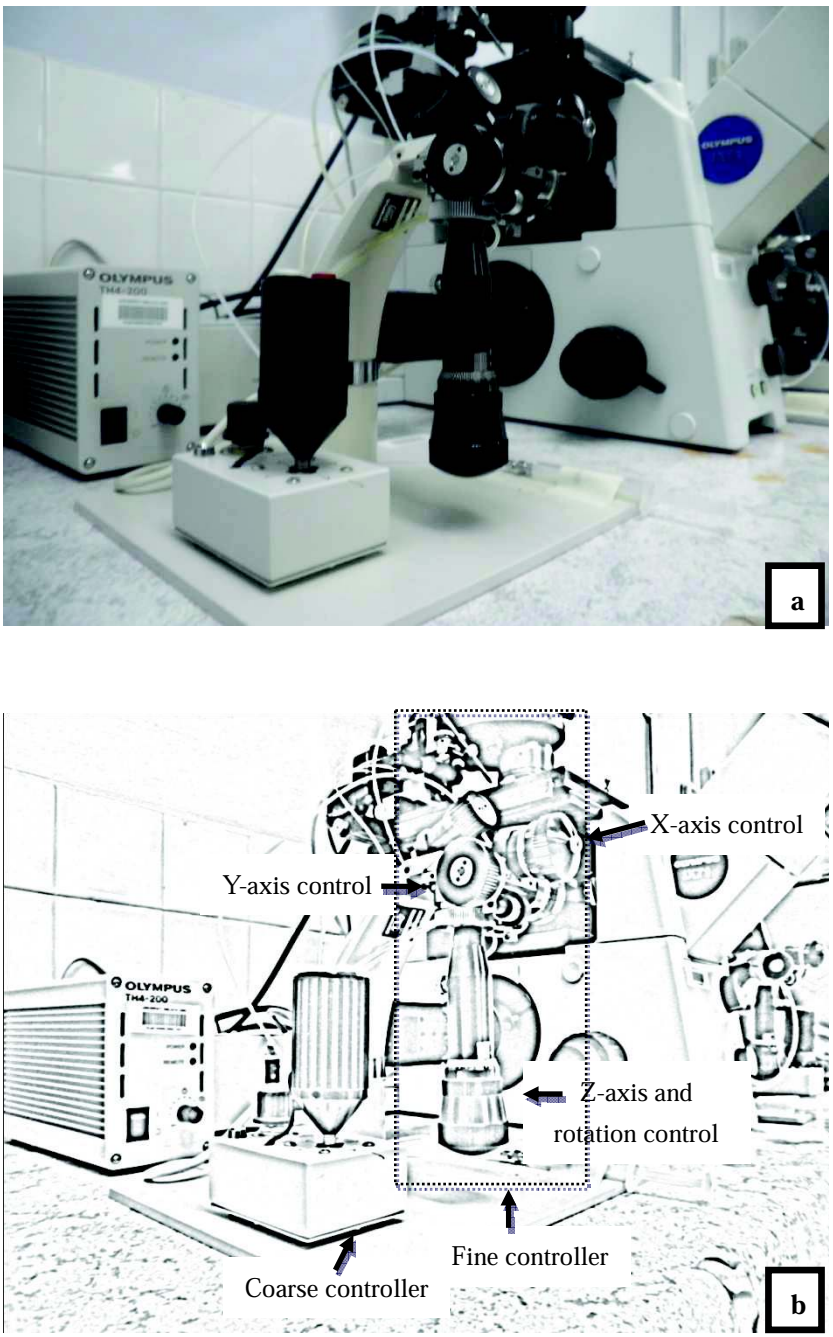

Figure 3.2: Fine and coarse joystick controls (Narishige, Japan). (a) Original photograph; (b) Labelled photograph.

3.3.2 Chemicals, Reagents and Media

Analytical grade laboratory chemicals and reagents were used in the preparation of all solutions and media. Chemicals, reagents and media used were purchased mainly from Sigma-Aldrich Co., from USA unless otherwise stated. A detailed list of the chemicals, reagents and media with catalogue number, manufacturer's and supplier's name is depicted in Appendix Table 1.2.

3.3.3 Labwares and Disposables

A list of labwares and disposables with manufacturer's name used in the study is tabulated in Appendix Table 1.3.

3.4 METHODOLOGY

3.4.1 General Maintenance of Research Laboratory

For successful IVP experiments, it is necessary to ensure that the laboratory activities and facilities adhere to strict cleanliness regimes and sterile techniques are followed throughout all experimental procedures. It is responsibility of the laboratory user to maintain the laboratory in a distraction and accident-free environment to optimise the outcome in any IVP endeavour. It is also utmost importance to minimise potential introduction of infection or contamination from any source particularly in areas or work space for oocyte or embryo handling and culture.

For all IVP experiments, this study used CO_2 (5%) in humidified air to maintain the correct physiological pH (pH 7.3 to 7.4) and a temperature of 38.5^{o}C. Monthly

cleaning regime of the CO_2 incubator involves wiping the inside wall, doors and racks with sterile reverse osmosis (RO) water and sterile towels or gauze. The tray and the RO water contained in it which to provide humidity should be sterile and changed with every cleaning regime. The CO_2 incubator must be monitored regularly and the LED display of temperature checked with independent thermometer readings. Repeated opening and closing of the CO_2 incubator should be kept to the minimum because it affects the stability of the oocyte or embryo culture environment.

All used glasswares and non-disposable items in the present study included glass bottles, beakers, volumetric flasks, measuring cylinders, conical flasks, magnetic stirrer, conical tubes, micropipette tips as well as microcentrifuge tubes. Cleaning solution (7X®-PF) was used as a detergent for glassware washing and was filled in a squirt bottle. The used glassware was rinsed in water to wash away traces of medium; the label was immediately removed and soaked in soap bath of 7X®-PF. The glassware was washed with diluted cleaning solution (7X®-PF) using a brush or sponge and immediately rinsed five times with water followed by five times with RO water. After rinsing was completed, the cap of glassware was placed loosely and covered snugly with a layer of aluminium foil. A piece of autoclave tape was placed on the foil. Alternatively, non-disposable items were placed in an autoclave bag and sealed; a piece of autoclave tape was placed on the seal bag. All items were allowed to be autoclaved for 20-25 minutes at 120^{o}C. After autoclaving was done, the glassware cap was tightened a little bit prevent any contaminants from entering and together with non-disposable items were transferred into the oven to dry before being

transferred in a clean and close glassware cabinet or appropriate storage cabinets. Also, the cap was not tightened completely until the glassware had cooled to prevent a vacuum forming in the glassware.

Before starting any experiment, the inside surfaces of the laminar flow work station, microscope stages and other equipment such as micropipettes were wiped with ethanol (70%). The residual traces of ethanol were allowed to evaporate for at least 20 minutes before commencing work. Any spillage were wiped immediately with dry tissue and if necessary, with ethanol (70%). When work finished, the inside surfaces of the laminar flow work station and all the equipment were wiped again with ethanol (70%). Also, the water bath was cleaned and water was changed frequently.

3.4.2 Preparation of Stocks and Media

All culture media used throughout the present study were prepared 'in-house' instead of purchased commercially. Thus, it was important to have a reliable source of clean water. Toxic contaminants in the culture medium ingredients, including the water, were always a major concern. The water used to prepare all culture media was sourced from a ultrapure water purification system; with treatments of particulate filtration, activated carbon filtration, reverse osmosis (RO) and electrodeionisation (EDI), ultraviolet oxidation system, followed by a Milli-Q PF Plus purification (18.2 MΩ-cm) and finally filtered through a membrane filter (0.22 µm) to eliminate trace particles as well as to prevent bacterial contamination from the environment.

Typically, fresh oocyte or embryo culture media were prepared weekly or biweekly. Preparation of different culture media requires accurate but time consuming measurements. Therefore, it is convenient to prepare media from a series of stock solutions (Nagy *et al.*, 2003). All fundamental stock solutions prepared were filter-sterilised using syringe filter (0.22 µm pore size), aliquot in microcentrifuge tubes and stored in the refrigerator (2-8^{o}C) or freezer (-20^{o}C) as appropriate. All the stock solutions and media were prepared under laminar air flow work station. For all the media, the pH was adjusted to 7.2 to 7.4 and osmolarity to 280 to 300 mOsm/kg (Tervit *et al.*, 1972; Brackett and Oliphant, 1975; Younis *et al.*, 1991; Takahashi and First, 1992).

3.4.2.1 Preparation of heparinised saline solution

The heparinised saline solution consisted of NaCl (4.5 g) and heparin (0.025 g) were weighted using a digital balance and dissolved in Milli-Q water (500 ml) by stirring gently. After preparation, the saline was sterilised by autoclaving and kept for 3 months in the refrigerator (4^{o}C) for future use.

Table 3.1: Composition of heparinised saline solution with a shelf life of 3 months (stored at 4^{o}C)

Chemical (catalogue number)	Concentraton	Quantity/500 ml
NaCl (S5886)	0.9 (w/v)	4.5 g
Heparin (H0777)	0.05 mg/ml	0.025 g

3.4.2.2 Preparation of flushing medium

The flushing medium for LOPU was connected to an aspiration system (a vacuum pump). It used for flushing microvolumes of fluid into the ovarian follicles on the

surface of the ovary; and the follicular fluids which containing oocyte from the follicles were subsequently aspirated. The aspirated contents were then collected in a sterile round-bottom test tube (14 ml) which was pre-warmed by a test tube heating system to be searched for oocytes under dissecting microscope. Typically, flushing medium (300 ml) was prepared within 12 hours before oocyte retrieval. The flushing medium consisted of DPBS supplemented with gentamicin sulfate salt (30 µg/ml) and heparin (52 IU/100 ml) as depicted in Table 3.2. The resulting medium was filtered-sterilised using syringe filter (0.22 µm pore size), aliquot into Terumo® luer slip syringe (50 ml) and maintained at 38.5°C prior to oocyte retrieval.

Table 3.2: Composition of flushing medium (300 ml)

Chemical components (catalogue number)	Final concentration	Quantity/300 ml
Phosphate-buffered saline (PBS) tablets (P4417)	1 tablet/100 ml	PBS (3 tablets) were dissolved in Milli-Q water (300 ml), sterilised by autoclaving.
Gentamicin sulfate salt (G3632)	30 µg/ml	Gentamicin sulfate salt (9 mg) was dissolved in PBS solution (300 ml) prior to use.
Heparin (H0777)	52 IU/100 ml	Heparin (1 mg*) was dissolved in PBS solution (300 ml) prior to use.

*1 mg of heparin contains 156 IU.

3.4.2.3 Preparation of oestrus goat serum (OGS)

The OGS is traditionally supplemented in oocyte or embryo culture media to provide additional unidentified beneficial growth factors, hormones and peptides.

3.4.2.3(a) Blood collection

To prepare the oestrus goat serum, blood samples (200 to 300 ml) were collected aseptically via the jugular vein from goats synchronised for oestrus using vacutainer® tubes (without heparin) with a needle (21G) and a venojector holder. Blood collected in vacutainer® tubes was left to clot for 30 to 60 minutes in laminar flow at room temperature to obtain the serum from clotted blood.

3.4.2.3(b) Heat-inactivation

The tubes containing clotted blood were centrifuged (500 x *g*, 10 minutes, 25°C), the supernatant (serum) was aspirated carefully into conical centrifuge tubes (15 ml). The serum was heat-inactivated in water bath (30 minutes, 56°C) to destroy components that might lead to cell lysis by antibody binding. After 30 minutes of treatment of 56°C, serum was removed from the water bath. Prolonged heat treatment could cause deterioration of some components of the serum. After cooling to room temperature, serum was centrifuged again (500 x *g*, 10 minutes, 25°C) to sediment residual erythrocytes. The OGS was aliquot in sterile microcentrifuge tubes (1.5 ml) and stored with a maximum shelf life of 6 months in the freezer (-20°C). OGS was thawed (room temperature) immediately prior to use.

3.4.2.4 Preparation of *in vitro* maturation (IVM) medium

In vitro maturation (IVM) medium was prepared a day before and equilibrated overnight in the CO_2 incubator (5%) prior to oocyte retrieval. A modified IVM

medium described by Jimenez-Macedo *et al.* (2005) was used which consisted of TCM-199 as a base medium supplemented with FSH (1 mg/ml), hCG (1 mg/ml), 17β-oestradiol (100 µg/ml), sodium pyruvate (2.75 mg/ml), gentamicin sulfate salt (10 µg/ml), cysteamine (10 mM) and heat-inactivated OGS. A list of stock solutions used to supplement IVM media with concentration, preparation method, storage temperature and shelf life is presented in Table 3.3.

Table 3.3: Preparation of stock solutions for IVM medium

Stock solution (catalogue number)	Concentration	Method of preparation (storage duration)
FSH (Folligon®)	1 mg/ml	Folligon® (5 mg) was dissolved in ultrapure water (5 ml), aliquot (110 µl) in microcentrifuge tube, sealed with parafilm, wrapped in aluminium foil and stored (-20°C). (12 months)
hCG (Chorulon®)	1 mg/ml	Chorulon® (5 mg) was dissolved in ultrapure water (5 ml), aliquot (110 µl) in microcentrifuge tube, sealed with parafilm, wrapped in aluminium foil and stored (-20°C). (12 months)
Oestradiol-17β (E4389)	100 µg/ml	Oestradiol-17β (1 mg) was dissolved in ultrapure water (10 ml), aliquot (110 µl) in microcentrifuge tube, sealed with parafilm, wrapped in aluminium foil and stored (-20°C). (12 months).
Gentamicin sulfate salt (G3632)	10 mg/ml	Gentamicin sulfate salt (10 mg) was dissolved in ultrapure water (1 ml), aliquot (110 µl) in microcentrifuge tube, sealed with parafilm, wrapped in aluminium foil and stored (-20°C). (12 months).
Cysteamine (M9768)	10 mM	Cysteamine (77.14 mg) was dissolved in ultrapure water (10 ml), aliquot (110 µl) in microcentrifuge tube, sealed with parafilm, wrapped in aluminium foil and stored (-20°C). (12 months)

(continued)

(continued)

Stock solution (catalogue number)	Concentration	Method of preparation (storage duration)
Sodium pyruvate (P3662)	2.75 mg/ml	Sodium pyruvate (2.75 mg) was dissolved in ultrapure water (1 ml), aliquot (110 µl) in microcentrifuge tube, sealed with parafilm, wrapped in aluminium foil and stored (4^{o}C). (2 weeks)

To prepare the IVM medium, TCM-199 (8.45 ml) was measured using a disposable plastic pipette (10 ml) and dispensed into a sterile conical tube (15 ml). Then FSH (100 µl), hCG (100 µl), gentamicin sulfate salt (50 µl), cycteamine (100 µl), sodium pyruvate (100 µl) and heat-inactivated OGS were added to the TCM-199 to make the final volume (10 ml) as shown in Table 3.3. After adding the stock solution, the pH of the medium was adjusted (pH 7.2 to 7.4) and finally filter-sterilised by using a syringe filter (0.22 µm pore size) into a sterile tube (15 ml). Oestradiol-17β (100 µl) was added last after the medium had filtered. With the cap of the tube loosened, the medium was incubated in presence of CO_2 (5%) in a humidified atmosphere of an incubator at 38.5^{o}C for overnight or at least 12 hours before preparing IVM microdroplets.

Table 3.4: Composition of IVM medium (10 ml)

Chemical component	Final concentration	Quantity/10 ml
TCM-199	Base medium	8.45 ml
FSH	10 µg/ml	0.10 ml
hCG	10 µg/ml	0.10 ml
Oestradiol-17β	1 µg/ml	0.10 ml
Cysteamine	100 µM	0.10 ml
Gentamicin sulfate salt	50 µg/ml	0.05 ml
Sodium pyruvate	275 µg/ml	0.10 ml
Heat-inactivated OGS	10% (v/v)	1.00 ml

3.4.2.5 Preparation of other solutions

A number of stocks and other solutions were prepared to use in sperm, ICSI and IVC media, oocyte denudation and activation. These are as follows:

3.4.2.5(a) Preparation of heparin stock solution

Heparin was used in the sperm medium to aid in capacitation of goat sperm. Heparin stock solution was prepared by dissolving heparin (5 mg) in sperm medium (5 ml) to make at 100X concentration (1 mg/ml) for convenience. The solution was mixed properly, aliquot (100 µl) in microcentrifuge tube, sealed with parafilm, wrapped in aluminium foil and stored for 6 months in the freezer (-20^{o}C).

Table 3.5: Composition of heparin stock solution with a shelf life of 6 months (stored at -20^{o}C)

Chemical (catalogue number)	Concentration (100X)	Quantity/5ml
Heparin (H0777)	1 mg/ml	5 mg

3.4.2.5(b) Preparation of HEPES buffer solution

HEPES buffer was used to maintain pH of a medium or solution at 7.2 to 7.4 outside the CO_2 incubator. The formula weight of HEPES is 238.3. Therefore, HEPES buffer stock solution (1 M) was prepared by dissolving HEPES powder (2.383 g) in Milli-Q water (10 ml). The solution was mixed properly, filter-sterilised using a syringe filter (0.22 µm pore size) into a sterile tube (15 ml), sealed with parafilm, wrapped in aluminium foil and stored for 6 months in the refrigerator (4^{o}C). The final concentration of HEPES buffer in any medium was 20 mM.

Table 3.6: Composition of HEPES buffer solution with a shelf life of 6 months (stored at 4°C)

Chemical (catalogue number)	Concentration	Quantity/10ml
HEPES (H7006)	1 M	2.383 g

3.4.2.5(c) Preparation of hyaluronidase solution

Hyaluronidase solution was used in the present study to denude COCs after maturation. A type IV-S hyaluronidase from bovine testes was used to prepare hyaluronidase solution. Hyaluronidase stock solution (750-1500 IU/mg) was prepared by dissolving hyaluronidase powder (1 mg) in Milli-Q water (1 ml) that was designated as 'Stock A'. The prepared solution was aliquot (100 µl) in microcentrifuge tubes, sealed with parafilm, wrapped in aluminium foil and stored for 6 months in the freezer (-20°C). On the day of treatment, one tube of 'Stock A' was withdrawn from the refrigerator and HEPES-buffered IVC medium (900 µl) was added to remove the cumulus cell of an oocyte.

Table 3.7: Composition of hyaluronidase stock solution with a shelf life of 6 months (stored at -20°C)

Chemical (catalogue number)	Concentration	Quantity/1ml
Hyaluronidase (H4272)	750-1500 IU/mg	1 mg

3.4.2.5(d) Preparation of sodium bicarbonate stock solution

Sodium bicarbonate solution is a common component of synthetic oviductal fluid (SOF) medium (Takahashi and First, 1992). $NaHCO_3$ stock solution (100 mM) is prepared by dissolving $NaHCO_3$ (4.2 g) in Milli-Q water (500 ml). The solution was stored for 6 months in the refrigerator (4°C).

Table 3.8: Composition of sodium bicarbonate stock solution with a shelf life of 6 months (stored at 4°C)

Chemical (catalogue number)	Concentration	Quantity/500ml
$NaHCO_3$ (S5761)	100 mM	4.2 g

3.4.2.5(e) Preparation of calcium ionophore (Ca^{2+} ionophore)

In the present study, Ca^{2+} ionophore was used to activate the goat oocytes after ICSI. A solution containing DMSO and absolute ethanol in 3:1 ratio was prepared and the whole content of Ca^{2+} ionophore vial (1 mg) was dissolved into DMSO-ethanol solution (1.34 ml), designated as 'Stock A'. The concentration of 'Stock A' solution was 1 mM. The 'Stock A' solution was aliquot in microcentrifuge tube (10 µl), wrapped in aluminium foil and store up to 6 months in the refrigerator (-20°C). On the day of treatment, one tube of 'Stock A' was withdrawn from the refrigerator and IVC medium (90 µl) was added. The solution (100 µl) was mixed properly and designated as 'Stock B' concentration of which was 100 µM. 'Stock B' solution (10 µl) was pipette and added to a IVC microdroplet (90 µl) to make a final concentration of 10 µM to activate oocytes.

Table 3.9: Composition of Ca^{2+} ionophore with a shelf life of 6 months (stored at -20°C)

Chemical (catalogue number)	Concentration	Quantity/1.34 ml
Ca^{2+} ionophore (I0634)	1 mM	1 mg

3.4.2.5(f) Preparation of 6-dimethylaminopurine (6-DMAP)

In the present study, 6-DMAP was used together with Ca^{2+} ionophore to activate the goat oocytes following ICSI. A solution containing DMSO and absolute ethanol in 3:1 ratio was prepared and 6-DMAP powder (0.03 g) was dissolved into DMSO-ethanol

solution (1.34 ml), designated as 'Stock A'. The concentration of 'Stock A' solution was 0.19 M. The 'Stock A' solution was aliquot in microcentrifuge tube (5 µl), wrapped in aluminium foil and store up to 6 months in the refrigerator (-20°C). On the day of treatment, one tube of 'Stock A' was withdrawn from the refrigerator and IVC medium (495 µl) was added. The solution (500 µl) was mixed properly (final concentration was 1.9 mM) prior to activate oocytes.

Table 3.10: Composition of 6-DMAP with a shelf life of 6 months (stored at -20°C)

Chemical (catalogue number)	Concentration	Quantity/1.34 ml
6-DMAP (D2629)	0.19 M	0.03 g

3.4.2.6 Preparation of modified SOF medium

In the present study, mSOF medium was used as the base medium for sperm wash and capacitation, ICSI and IVC. For convenience, a concentrated stock of the components of mSOF at 10X concentration designated as mSOF stock medium was first prepared followed by the mSOF working solution (1X concentration).

3.4.2.6(a) Preparation of mSOF stock medium

The mSOF stock medium contained NaCl (3.15 g), KCl (266.90 mg), KH_2PO_4 (80.90 mg; BDH Laboratory Supplies, England), $CaCl_2.2H_2O$ (125.70 mg), $MgCl_2.6H_2O$ (49.80 mg) and phenol red (25 µl; Gibco BRL, USA). The concentration and volume/weight of each of the components of the medium is depicted in Table 3.11. To prepare mSOF stock solution (50 ml), a sterile conical flask (50 ml) was filled with Milli-Q water (approximately 20 ml). All the chemicals were weight using a digital balance and added stepwise as well as dissolved in Milli-Q water by gently swirling

the flask. Milli-Q water was added to dissolve the salts to make a total volume (approximately 40 ml) and followed by the addition of phenol red (0.5%) solution. Then, the volume of solution was topped up to 50 ml. Finally, the solution was filter-sterilised using syringe filter (0.22 µm pore size) and stored in a Schott bottle (50 ml) for a shelf life of 3 months in the refrigerator (4°C).

Table 3.11: Composition of mSOF stock medium (10X) with a shelf life of 3 months (stored at 4°C)

Chemical (catalogue number)	Concentration (10X)	Quantity/50 ml
NaCl (S5886)	107.70 mM	3.15 g
KCl (P5404)	7.16 mM	0.2669 g
KH_2PO_4 (Prod29608)	1.19 mM	0.8090 g
$CaCl_2.2H_2O$ (C7902)	1.71 mM	0.1257 g
$MgCl_2.6H_2O$ ((M2393)	0.49 mM	0.0498 g
Phenol red (15100-43)	1 µg/ml	0.025 ml
Milli-Q water	-	49.98 ml

3.4.2.6(b) Preparation of mSOF working solution

The mSOF working solution was prepared with mSOF stock medium at 1X concentration as presented in Table 3.12. Typically, to prepare the mSOF working solution (100 ml), mSOF stock medium (10 ml) and sodium bicarbonate stock solution (25.07 ml) were measured using a disposable serological pipette and dispensed into a sterile conical flask (100 ml). All the chemicals were weighed using a digital balance. Sodium pyruvate (3.6 g), gentamicin sulfate salt (5 mg) and L-glutamine (14.6 mg) were added stepwise and dissolved in the solution by gently swirling the flask. Sodium DL-lactate 60% syrup (54.8 µl), BME (2 ml) and MEM (1 ml) were measured using micropipettes and dispensed in the solution mixture. The resulting solution was topped up to 100 ml with Milli-Q water. The resulting mSOF

working medium was filter-sterilised by syringe filter (0.22 µm pore size), stored in the Schott bottle (100 ml) with a shelf life of 2 weeks in the refrigerator (4°C).

Table 3.12: Composition of mSOF working solution (1X) with a shelf life of 2 weeks (stored at 4°C)

Component (catalogue number)	Concentration (1X)	Quantity/100 ml
mSOF stock solution	1X	10 ml
$NaHCO_3$	25.07 mM	25.07 ml
Sodium pyruvate (P3662)	0.30 mM	3.6 g
Sodium DL-lactate (60% syrup) (L4263)	3.30 mM	0.0548 ml
Gentamicin sulfate salt (G3632)	50 µg/ml	0.005 g
L-glutamine (M3126)	1.00 mM	0.0146 g
BME amino acids solution (B6766)	1X	2 ml
MEM non-essential amino acids solution (M7145)	1X	1 ml
Milli-Q water	-	61.7 ml

3.4.2.7 Preparation of sperm wash and capacitation medium

mSOF medium was used as base medium to prepare sperm wash and capacitation medium. Sperm wash and capacitation medium was prepared a day or at least 12 hours before ICSI experiment, filter sterilised using syringe filter (0.22 µm pore size) and equilibrated overnight in CO_2 (5%) at 38.5°C prior to use.

3.4.2.7(a) Preparation of sperm wash medium

The sperm wash medium (6 ml) was consisted of mSOF working solution supplemented with OGS (20%) which is depecired in Table 3.13(a). mSOF working solution (4.8 ml) was dispensed using a disposable serological pipette (5 ml) into a sterile conical tube (15 ml). Then OGS (1.2 ml) was pipette into the conical tube to make the final volume (6 ml). The resulting medium was filter-sterilised by syringe filter (0.22 µm pore size) and equilibrated overnight in the CO_2 incubator (5%) at

least for 12 hours or overnight prior to use.

Table 3.13(a): Composition of sperm wash medium (6 ml)

Composition	Concentration (1X)	Quantity/6 ml
mSOF working solution	1X	4.8 ml
OGS	20% (v/v)	1.2 ml

3.4.2.7(b) Preparation of sperm capacitation medium

The sperm capacitation medium consisted of mSOF working solution supplemented with heparin (Section 3.4.2.5a) and OGS (20%), which is depicted in Table 3.13(b). mSOF working solution (1.4 ml) was pipette into a sterile conical tube (15 ml). Heparin (200 µl) and OGS (400 µl) was then pipette into the conical tube to make the final volume (2 ml). The resulting medium was filter-sterilised by syringe filter (0.22 µm pore size) and incubated in the CO_2 incubator (5%) at least for 12 hours or overnight prior to use.

Table 3.13(b): Composition of sperm capacitation medium (2 ml)

Composition	Concentration (1X)	Quantity/2 ml
mSOF working solution	1X	1.4 ml
Heparin	50 µg/ml	0.2 ml
OGS	20% (v/v)	0.4 ml

3.4.2.8 Preparation of ICSI medium

ICSI medium consisted of mSOF working solution and HEPES buffer (Section 3.4.2.5b) supplemented with OGS (10%) which is depicted in Table 3.14. The medium was prepared fresh or few hours before ICSI. mSOF working solution, HEPES buffer and OGS were pipette and dispensed into a sterile conical tube (15 ml). The resulting medium was filter-sterilised by syringe filter (0.22 µm pore size) and then kept in the refrigerator (4°C). The medium temperature was raised to 38.5°C in

water bath prior to use.

Table 3.14: Composition of ICSI medium (3 ml)

Composition	Concentration (1X)	Quantity/3 ml
mSOF working solution	1X	2.64 ml
OGS	20% (v/v)	0.30 ml
HEPES buffer		0.06 ml

3.4.2.9 Preparation of IVC medium

The IVC medium was used for IVC of embryo (embryo washing and culture) in the present study. The medium was mSOF working solution supplemented with heat-inactivated OGS (10%) as shown in Table 3.15. The medium was prepared fresh at least 12 hours before ICSI throughout the experimental period. mSOF working solution (4.5 ml) was measured using a disposable serological pipette (5 ml) and dispensed into a sterile conical tube (15 ml). The IVC medium supplemented with heat-inactivated OGS (0.5 ml). The resulting medium was filter-sterilised using syringe filter (0.22 µm pore size) and equilibrated overnight in the CO_2 incubator (5%) prior to use.

Table 3.15: Composition of IVC medium (5 ml)

Composition	Concentration (1X)	Quantity/5 ml
mSOF working solution	1X	4.5 ml
OGS	10% (v/v)	0.5 ml

3.4.3 Preparation of Hand-controlled Pipette and Microneedles

A hand-controlled pipette was used for handling the oocyte and embryos. It was constructed as shown in Figure 3.3, consisting of a pulled-glass Pasteur pipette attached to a pipette pump. Microneedles were used for ICSI included holding and injection needles. All the glass pipettes and needles used in different experiments

were prepared 'in-house' in the laboratory. For the preparation of needles for ICSI, this aspect should not be underestimated and it takes a lot of training and patience to make a good ICSI needles.

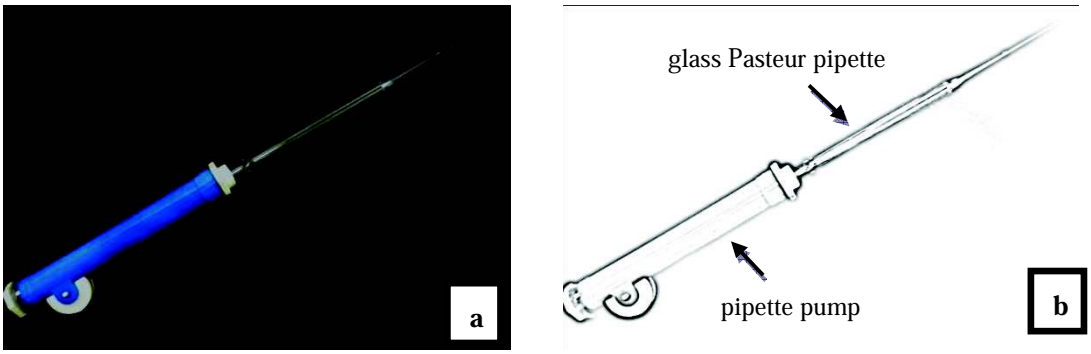

Figure 3.3: Hand-controlled pipette. (a) Original photograph; (b) Labelled photograph.

3.4.3.1 Capillary cleaning and sterilisation

The hand-controlled pipette and borosilicate capillaries for making holding pipette and injection pipette were soaked in hydrochloric acid solution (10%) for 24 hours in a glass cylinder and rinsed with Milli-Q water twenty times to remove all traces of the acid. The glass Pasteur pipettes were sterilised by autoclaving and thereafter, dried thoroughly in the oven (56^{o}C) overnight; whilst the borosilicate capillaries were then dried in an oven at 150^{o}C for 4 hours.

3.4.3.2 Preparation of hand-controlled pipette

The glass Pasteur pipette was softened by rotating the middle portion in a fine spirit burner flame until the glass became soft. The glass was immediately withdrawn from the heat and both ends were quickly pulled smoothly in opposite directions so that the middle portion had an inner diameter (ID) of approximately 200 and 500 µm for

embryo and cumulus-oocyte complex, respectively. For the neat break, the pulled portion of the capillary was scribed with a diamond stone and snapped at the scribed portion. It was important to achieve an evenly snapped straight tip because a jagged capillary end may potentially cause damage to the zona pellucida during oocyte or embryo handling. In addition, sharp edges of the pipette were easily caught by the plastic surface of the culture dish, causing the pipette to break and tend to collect more debris particularly in handling the cumulus-oocyte complexes. The tip of the pipette was fire-polished by quickly touching the flame quickly to achieve a smooth edge of the pipette tip.

3.4.3.3 Preparation of microneedles

Injection of sperm into the ooplasm is the most invasive micromanipulation technique in assisted reproductive technology (ART). Hence, the design and quality of the microneedles especially the injection needle is of utmost important, as the size and shape of this needle will determine the success or failure of a micromanipulation procedure. The holding and injection needles were made from thin-walled borosilicate capillaries (Drummond, Broomall, USA) with an inner and outer diameter of 0.69 and 0.97 mm, respectively, and a length of 10 cm. Three instruments are necessary to prepare both holding and injection needles are micropuller, microforge and microgrinder.

A horizontal micropuller (P-97, Sutter Instrument, USA) is preferred to the vertical as the former produced needles of uniform shapes as shown in Figure 3.4. The

quantity of heat, pull speed and strength have been established and stored in the programs. The desired shape of the needle should be one that has a long and uniform tapering end with a length of approximately 10 mm. After pulling, microforge and microgrinder are used to finalise the preparation of the injection needle (Figure 3.4). A microforge is an instrument to make injection and holding needles of a specific size. Before needle processing can begin, it was necessary to place a small bead of glass on top of the heater element. It was to ensure the filament itself never came into direct contact with the needle. The filament glowed dull red with the heater control adjusted, and then a needle lowered on to the hot filament. The needle was lowering continuously as the glass melted until a bead of approximately 20 to 30 µm diameter has formed; and the heat switched off immediately. The needle closed to the bead was broke with a pair of fine forceps, and then the broken pipette withdrawn as well as discarded. Finally, the bead heated gently until the jagged portion absorbed. The microforge was now ready to process holding and injection pipettes.

Injection needle was prepared by cutting the tip of a pulled capillary on a heated filament of the microforge at an inner and outer diameter of approximately 8 to 9 µm and 9 to 10 µm, respectively. The needle tip was ground to produce a bevelled edge with a microgrinder (Figure 3.5) at 45^{o} desired angle for approximately 3 minutes. The dust accumulated in the needle was washed away with hydrofluoric acid (10%) for 5 seconds only and washed thoroughly with Milli-Q water. The injection and holding needles were bent at 30 to 35^{o} with microforge to allow a horizontal displacement on the microscope stage.

The holding needle is a tool that holds an oocyte firmly for a micromanipulation procedure. Basically, holding needle was prepared by scoring the pulled capillary with an ampoule cutter, breaking it and fire polishing the tips with a microforge. The inner and outer diameter of the holding needle was approximately 25 µm; and 150 to 180 µm, respectively. Prepared injection and holding needles were packed in the needle holders and sterilised by the application of ultraviolet (UV) light for 30 minutes inside the laminar flow cabinet on the day of manipulation.

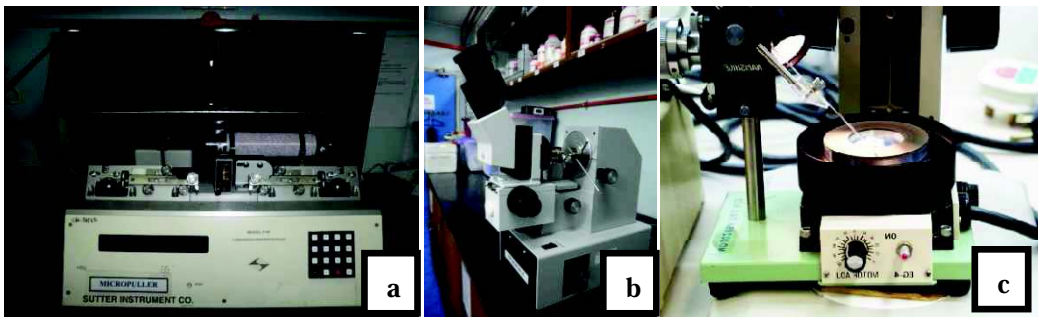

Figure 3.4: Preparation of microneedles. (a) Micropuller, (b) Microforge, and (c) Microgrinder.

3.4.4 Preparation of Mineral Oil

Mineral oil was used throughout the IVP experiments to layer microdroplets as it was embryo tested and less toxic to the oocytes and embryos. It was necessary to overlay the microdroplets of culture medium with equilibrated oil as the oil serves as a physical barrier that separates microdroplets from the atmosphere and airborne particles or pathogens. It was also to prevent evaporation and delay gas diffusion, thus stabilising the pH, temperature and osmolarity of the microenvironment surrounding the oocyte/embryo during handling outside CO_2 incubator (5%). This was done by pouring IVM or IVC medium (20 ml) in a sterile tissue culture flask (50 ml) followed

by mineral oil (30 ml) in the ratio of 3:2 (medium to mineral oil). The oil was then equilibrated with the medium (free of serum) by incubating overnight in the CO_2 incubator (5%) with the cap loosened.

3.4.5 Protocols for Donor Goat Preparation

A constant supply of goat oocyte samples used throughout this study was from superovulated goats. Therefore, donor goats that were selected to provide oocytes were prepared to undergo oestrus synchronisation, superovulation and finally laparoscopic oocyte pick-up (LOPU). A schematic representation of the processes involved in donor goat is shown in Figure 3.5.

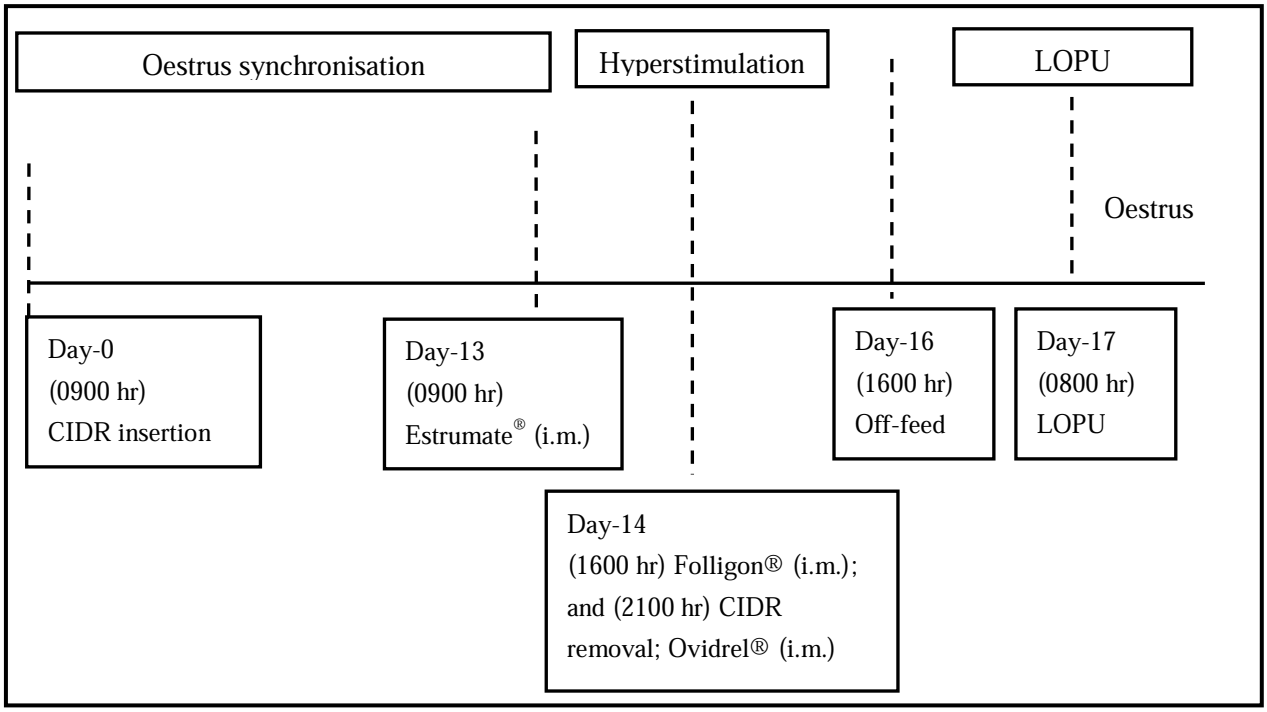

Figure 3.5: A schematic representation of the process involved in donor goat preparation: oestrus synchronisation, superovulation and finally LOPU at the onset of oestrus. Dosage of hormone treatments was described in the text.

3.4.5.1 Oestrus synchronisation of donor goats

Oestrus synchronisation is a procedure for manipulating the oestrous cycle of donor goat so that it is possible to plan the date and time of oocyte retrieval during LOPU. To synchronise the oestrous cycle of a donor goat, a Controlled Intravaginal Drug Release device (CIDR®, 0.3 g progesterone) was inserted into the vagina with the help of a sterile CIDR applicator and a veterinary obstetrical lubricant (K-Y Jelly) for 14 days (at 0900 hr on Day-0) before being removed. The CIDR is made of an inert silicone elastomer that is non-porous and does not readily absorb bodily fluids; once properly inserted deep into the donor's vagina, the CIDR unfolds into a 'T' like formation that aids in retention. Daily monitoring of the device was performed to confirm that it had not been inadvertently removed. At approximately 36 hours prior to CIDR removal, a luteolytic treatment of Cloprostenol (Estrumate®, 125 µg) was administered intramuscularly (at 0900 hr on Day-13) to regress corpus luteum that facilitates initiation of pro-oestrus and eventually resulted in oestrogen surge for a rapid and highly visible onset of heat (oestrus).

3.4.5.2 Superovulation of donor goats

Superovulation is a procedure conducted following oestrus synchronisation to recruit the multiple growths of follicles in the ovary. Morphologically, the effect of superovulation is seen as several fluid-filled 'pimple-like' protrusions on the surface of the ovary. Folligon® (1200 IU or 1500 IU FSH; depending on the type of experiments) was administered intramuscularly (at 1600 hr on Day-14) prior to CIDR

removal. Upon removal of CIDR (at 2100 hr on Day-14), the donor goats were superovulated by administering Ovidrel® (250 IU CG) intramuscularly at 60 hours prior to LOPU. At approximately 48 hours after FSH and hCG administration, the donor goats were observed for the onset of oestrus behaviour such as twitching of the tail, increased vaginal secretion and eagerness to be mounted on by a teaser buck. The onset of oestrus marked the urgency to initiate LOPU procedure.

3.4.6 Laparoscopic Oocyte Pick-Up (LOPU)

Laparoscopic oocyte pick-up (LOPU) is a microsurgical procedure to retrieve oocytes from mature follicles seen as 'pimple-like' protrusions on the surface of the ovary via four incisions on the abdomen of the donor goat. Oocytes were retrieved by aspiration of follicular contents under laparoscopic observation. Before the actual oocyte retrieval process began, the donor goat was sedated and anaesthetised, surgical instruments and accessories were disinfected. During oocyte retrieval, test tubes (each containing 1 to 3 ml of follicular fluid) were passed to embryologists to be scanned under dissecting microscopes for oocytes. After oocyte retrieval, the donor goat underwent post-surgical treatment.

3.4.6.1 Sedation and anaesthetisation of donor goats

The donor goats were deprived of food and water (at 1600 hr on Day-16) for 16 hours prior to LOPU. On the day of performing LOPU (at 0800 hr on Day-17), the donor goat was sedated and anaesthetised with xylazine hydrochloride (0.22 mg/kg body

weight) followed by ketamine hydrochloride (11 mg/kg body weight) via intramuscular (i.m.) injection. The donor goat was maintained under anaesthesia with administration (i.m.) ketamine hydrochloride (0.1 mg/kg body weight) at regular intervals of approximately 30 minutes or as required while LOPU was in progress.

3.4.6.2 Disinfection of surgical instruments, and skin area of goat for microsurgery

A well-organised and consistent surgical preparation system could avoid errors and facilitate surgery. On the day prior to surgery, non-autoclavable surgical instruments such as atraumatic grasper, trocar and cannula, fibre optic cable, light probe for endoscope, silicone tubing of flushing system and gas tubing with luer lock at both ends for CO_2 system were disinfected by immersing completely in Gigasept® solution (10%) for 10 minutes and subsequently rinsed in sterile autoclaved distilled water before placing on a sterile surgical table-cum-trolley. Autoclavable surgical instruments were ready to use after being autoclaved and dried completely in the oven (56^oC). The outer wrapping of the surgical pack was opened and unfolded carefully without touching the sterilised instruments inside. All the surgical instruments were assembled and arranged in a consistent order on a sterile surgical table-cum-trolley which was already covered with a sterile drape.

When the donor goat had immobilised, it was placed on a clean small ruminat restraining cradle. The restraining table was set at 45^o angle with the head of the goat lowered to facilitate the laparoscopy procedure. The abdominal area of the donor goat

was disinfected with diluted Hibiscrub (10%) using clean gauzes and the hair shaved. After shaving, the bare skin was wiped with undiluted Hibiscrub and subsequently with weak iodine solution. The donor goat was then covered with sterile drape with an opening that revealed the disinfected bare skin and was ready for oocyte retrieval surgery. The drape was positioned without the fabric dragging across a non-sterile surface and secured in place with towel clamps at four corners of the surgical site.

3.4.6.3 Oocyte retrieval and search

The light probe was connected to a light system via fibre optic cable. The light system, in turn, was connected to a CCD camera and monitor. The lens of the light probe and the entire length of the fibre optic cable were disinfected with ethanol (70%) and white balance was performed. A small incision was made on the disinfected abdominal area and a trocar connected to a CO_2 tank via the CO_2 insufflator was inserted into the incision to insufflate the abdominal cavity with CO_2. Once the peritoneum was expanded in order to facilitate visualisation of the reproductive tract, three small incisions (3-5 mm) were made, one near the umbilicus to facilitate insertion of trocar for passing the laparoscope, one on the right side of lower-ventral abdomen to insert the trocar for passing the grasping forceps and one on the left side of lower-ventral abdomen for passing oocyte retrieval needle. The light probe and grasper were inserted into each trocar, respectively; and finally the oocyte retrieval needle was inserted directly into the left side of the abdominal cavity.

The collection medium (Table 3.2) for oocyte retrieval was pre-warmed (38.5^{o}C)

and filled in a sterile luer slip syringe (50 ml) and placed horizontally in the aspiration system. The collection tubes (disposable round-bottom test tubes, 14 ml) were placed in a test tube heating system to maintain constant temperature (38.5^{o}C) during LOPU. The oocyte retrieval needle was sterilised by application of UV light for 30 minutes inside the laminar flow cabinet and rinsed with collection medium prior to use.

The uterine horns were gently manipulated to allow visualisation of each stimulated ovary using the grasper. Once an ovary was identified, the ovarian ligament was grasped, without damaging the oviduct, to effectively stabilise the ovary for follicular puncture. The ovarian characteristics were recorded for each donor goat prior to the follicle puncture procedure. During the surgery, the ovaries were exposed by pulling the fimbria in different directions using the grasping forceps and the follicular contents were aspirated from all follicles visible on the surface of the ovaries by puncturing follicles with the aspiration needle. The collection tubes containing aspirated fluid (1 to 3 ml) were then passed to embryologists for oocyte search. Before oocyte search began, it was necessary to ensure that the microscope stage was warmed to 38.5^{o}C. During the oocyte search, the follicular contents were dispensed into a sterile petri dish (90 mm) for oocyte isolation and evaluation under a stereomicroscope (magnification 20X and 40X). Oocytes were then washed in flushing medium and cultured according oocyte grades for subsequent IVM-ICSI procedures.

After oocyte retrieval, the ovary was rinsed with warm heparinised (38.5^{o}C) physiological saline using an insemination gun introduced through one of the trocar to

aid in reducing adhesions following oocyte aspiration. The ovary was then released and the incisions on the abdomen were sutured; and finally the donor goat was carefully removed from the cradle. The sutured incision area was sprayed with antiseptic and insecticide containing cyphenothrin. The donor goat was administered with oxytetracycline (20 mg/kg body weight) via intramuscular injection once in four days within the duration of 2 weeks to prevent possible post-surgical infection. Generally, the oocyte retrieval procedure needed 30 to 40 minutes to complete as well as depending on the number of follicles to be aspirated.

After oocyte retrieval surgery was completely, all surgical instruments were washed in diluted 7X®-PF solution using a sponge and immediately rinsed five times with running tap-water followed by two times with RO water. Each instrument was inspected to ensure that all debris (especially blood clot) had been removed. After physical cleaning, instruments were drained dry. The outer surface of the aspiration needle was disinfected with ethanol (70%) and the openings were flushed with ethanol (70%) using a syringe (20 ml) attached to a needle (18G), then drained dry and packed in a surgical bag. Autoclavable surgical instruments were not too densely packed in the autoclave bag to allow for adequate steam penetration in the autoclave machine. An indicator test strip was placed on the surgical pack which recorded the date of sterilisation. Non-autoclavable surgical instruments were drained dry and packed individually in a clean transparent bag before proceeding to UV sterilisation (30 minutes).

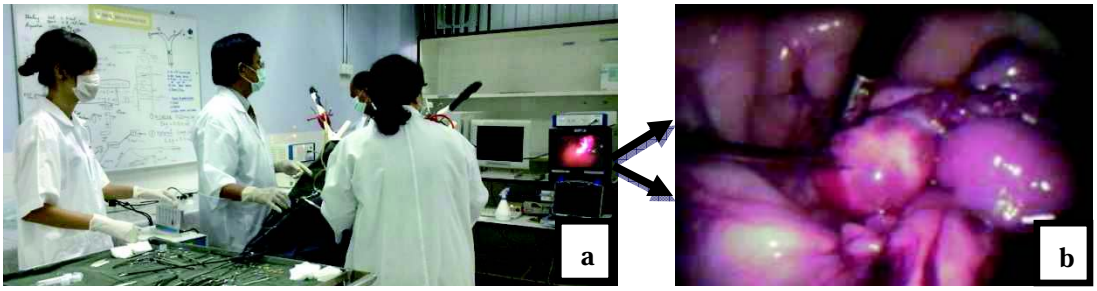

Figure 3.6: Laparoscopic oocyte pick-up (LOPU) procedure. (a) LOPU was carried out by placing the donor goat on a cradle for laparoscopic procedure and (b) The follicle puncture and follicular fluid aspiration were performed.

3.4.7 *In Vitro* Production (IVP) of Goat Embryos

The IVP embryos involved the retrieval of immature oocytes from the ovarian follicles of hyperstimulated-donor goats underwent the IVM process, followed by ICSI and subsequent IVC of embryos. The entire *in vitro* experiments took approximately 10 days.

3.4.7.1 *In vitro* maturation (IVM)

IVM microdroplets (50 µl or 100 µl sized) were prepared and overlaid with equilibrated light mineral oil in a petri dish (35 mm) as well as labelled with the author's initials, date and type of experiment. The dish was equilibrated overnight and kept in CO_2 incubator (5%) throughout the oocyte retrieval surgery to permit temperature and gas equilibration. Before oocyte retrieval, the stereoscope was attached to a stage warmer at 38.5^{o}C prior to embryo search due to the oocytes were very sensitive to temperature. Follicular contents in the collection tube were dispensed into a petri dish (90 mm) and examined under the microscope. The cumulus-oocyte

complex (COC) was located and picked using a hand-controlled pipette. The COC was immediately washed in the washing IVM microdroplets (50 µl) for three times to remove debris and subsequently transferred into the IVM microdroplets (10-12 oocytes/50 µl and 20-25 oocytes/100 µl). The COCs were graded and cultured in separate microdroplets based on their cumulus cell (CC) investment and morphology of the oocyte as described by Rahman *et al.* (2007a) which is depicted in Table 3.16

Table 3.16: Grading of the recovered oocytes according to the cumulus cell investment and morphology of the oocyte

Characteristics of COCs and CFOs	Grades
COCs with more than 5 complete layers of cumulus cells (CCs), finely granulated homogenous ooplasm and normal morphological features.	Grade A
COCs with 3-5 complete layers of CCs, finely granulated homogenous ooplasm and normal morphological features.	Grade B
COCs with 1-2 complete layers of CCs or COCs with 3-5 partially invested CC layers, finely granulated homogenous ooplasm and normal morphological features.	Grade C
CFOs or oocyte with incomplete investment of CCs (1-2 layers), finely granulated homogeneous ooplasm and normal morphological features.	Grade D
Degenerating oocyte or oocyte with abnormal, size, shape and heterogeneous ooplasm, or apoptotic in jelly-like CC investment or very small oocytes.	Grade E

Adapted from Rahman *et al.* (2007a).

IVM was performed at 38.5°C in humidified atmosphere with CO_2 (5%) humidified air for stipulated durations ranging from 22-29 hours depending on the experimental treatment. At the end of the maturation culture, cumulus expansion was scored into two categories, i.e. either complexes floating in the maturation medium and surrounded by a light-coloured fully expanded cumulus mass (were defined as 'cumulus-expanded'), or vice versa (were defined as 'cumulus-unexpanded).

3.4.7.2 Intracytoplasmic sperm injection (ICSI)

The ICSI procedure involved preparation of oocytes, sperm and microinjection dish, alignment of injection and holding needles; and sperm immobilisation.

3.4.7.2(a) Preparation of oocytes for ICSI

After maturation, cumulus cells were removed from oocytes by repeated pipetting in HEPES-buffered IVC medium containing hyaluronidase (100 µl; 80 IU/ml). The speed of removing the cumulus cells was hastened when the COC was first manually aspirated in and out of a sterile Pasteur pipette. When most of the cumulus cells have been removed, the oocyte was further aspirated with drawn-out Pasteur pipettes with an opening of about 250 µm and 200 µm in diameter, respectively, under a dissecting microscope. These denuded oocytes were washed through 3 microdroplets of HEPES-buffered IVC medium and accessed for maturation under an inverted microscope. Oocytes with a clear first polar body (PB-1) were considered as metaphase II (MII) stage and meiotic competent. These oocytes were transferred to another dish and washed through three microdroplets (50 µl) of IVC medium and then left in the final microdroplets; and incubated in the presence of CO_2 (5%) at 38.5^{o}C until ICSI. Oocytes without PB-1 were considered immature and transferred to another dish and washed through three microdroplets (50 µl) of IVM medium and then left in the final microdroplets; and incubated in the presence of CO_2 (5%) at 38.5^{o}C until second assessment for maturation and ICSI.

3.4.7.2(b) Preparation of sperm for ICSI

Sperm preparation of frozen-thawed semen was performed at approximately 90 minutes the before IVM duration ended. The source of frozen semen was from a sperm bank of proven fertility. First, a frozen straw of semen was removed aseptically from the liquid nitrogen (LN_2) tank and pre-thawed at room temperature (25^{o}C, 1 minute) followed by thawing in a water bath (37^{o}C, 1 minute). The straw was removed from the water bath and outer surface of the straw was disinfected with ethanol (70%) and allowed to dry. Equilibrated overnight sperm wash medium (2 ml) was poured into a sterile conical tube (15 ml). Using a pair of sterile scissors, the straw was cut at one end (sealed end) and then inserted into the cut pipette tip attached to a syringe (1 ml). The other end (cotton-plugged end) of the straw was then cut and gently passed through the medium and semen was released slowly into the bottom of the tube. A drop of the semen residue left in the straw was placed onto a glass slide and checked under the microscope to make sure that the sperm were alive and actively moving. With the tube tightly capped, the tube was centrifuged (200 x *g*, 10 minutes) and the supernatant was discarded. Fresh sperm wash medium was added to the pellet to a total volume of 2 ml. The tube was flicked to mix the pellet with fresh sperm wash medium. With the tube tightly capped, the tube was again centrifuged (200 x *g*, 10 minutes) and the supernatant was discarded carefully without agitating the sperm pellet. After two times washing, the sperm pellet was resuspended in sperm wash and sperm capacitation medium containing heparin (1:1); and incubated (38.5^{o}C in 5% CO_2 in air, 15 minutes). The capacitation-treated sperm tube was withdrawn from the

CO_2 incubator and kept in the rack inside the laminar flow cabinet prior to ICSI procedure.

3.4.7.2(c) Preparation of microinjection dish

The microinjection dish was prepared on the cover of a petri dish (35 mm). Four microdroplets (5 µl) of ICSI medium were placed on the right side, closed to the centre of the dish. These microdroplets were numbered as 1, 2, 3 and 4 to avoid confusion during ICSI. One elongated microdroplet (10 µl) of PVP (10%) was placed at the centre of the dish. The PVP microdroplet was spread longitudinally to make it completely flattened so that sperm can be immobilised easily. Two elongated microdroplets (5 µl) of sperm medium were prepared on the left side of PVP microdroplet. The whole dish was then over-laid with mineral oil as soon as possible and the oil should just over the microdroplets completely to avoid evaporation. The microdroplets of ICSI medium were prepared not too close to the edge of dish or to microdroplets of sperm and PVP to avoid mixing. Microinjection dish was equilibrated on the stage warmer (38.5^{o}C, 15 minutes).

3.4.7.2(d) Alignment of injection and holding needles

Aligning the injection and holding needles over the microinjection dish so that they looked 'straight' under the microscope would ensure a smooth and easy performance of the ICSI procedure. All the knobs (X-, Y- and Z-control) and the syringes (3 ml) were adjusted to the centre of the scale. Alignment of the needles was done over a

petri dish cover (35 mm). Two ‘flatten” elongated microdroplets were prepared containing ICSI medium (5 µl) and PVP (10%; 5 µl) over-laid with mineral oil at the centre of the petri dish cover. This dish was used to align and stabilise the pressure within the needle. Alignment was started with the injection needle followed by holding needle. The injection needle was inserted to the needle holder (right micromanipulation), tightened well and placed above the PVP microdroplet. The tip of the injection and holding needle was left in the mineral oil for few minutes to allow equilibration to enhance the control within the needles before placed into ICSI and PVP microdroplet, respectively. Then the outline of the PVP microdroplet was sharply focused before the injection needle was lowered into the microdroplet. The injection needle was then checked for smooth X-, Y- and Z- movement along the bottom of the dish. Smooth and gentle aspiration and expulsion of the PVP was also checked. The injection needle was left in the PVP microdroplet for a few minutes to allow equilibration to establish by capillary action. The holding needle was mounted on the left micromanipulator and controlled by the right joystick, was placed above the ICSI microdroplet and adjusted to be ‘straight’. The holding needle should not be placed into the PVP microdroplet because shrinkage of the oocyte can occur if the oocyte has intimate contact with PVP. The holding needle was then focused with the injection needle and was aligned so that the working tips were parallel to the microscope stage under low magnification (4X objective). Finally both the holding and injection needles were checked under high magnification (10X objective) to assure the accurate alignment (sharply in focus) and parallel.

3.4.7.2(e) Transfer of gametes onto the microinjection dish

Capacitated and washed sperm suspension (1 to 2 µl) was placed on the left side of two elongated sperm microdroplets. This allowed sperm to swim towards the right side of the microdroplets in a few minutes. Four MII oocytes were washed three times in ICSI medium and then transferred one in each of the microdroplet of ICSI medium prepared on microinjection dish. The ICSI procedure was carried out on the heated stage (38.5^{o}C) of the microscope with magnification of 4X, 10X and 20X objectives, respectively.

3.4.7.2(f) Sperm immobilisation

The most motile sperm will be found at the periphery of the sperm microdroplet. Therefore, the injection needle was lowered to the edge of the sperm microdroplet so that its open end faced into it. The motile sperm were then either swam into the injection needle or be aspirated into it by capillary action. Once sufficient sperm (3 to 5) for injection have entered the injection needle, they were deposited into the PVP microdroplet. The injection needle was lowered; the sperm inside the needle were focused and then released into the PVP microdroplet slowly. A morphologically normal motile sperm was aspirated again, head-first, into the injection needle and brought to a more flattened area and released. The sperm was rotated until it was aligned vertical to the injection needle. Then, the injection was raised slightly above the bottom of the microinjection dish, passed its tip over the sperm tail, lowered it until it made contact with the bottom of the microinjection dish, and drew it rapidly

across the sperm tail. The immobilised sperm was confirmed if the tail of the sperm was clearly kinked following the procedure. Once immobile, the sperm was aspirated tail first into the injection needle.

3.4.7.2(g) Sperm injection into the oocytes

The injection needle with the immobilised sperm was brought to the microdroplet containing the oocyte. The polar body was placed at 6 or 12 o'clock and achieved by rotating the oocyte at the tip of the holding needle with the injection needle or by gentle suction and releasing of the oocyte by the holding needle. While the oocyte was held firmly by the holding needle, the injection needle was brought close to the oocyte. The injection needle was then focused to the same focal plane as the oolemma, and the sperm was then gently pushed towards the tip of the injection needle. When the sperm was sufficiently close to the tip (20 µm), the injection needle was then slowly advanced through the zona pellucida and into the ooplasm at 3 o'clock position. A small amount of ooplasm (1 to 2 pl) was gently aspirated into the needle when the injection needle almost touched the opposite sire at 9 o'clock position. The ooplasm was sucked in and out vigorously but gently to break the oolemma. A sudden flux of ooplasm into the needle confirmed the oolemma breakage, thereby facilitating sperm injection. A single sperm was gently deposited into the ooplasm with a minimum volume of PVP (<5 pl). After the injection, the needle was gently removed and the oocyte was released from the holding needle. The whole process was repeated until injection of all four oocytes in each ICSI microdroplets. Sham injections were

performed in a similar manner. The oolemma was ruptured and the ooplasm was aspirated into the injection pipette and re-injected into the oocyte with a minimum volume of medium without carrying a sperm.

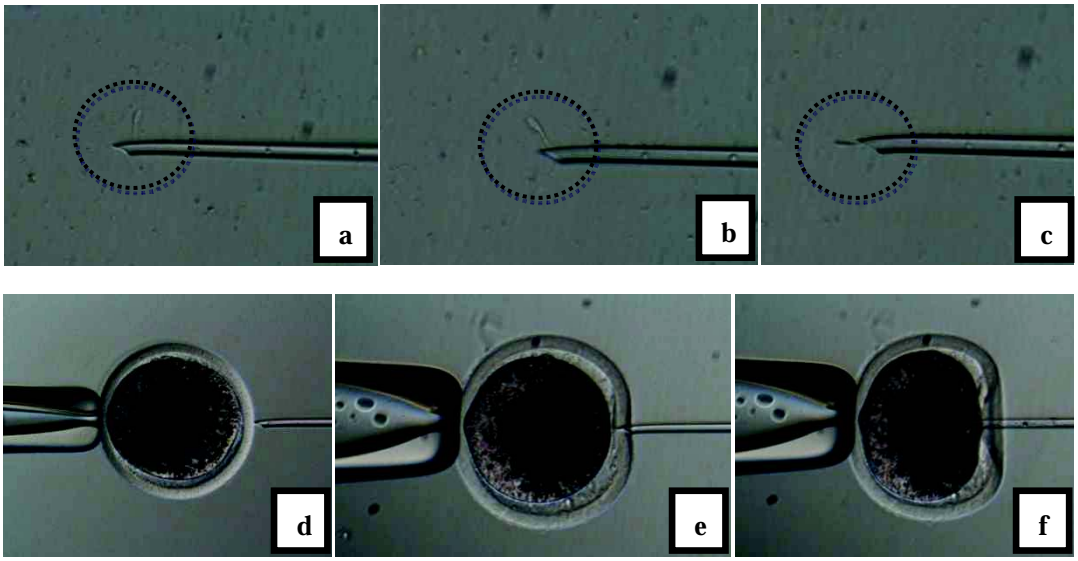

Figure 3.7 (a-f): Sperm immobilisation, aspiration and injection process. (a) Crushing the mid-piece of sperm tail with ICSI needle; (b) crushed tail with evidenced by a kink (point of break) on the tail, (c) aspiration of immobilised sperm in tail-first manned into the ICSI needle; (d) a MII oocytes is held with PB-1 at 12 o'clock with holding needle and ICSI needle is positioned at 3 o'clock with sperm inside; (e)ICSI needle is pushed through the zona pellucida and oolemma; and (f) ooplasm aspiration, reinjection and deposition of sperm.

3.4.7.2(h) Chemical treatments of goat oocytes

Three different chemical treatments were described as follow for both ICSI and sham-injection groups: 1) control ICSI or sham injection without chemical treatment, 2) treated with calcium ionophore (Ca^{2+} ionophore) after ICSI or sham injection and 3) treated with Ca^{2+} ionophore followed by 6-dimathylaminiourine (6-DMAP) after ICSI and sham injection. For the group of injected oocytes were treated with Ca^{2+} ionophore, oocytes were washed three times in IVC medium and then exposed to Ca^{2+}

inophore (10 µM) for 5 minutes at 38.5°C. Meanwhile, for the group of injected oocytes were treated with Ca^{2+} ionophore followed by 6-DMAP, oocytes were treated with Ca^{2+} inophore (10 µM) for 5 minutes and were then transferred to a microdroplet of 6-DMAP (1.9 mM) for an additional 3 hours; and incubated in the presence of CO_2 (5%) at 38.5°C.

3.4.7.2(i) In vitro embryo culture

The culture medium used for *in vitro* embryo culture was described in Table 3.15. Microdroplets of embryo culture (50 µl) were prepared on a petri dish (35 mm) and equilibrated overnight under purified mineral oil to permit temperature and gas equilibration. All injected, sham-injected, Ca^{2+} ionophore-treated or Ca^{2+} ionophore followed by 6-DMAP treated oocytes were washed three times with IVC medium before being transferred in the final IVC microdroplets; and cultured at 38.5°C in 55% CO_2 and humidified air for 9 days. The embryos were evaluated daily for embryonic development.

3.5 EXPERIMENTAL DESIGN

In the present research, the author attempted to produce *in vitro* goat embryos through ICSI technique from LOPU-derived oocytes. The present study was carried out to investigate the effects of: a) LOPU-derived oocytes from superovulated-donor goats on quantity and quality of oocytes obtained, b) different dosages of PMSG, c) different durations of IVM; and d) different chemical treatments on the ability of these

oocytes to be fertilised through ICSI as well as underwent embryonic development *in vitro*.

3.5.1 Effect of Goat Genotype, Body Weight, Age, Oocyte Retrieval Cycle and Ovarian Stimulation Response after Superovulation on Quantity and Quality of Oocytes (Experiment 1)

A total of 84 female goats were selected compromising of Boer crossbred and Jamnapari breed goats. These goats were sourced from the ISB Mini Livestock Farm or purchased from local farm. To study the effect of superovulation on the quantity and quality of oocytes, donor goats were synchronised (CIDR device and Estrumate®) and stimulated with gonadotrophins (Folligon® and Ovidrel®) [Sections 3.4.5.1 and 3.4.5.2]. The OR surgery was conducted at the onset of oestrus by LOPU technique [Section 3.4.6]. A total of 42 surgery sessions were performed for retrieval of immature oocytes. In the OR surgery, only one side (either left or right) of the ovary from a donor goat was allocated for the present study and the other side was for the other study of cloning. However, on some occasions, both ovaries from one donor goat were assigned for the present study.

For the genotype group, two breeds of goat were investigated on the quantity and quality of oocytes retrieved per ovary from superovulated donors. Female donors were from Boer crossbred and Jamnapari breed goats. Purchased goats were quarantined for at least 1 month to confirm the goats were disease-free prior to use for experiments. For the body weight group, the donor goats were divided into three body weight group: light (<20 kg), medium (20-29 kg) and heavy (>29 kg). For the age

group, female donors were ranging from 6 to 42 months old. The donor goats were divided into three age groups: Group 1 (<18 months), Group 2 (18 to 24 months) and Group 3 (>24 months). To standardise the experiment, effects of body weight and age were investigated based on OR1 cycle, in which, the data of oocyte retrieved from the OR2 and OR3 cycles were omitted. This was due to avoid the reasons such as animal's health status, low response of donor goats to superovulation or physically abnormal ovary (adhesion or degenerative ovary) in the quantity and quality oocytes retrieved from repeated OR cycles. For the OR cycle group, female donor with various OR cycle background were investigated, i.e. OR1 (first OR cycle) and OR2 (second repeated OR cycle), and data of oocyte retrieved from the OR3 cycle were omitted due to the limited number of donor goats from OR3 cycle allocated for the present study. Donor goats were reused for the following OR surgery in an interval of 3 months between the surgeries. For the stimulation response group, the ovaries were categorised into three stimulation response groups: excellent (>10 follicles), good (5 to 10 follicles) and satisfactory (<5 follicles).

The quantity and quality of oocytes retrieved per ovary was recorded based on different oocyte grades (Table 3.16). Oocytes from different factor groups and different oocytes grades were cultured separately in microdroplets of IVM in the CO_2 incubator in the presence of CO_2 (5%) at 38.5^{o}C. The effects of genotype, body weight, age, OR cycle and stimulation response on quantity and quality of immature oocytes retrieved per ovary were analysed by analysis of variance (ANOVA), followed by Duncan's multiple range tests (DMRT). Correlation coefficients (r)

between body weight and age in Boer crossbred goats were performed. Meanwhile, body weight and age on the number of oocytes retrieved in goats were analysed statistically with regression coefficients (r^2).

3.5.2 Effect of PMSG Dosage on Quantity and Quality of Oocyte, IVM Rate and Subsequent Embryo Development Following ICSI in Goat (Experiment 2)

In this experiment, the effect of PMSG dosage on the quantity and quality oocyte retrieved; and developmental competence of *in vitro* matured-fertilised embryos were studied. A total of 82 healthy donor goats were randomly selected and treated with reproductive hormones for oestrus synchronisation (CIDR device and Estrumate®), followed by superovulation (PMSG either 1200 IU or 1500 IU; and Ovidrel®) [Sections 3.4.5.1 and 3.4.5.2]. The quantity and quality of oocytes retrieved per ovary was recorded based on different oocyte grades. Immature oocytes were then cultured in IVM microdroplets (based on different grades) for stipulated durations ranging from 22-29 hours depending on the experimental treatment [Section 3.4.7.1]. At the end of IVM culture, the COCs were denuded using hyaluronidase to identify the MII oocytes [Section 3.4.7.2(a)]. The maturation rate was recorded as percentage calculated from the number of oocytes used in IVM culture, in the respective oocyte grades.

Only matured oocytes of Grades A, B and C were subsequently used for the ICSI experiment [Section 3.4.7.2(g)] and the sperm-injected oocytes were cultured in IVC

medium in CO_2 (5%) in humidified air at 38.5°C [Section 3.4.7.2(i)]. The cleavage rate (2-cell) was recorded as percentage calculated from the number of oocytes used in ICSI experiment, in the respective oocyte grades. The embryos were cultured for daily evaluation on developmental competence. The developmental rate was recorded as percentage calculated from the number of 2-cell stage embryos cultured in IVC, in the respective oocyte grades.

The quantity and quality of oocyte retrieved per ovary, rate of maturation, cleavage and developmental were recorded in percentage for the respective grades. Data were analysed by analysis of variance (ANOVA), followed by Duncan's multiple range tests (DMRT).

3.5.3 Effect of Oocyte Quality and Duration of IVM on the Rates of Maturation, Cleavage and Developmental Competence of *In Vitro* Produced Goat Embryos by ICSI Technique (Experiment 3)

In this experiment, the effect of IVM duration on LOPU-derived goat oocytes toward embryo yield through ICSI technique was investigated. The parameters of IVM duration tested were 22 to 25 hours and 26 to 29 hours. Briefly, donor goats were oestrus-synchronised, superovulated and the oocytes retrieved via LOPU [Sections 3.4.5 and 3.4.6]. There were five grades of oocytes (Grades A, B, C, D and E) based on cellular vestments and cytoplasm uniformity (Table 3.16).

The oocytes were washed in the washing IVM microdroplets (50 µl) for three times and subsequently transferred into the IVM microdroplets (10-12 oocytes/50 µl and 20-25 oocytes/100 µl). Separate batches of oocytes were IVM-cultured for 22 to

25 hours and 26 to 29 hours, respectively, in CO_2 (5%) in humidified air at 38.5°C [Section 3.4.7.1]. The maturation rate was recorded as percentage calculated from the number of oocytes used in IVM culture, in the respective oocyte grades.

After completing their respective IVM duration, the oocytes were sperm-injected [Section 3.4.7.2(g)] and cultured in IVC medium [Section 3.4.7.2(i)] for daily evaluation on developmental competence. The cleavage rate (2-cell) was recorded as percentage calculated from the number of oocytes used in ICSI experiment, in the respective oocyte grades. Meanwhile, the developmental rate was recorded as percentage calculated from the number of 2-cell stage embryos cultured in IVC, in the respective oocyte grades.

The rates of maturation, cleavage and developmental were recorded in percentage for the respective oocyte grades. Data were analysed by analysis of variance (ANOVA), followed by Duncan's multiple range tests (DMRT).

3.5.4 Effect of Chemical Treatments on the Developmental Competence of *In Vitro* Produced Goat Embryos Fertilised by ICSI Technique (Experiment 4)

This study was designed to compare the chemical treatments and embryo developmental competence of LOPU-derived *in vitro* matured oocytes fertilised by ICSI or sham injection (without a sperm). At the end of maturation culture, oocytes were stripped of their cumulus cells by pipetting in HEPES-buffered IVC medium containing hyaluronidase (100 µl; 80 IU/ml) [Section 3.4.7.2(a)]. Denuded MII

oocytes were selected, washed and cultured prior to ICSI experiment. Only matured oocytes of Grades A, B and C were used in this experiment since the availability of small number of IVM oocytes of Grades D and E. Frozen semen was taken from male donors of proven fertility. In brief, MII oocyte was injected with one sperm into the ooplasm with a minimum volume of medium (<5 pl). Sham injection was performed in a similar manner without sperm cell but expelling a similar volume of PVP as in ICSI. The oolemma was ruptured and the ooplasm was aspirated into the injection needle and re-injected into the oocyte with a minimum volume of medium [Section 3.4.7.2(g)]. Three different chemical treatments were followed for both ICSI and sham injected groups: 1) control ICSI or sham injection without chemical treatment: in this treatment, denuded oocytes were subjected to sperm or sham injection followed by cultured in IVC without treatment; 2) treated with calcium ionophore (Ca^{2+} ionophore) after ICSI or sham injection: injected oocytes were exposed to Ca^{2+} inophore (10 µM) for 5 minutes at 38.5°C and cultured; and 3) treated with Ca^{2+} ionophore followed by 6-dimathylaminiourine (6-DMAP) after ICSI and sham injection: injected oocytes were treated with Ca^{2+} inophore (10 µM) for 5 minutes and were then transferred to a microdroplet of 6-DMAP (1.9 mM) for an additional 3 hours in the presence of CO_2 (5%) at 38.5°C [Section 3.4.7.2(h)]. All the injected oocytes were washed three times before being cultured in IVC medium and checked every 24 hours to record the developmental stage [Section 3.4.7.2(i)]. The cleavage rate (2-cell) was recorded as percentage calculated from the number of oocytes used in ICSI experiment, in the respective oocyte grades. Meanwhile, the developmental

rate was recorded as percentage calculated from the number of 2-cell stage embryos cultured in IVC, in the respective oocyte grades.

The rates of cleavage and developmental were recorded in percentage for the respective chemical treatments. Data was analysed by analysis of variance (ANOVA), followed by Duncan's multiple range tests (DMRT).

3.6 STATISTICAL ANALYSIS

The effects of goat genotype, body weight, age, oocyte retrieval cycle and stimulation response after superovulation on quantity and quality of oocytes (Experiment 1); PMSG dosage on quantity, quality, IVM and subsequent embryo development following ICSI in goat (Experiment 2); oocyte quality and duration of IVM on the rates of maturation, cleavage and developmental competence of *in vitro* produced goat embryos (Experiment 3); as well as chemical treatments on the developmental competence of *in vitro* produced goat embryos fertilised by ICSI technique (Experiment 4), were all analysed by using one-way analysis of variance (ANOVA). The effects of different factors on LOPU, IVM, cleavage and embryo development following ICSI, specific in each experiment, were compare and the significant differences between the means were further analysed using Duncan's Multiple Range Test (DMRT) to show the specific differences among the factors on the parameter measured and $P<0.05$ was considered significant for all statistical tests. In Experiment 1, correlation coefficients (r) between body weight and age in Boer crossbred goats were performed using SPSS programme. Meanwhile, body weight and age on the

number of oocytes retrieved in goats were determined with regression coefficients (r^2). A 5% significance level was used for the statistical tests. The data presented in various experiments were as mean± standard error of means (mean±SEM). The analysis was carried out with the SPSS (Statistical Packages for Social Science) version 17, SPSS Inc, USA.

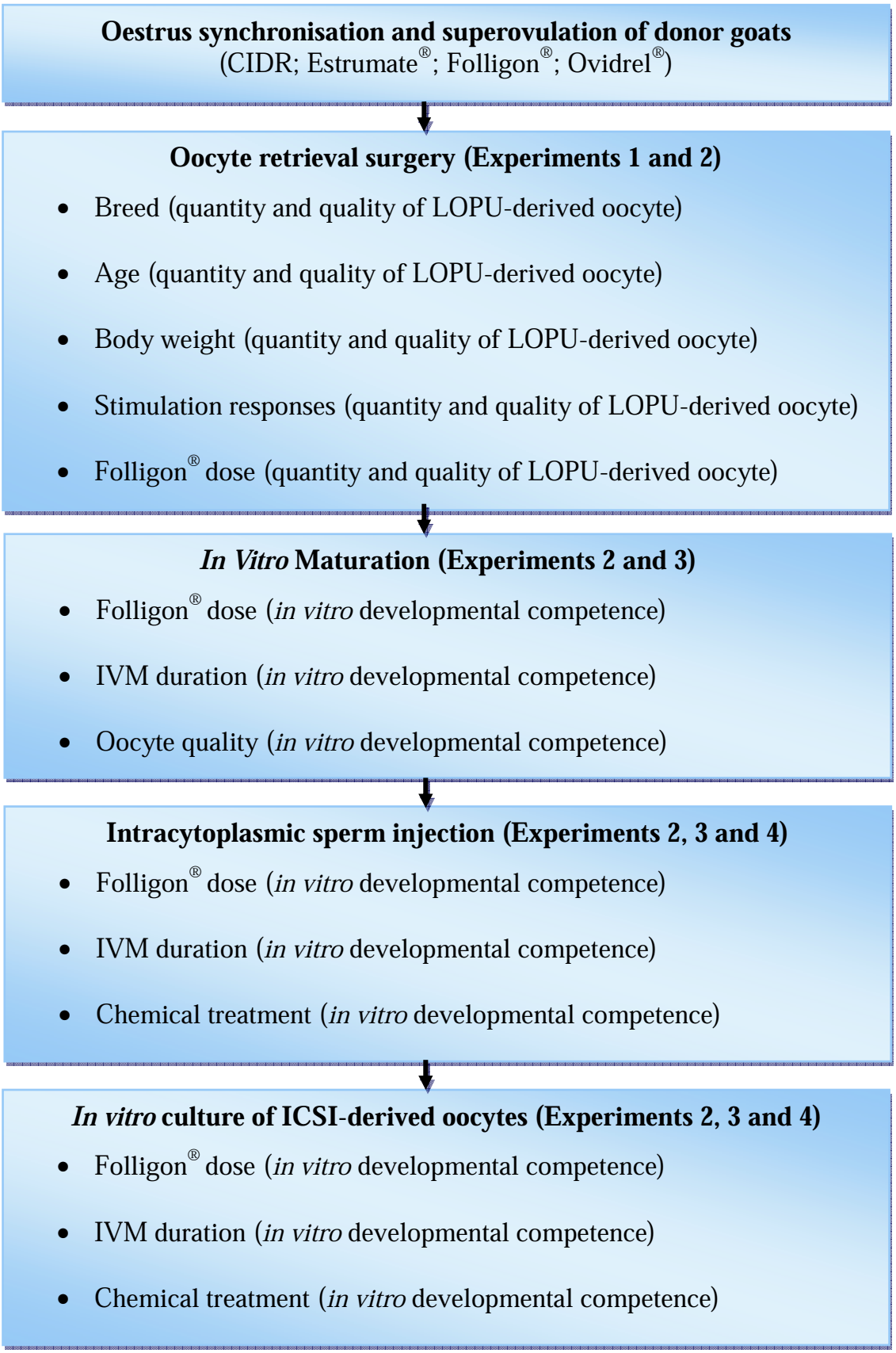

Figure 3.8: A schematic overview of the experimental design for *in vitro* produced ICSI-derived embryos.

Chapter 4

4.0 RESULTS

Chapter 4

4.0 RESULTS

4.1 EFFECT OF GOAT GENOTYPE, BODY WEIGHT, AGE, OOCYTE RETRIEVAL CYCLE AND OVARIAN STIMULATION RESPONSE AFTER SUPEROVULATION ON QUANTITY AND QUALITY OF OOCYTES (EXPERIMENT 1)

For the goat genotype group (Tables 4.1 and 4.2), a total of 420 oocytes were retrieved from 84 ovaries with an average of 4.92±0.32 oocytes retrieved per ovary. A total of 43 oocytes were retrieved from 12 ovaries of Jamnapari breed goats and 377 oocytes from 72 ovaries of Boer crossbred goats. An average of 3.58±0.56 and 5.14±0.35 oocytes were retrieved per ovary from the Jamnapari breed and Boer crossbred goats, respectively. No significant differences ($P>0.05$) were observed in the percentage and number of oocytes retrieved per ovary between the two genotypes, in the respective grades. However, there were significant differences ($P<0.05$) in the percentage and number of oocytes retrieved per ovary among the oocyte grades within each goat breed. Grades A, B and C (80.04%, average of 4.20 oocytes per ovary) yielded higher number of oocytes retrieved compared to Grades D and E (19.96%, average of 0.80 oocytes per ovary). The average percentages for oocytes of Grades A, B, C, D and E retrieved per ovary were 23.52±2.95%, 19.51±2.60%, 37.01±3.13%, 9.56±1.91% and 10.40±2.71%, respectively. Correspondingly, the average numbers of oocytes retrieved per ovary were 1.33±0.18, 0.99±0.13, 1.88±0.17, 0.44±0.08 and 0.36±0.09, respectively.

For the body weight group (Tables 4.3 and 4.4), a total of 272 oocytes were retrieved from 52 ovaries with an average of 5.17±0.44 oocytes retrieved per ovary. To standardise the experiment, effect of body weight was studied from the OR1 cycle, in which, the data retrieved from the OR2 and OR3 cycles were omitted. A total of 137, 108 and 27 oocytes were obtained from 21, 23 and 8 ovaries of light, medium and heavy body weight goats,

respectively. A significant difference (P<0.05) was observed in the number of oocytes retrieved per ovary between light body weight (6.52±0.75) versus heavy body weight (3.38±0.44) goats. No significant difference (P>0.05) was observed in the number of oocytes retrieved per ovary among light and medium body weight goats; and medium and heavy body weight goats. However, a significant difference (P<0.05) was observed in the number of oocytes retrieved per ovary from Grades A, B and C between light body weight goats (1.86±0.39, 1.29±0.29 and 2.71±0.35, respectively) and corresponding oocytes grades for heavy body weight goats (0.25±0.16, 0.25±0.25 and 1.25±0.53, respectively). For Grade E oocytes, there were significant differences (P<0.05) between light body weight (2.78±2.39%, average of 0.10±0.07 oocytes per ovary) and heavy body weight (29.17±15.98%, average of 0.75±0.41 oocytes per ovary) goats. Most of the oocytes retrieved from light, medium and heavy goats were of Grades A and C (68.77%, average of 4.57 oocytes per ovary), Grades A and C (51.55%, average of 2.74 oocytes per ovary) and Grades C and D (55.84%, average of 2.13 oocytes per ovary), respectively.

For the age group (Tables 4.5 and 4.6), a total of 228 oocytes were retrieved from 38 ovaries with an average of 6.00±0.52 oocytes retrieved per ovary. To standardise the experiment, effect of age was studied from the OR1 cycle, in which, the data retrieved from the OR2 and OR3 cycles were omitted. A total of 82, 87 and 59 oocytes were retrieved from 14, 14 and 10 ovaries of Group 1, 2 and 3 age goats, respectively. Averages of 5.86±0.75, 6.21±1.03 and 5.90±0.52 oocytes per ovary were retrieved from Group 1, 2 and 3 age goats, respectively. No significant differences (P>0.05) were observed in the percentage and number of oocyte retrieved per ovary among the three age groups, in the respective grades. However, there were significant differences (P<0.05) in the percentage and number of oocytes retrieved per ovary between oocyte grades of Grades A, B or C versus Grades D and E. Grades A, B and C (85.62%, average of 5.31 oocytes per ovary) yielded higher number of

oocytes retrieved compared to Grades D and E (14.38%, average of 0.68 oocytes per ovary). The average percentages for oocytes of Grades A, B, C, D and E retrieved per ovary were 26.60±4.10%, 21.38±3.40%, 37.64±3.73%, 8.90±2.39% and 5.48±3.15%, respectively. Correspondingly, the average numbers for oocytes retrieved per ovary were 1.68±0.32, 1.26±0.20, 2.37±0.28, 0.50±0.14 and 0.18±0.10, respectively.

For the OR cycle group (Tables 4.7 and 4.8), a total of 400 oocytes were retrieved from 80 ovaries with an average of 5.00±0.33 oocytes retrieved per ovary. To standardise the experiment, effect of OR cycle was studied from the OR1 and OR2 cycles, in which, the data retrieved from the OR3 cycle was omitted. A total of 272 and 128 oocytes were retrieved from 52 and 28 ovaries of goats with OR1 and OR2 cycles, respectively. Average of 5.23±0.44 and 4.57±0.49 oocytes per ovary were retrieved from goats with OR1 and OR2 cycles, respectively. No significant differences ($P>0.05$) were observed in the percentage and number of oocytes retrieved per ovary between the two OR cycles, in the respective grades. However, there were significant differences ($P<0.05$) in the percentage and number of oocytes retrieved per ovary between oocyte grades of Grades A, B or C versus Grades D or E. For the all two OR cycles, average percentages of oocytes retrieved of Grades A, B, C, D and E retrieved per ovary were 24.28±3.06%, 19.78±2.69%, 35.86±3.22%, 9.42±1.97% and 10.67±2.83%, respectively. Correspondingly, the average numbers of oocytes retrieved were 1.38±0.18, 1.00±0.13, 1.83±0.18, 0.44±0.08 and 0.36±0.09, respectively.

For the ovarian stimulation response group (Tables 4.9 and 4.10), a total of 420 oocytes were retrieved from 84 ovaries with an average of 5.00±0.32 oocytes retrieved per ovary. A total of 73, 292 and 55 oocytes were retrieved from 11, 57 and 16 ovaries of excellent, good and satisfactory ovarian stimulation responses. No significant difference ($P>0.05$) was observed in the number of oocytes retrieved per ovary between excellent and good ovarian stimulation response groups. However, a significant difference ($P<0.05$) was

observed in the number of oocytes retrieved per ovary between excellent (6.64±0.81) and satisfactory (3.44±0.58) ovarian stimulation response groups. No significant difference ($P>0.05$) was observed in the number of Grades B (average number of 0.99±0.13), D (average number of 0.44±0.08) and E (average number of 0.36±0.09) oocytes retrieved per ovary between excellent, good and satisfactory ovarian stimulation response groups. For Grades A and C oocytes, there was significant difference ($P<0.05$) among excellent (average of 2.09±0.76 and 2.55±0.46 oocytes per ovary, respectively) and satisfactory (average of 0.81±0.32 and 1.19±0.28 oocytes per ovary, respectively) ovarian stimulation response groups. There were also significant differences ($P<0.05$) in the percentage and number of oocytes retrieved per ovary among the oocytes grades within each ovarian stimulation response group. Most of the oocytes retrieved from excellent, good and satisfactory ovarian stimulation response groups were Grades A and C (67.51%, average of 4.64 oocytes per ovary, 60.77%, average of 3.28 oocytes per ovary and 54.90%, average of 2.00 oocytes per ovary, respectively).

The correlation coefficients (r) between body weight and age in Boer crossbred goats are presented in Table 4.11. There was a significant ($P<0.01$) positive correlation between the body weight and age (r=0.56). Regression coefficients (r^2) for the body weight and age on the number of oocytes retrieved in goats are presented in Table 4.12. The corresponding regression coefficients (r^2) between number of retrieved oocytes and body weight, and between number of retrieved oocytes and age of goats were 0.14 ($P<0.05$) and 0.00 ($P>0.05$) with the equations of prediction of y=-0.16x+8.80 and y=-0.00x+6.03, with y being body weight or age and x being number of oocytes retrieved as shown in Figures 4.1 and 4.2, respectively. Regression coefficients (r^2) for the body weight on the number of oocytes retrieved among the oocyte grades in goats are summarised in Table 4.13. No significant difference ($P>0.05$) was observed between body weight and number of Grades A (r^2=0.07)

and D (r^2=0.00) oocytes retrieved. However, there was a significant (P<0.05) regression correlation between body weight and number of Grades B and E oocytes retrieved (r^2=0.11 and r^2=0.14, respectively). For Grade C oocytes retrieved, there was a significant (P<0.01) high regression correlation with body weight (r^2=0.17). The number of oocytes retrieved among the oocyte grades and the body weight are depicted in Figures 4.3, 4.4, 4.5, 4.6 and 4.7. The corresponding regression coefficients (r^2) between the oocytes of Grades A, B, C, D and E retrieved, and the body weight was described with the following regression equations of y=-0.06x+2.70, y= 0.05x+2.17, y=-0.09x+4.08, y=0.01x+0.41 and y=0.04x-0.56, with y being body weight and x being number of oocyte grades retrieved, respectively.

Table 4.1: Percentage (%, mean±SEM) of oocytes retrieved from goat genotypes

Genotypes	No. of ovaries	No. of oocytes retrieved per ovary	Percent of oocytes retrieved per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
Jamnapari	12	3.58±0.56[a] (n=43)	19.65±9.47[az] (n=9)	10.69±4.97[az] (n=5)	34.58±10.99[az] (n=14)	14.93±6.31[az] (n=7)	20.14±9.81[az] (n=8)
Boer crossbred	72	5.14±0.35[a] (n=377)	24.17±3.08[ay] (n=103)	20.98±2.89[ay] (n=78)	37.41±3.20[az] (n=144)	8.67±1.96[ax] (n=30)	8.78±2.69[ax] (n=22)
Average	42 (n=84)	4.92±0.32 (n=420)	23.52±2.95[y] (n=112)	19.51±2.60[y] (n=83)	37.01±3.13[z] (n=158)	9.56±1.91[x] (n=37)	10.40±2.71[x] (n=30)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[xyz] Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.2: Number (n, mean±SEM) of oocytes retrieved from goat genotypes

Genotypes	No. of ovaries	No. of oocytes retrieved per ovary	No. of oocytes retrieved per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
Jamnapari	12	3.58±0.56[a] (n=43)	0.75±0.31[az] (n=9)	0.42±0.19[az] (n=5)	1.17±0.37[az] (n=14)	0.58±0.23[az] (n=7)	0.67±0.31[az] (n=8)
Boer crossbred	72	5.24±0.35[a] (n=377)	1.43±0.20[ay] (n=103)	1.08±0.14[ay] (n=78)	2.00±0.19[az] (n=144)	0.42±0.08[ax] (n=30)	0.31±0.09[ax] (n=22)
Average	42 (n=84)	4.92±0.32 (n=420)	1.33±0.18[y] (n=112)	0.99±0.13[y] (n=83)	1.88±0.17[z] (n=158)	0.44±0.08[x] (n=37)	0.36±0.09[x] (n=30)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[xyz] Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.3: Percentage (%, mean±SEM) of oocytes retrieved from different body weight groups

Body weight*	No. of ovaries	No. of oocytes retrieved per ovary	Percent of oocytes retrieved per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
Light	21	6.52±0.75^{b} (n=137)	24.39±3.96^{ay} (n=39)	18.99±4.44^{axy} (n=27)	44.38±4.65^{az} (n=57)	9.46±3.15^{awx} (n=12)	2.78±2.39^{aw} (n=2)
Medium	23	4.70±0.61^{ab} (n=108)	23.67±6.43^{az} (n=28)	24.87±5.50^{az} (n=24)	27.88±5.86^{az} (n=35)	10.91±3.90^{az} (n=10)	12.68±5.69^{abz} (n=11)
Heavy	8	3.38±0.44^{a} (n=27)	8.75±6.39^{az} (n=2)	6.25±6.25^{az} (n=2)	28.19±9.21^{az} (n=10)	27.65±11.85^{bz} (n=7)	29.17±15.98^{bz} (n=6)
Average	17.33 (n=52)	5.17±0.44 (n=272)	21.66±3.44^{y} (n=69)	19.63±3.24^{xy} (n=53)	34.59±3.61^{z} (n=102)	12.90±2.87^{xy} (n=29)	11.22±3.75^{x} (n=19)

*Body weight: light (<20 kg), medium (20-29 kg) and heavy (>29 kg)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)

^{wxyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.4: Number (n, mean±SEM) of oocytes retrieved from different body weight groups

Body weight*	No. of ovaries	No. of oocytes retrieved per ovary	No. of oocytes retrieved per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
Light	21	6.52±0.75^{b} (n=137)	1.86±0.39^{by} (n=39)	1.29±0.29^{bxy} (n=27)	2.71±0.35^{bz} (n=57)	0.57±0.20^{awx} (n=12)	0.10±0.07^{aw} (n=2)
Medium	23	4.70±0.61^{ab} (n=108)	1.22±0.40^{abyz} (n=28)	1.04±0.23^{abyz} (n=24)	1.52±0.35^{abz} (n=35)	0.44±0.14^{ay} (n=10)	0.48±0.21^{aby} (n=11)
Heavy	8	3.38±0.44^{a} (n=27)	0.25±0.16^{az} (n=2)	0.25±0.25^{az} (n=2)	1.25±0.53^{az} (n=10)	0.88±0.35^{az} (n=7)	0.75±0.41^{bz} (n=6)
Average	17.33 (n=52)	5.17±0.44 (n=272)	1.33±0.25^{y} (n=69)	1.02±0.16^{xy} (n=53)	1.96±0.24^{z} (n=102)	0.56±0.12^{wx} (n=29)	0.37±0.12^{w} (n=19)

*Body weight: light (<20 kg), medium (20-29 kg) and heavy (>29 kg)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)

^{wxyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.5: Percentage (%, mean±SEM) of oocytes retrieved from different age groups

Age group *	No. of ovaries	No. of oocytes retrieved per ovary	Percent of oocytes retrieved per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
Group 1	14	5.86±0.75^{a} (n=82)	26.03±4.67^{ayz} (n=22)	21.55±5.60^{axy} (n=16)	38.11±5.33^{az} (n=33)	10.15±4.13^{awx} (n=9)	4.17±3.58^{aw} (n=2)
Group 2	14	6.21±1.03^{a} (n=87)	22.93±5.87^{axy} (n=24)	26.82±6.43^{ayz} (n=24)	40.01±6.52^{az} (n=33)	10.23±4.12^{awx} (n=6)	0.00±0.00^{aw} (n=0)
Group 3	10	5.90±0.52^{a} (n=59)	32.52±11.95^{az} (n=18)	13.52±4.73^{ayz} (n=8)	33.68±8.47^{az} (n=24)	5.29±4.29^{ay} (n=4)	15.00±10.67^{ayz} (n=5)
Average	12.67 (n=38)	6.00±0.52 (n=228)	26.60±4.10^{y} (n=64)	21.38±3.40^{y} (n=48)	37.64±3.73^{z} (n=90)	8.90±2.39^{x} (n=19)	5.48±3.15^{x} (n=7)

*Age group: group 1 (<18 months), group 2 (18-24 months) and group 3 (>24 months)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

[wxyz] Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.6: Number (n, mean±SEM) of oocytes retrieved from different age groups

Age group *	No. of ovaries	No. of oocytes retrieved per ovary	No. of oocytes retrieved per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
Group 1	14	5.86±0.75^{a} (n=82)	1.57±0.34^{ayz} (n=22)	1.14±0.28^{ay} (n=16)	2.36±0.46^{az} (n=33)	0.64±0.29^{axy} (n=9)	0.14±0.10^{ax} (n=2)
Group 2	14	6.21±1.03^{a} (n=87)	1.71±0.54^{az} (n=24)	1.71±0.41^{az} (n=24)	2.36±0.46^{az} (n=33)	0.43±0.14^{ay} (n=6)	0.00±0.00^{ay} (n=0)
Group 3	10	5.90±0.52^{a} (n=59)	1.80±0.85^{ayz} (n=18)	0.80±0.29^{ay} (n=8)	2.40±0.62^{az} (n=24)	0.40±0.31^{ay} (n=4)	0.50±0.34^{ay} (n=5)
Average	12.67 (n=38)	6.00±0.52 (n=228)	1.68±0.32^{y} (n=64)	1.26±0.20^{y} (n=48)	2.37±0.28^{z} (n=90)	0.50±0.14^{x} (n=19)	0.18±0.10^{x} (n=7)

*Age group: group 1 (<18 months), group 2 (18-24 months) and group 3 (>24 months)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

[xyz] Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.7: Percentage (%, mean±SEM) of oocytes retrieved from different OR cycles

OR cycle	No. of ovaries	No. of oocytes retrieved per ovary	Percent of oocytes retrieved per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
OR1	52	5.23±0.44^{a} (n=272)	21.66±3.44^{ay} (n=69)	19.63±3.24^{axy} (n=53)	34.59±3.61^{az} (n=102)	12.90±2.87^{bxy} (n=29)	11.22±3.75^{ax} (n=19)
OR2	28	4.57±0.49^{a} (n=128)	29.14±5.97^{ayz} (n=41)	20.05±4.87^{axy} (n=27)	38.21±6.37^{az} (n=44)	2.95±1.11^{aw} (n=6)	9.65±4.20^{awx} (n=10)
Average	40 (n=80)	5.00±0.33 (n=400)	24.28±3.06^{y} (n=110)	19.78±2.69^{y} (n=80)	35.86±3.22^{z} (n=146)	9.42±1.97^{x} (n=35)	10.67±2.83^{x} (n=29)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.8: Number (n, mean±SEM) of oocytes retrieved from different OR cycles

OR cycle	No. of ovaries	No. of oocytes retrieved per ovary	No. of oocytes retrieved per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
OR1	52	5.23±0.44^{a} (n=272)	1.33±0.25^{ay} (n=69)	1.02±0.16^{axy} (n=53)	1.96±0.24^{az} (n=102)	0.56±0.12^{bwx} (n=29)	0.37±0.12^{aw} (n=19)
OR2	28	4.57±0.49^{a} (n=128)	1.46±0.26^{ayz} (n=41)	0.96±0.23^{ay} (n=27)	1.57±0.24^{az} (n=44)	0.21±0.08^{ax} (n=6)	0.36±0.14^{ax} (n=10)
Average	40 (n=80)	5.00±0.33 (n=400)	1.38±0.18^{y} (n=110)	1.00±0.13^{y} (n=80)	1.83±0.18^{z} (n=146)	0.44±0.08^{x} (n=35)	0.36±0.09^{x} (n=29)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{wxyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.9: Percentage (%, mean±SEM) of oocytes retrieved from different ovarian stimulation response groups

Ovarian stimulation response	No. of ovaries	No. of oocytes retrieved per ovary	Percent of oocytes retrieved per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
Excellent	11	6.64±0.81^{b} (n=73)	30.21±9.82^{ayz} (n=23)	18.05±5.98^{axy} (n=12)	37.30±5.55^{az} (n=28)	9.89±4.23^{ax} (n=7)	4.55±4.55^{ax} (n=3)
Good	57	5.12±0.39^{ab} (n=292)	22.40±3.04^{ay} (n=76)	21.99±3.12^{ay} (n=64)	38.37±3.93^{az} (n=111)	10.20±2.57^{ax} (n=25)	7.05±2.59^{ax} (n=16)
Satisfactory	16	3.44±0.58^{a} (n=55)	22.92±9.09^{azy} (n=13)	11.67±6.65^{ayz} (n=7)	31.98±7.94^{az} (n=19)	7.08±3.03^{ay} (n=5)	26.35±9.63^{byz} (n=11)
Average	28 (n=84)	5.00±0.32 (n=420)	23.52±2.95^{y} (n=112)	19.51±2.60^{y} (n=83)	37.01±3.13^{z} (n=158)	9.56±1.91^{x} (n=37)	10.40±2.71^{x} (n=30)

*Ovarian stimulation response: excellent (>10 follicles), good (5-10 follicles) and satisfactory (<5 follicles)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)

^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.10: Number (n, mean±SEM) of oocytes retrieved from different ovarian stimulation response groups

Ovarian stimulation response	No. of ovaries	No. of oocytes retrieved per ovary	No. of oocytes retrieved per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
Excellent	11	6.64±0.81^{b} (n=73)	2.09±0.76^{byz} (n=23)	1.09±0.34^{axy} (n=12)	2.55±0.46^{bz} (n=28)	0.64±0.28^{ax} (n=7)	0.27±0.27^{ax} (n=3)
Good	57	5.12±0.39^{ab} (n=292)	1.33±0.19^{aby} (n=76)	1.12±0.16^{ay} (n=64)	1.95±0.22^{abz} (n=111)	0.44±0.10^{ax} (n=25)	0.28±0.10^{ax} (n=16)
Satisfactory	16	3.44±0.58^{a} (n=55)	0.81±0.32^{ayz} (n=13)	0.44±0.22^{ay} (n=7)	1.19±0.28^{az} (n=19)	0.31±0.12^{ay} (n=5)	0.69±0.22^{ayz} (n=11)
Average	28 (n=84)	5.00±0.32 (n=420)	1.33±0.18^{y} (n=112)	0.99±0.13^{y} (n=83)	1.88±0.17^{z} (n=158)	0.44±0.08^{x} (n=37)	0.36±0.09^{x} (n=30)

*Ovarian stimulation response: excellent (>10 follicles), good (5-10 follicles) and satisfactory (<5 follicles)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)

^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.11: Correlation coefficients between body weight and age in Boer crossbred goats

	Body weight	Age
Body weight	-	0.56** (n=37)
Age		-

**Significant at P<0.01

Table 4.12: Regression coefficients of body weight and age on the number of oocytes retrieved in goats

Factors	No. of ovaries	Regression equation	Regression coefficient (r^2)	P value
Body weight (kg)	52	y =-0.16x+8.80	0.14	0.01*
Age (months)	38	y =-0.00x+6.03	0.00	0.98

y: factor and x: number of oocytes retrieved
*Significant at P<0.05

Table 4.13: Regression coefficients of body weight on number of oocytes retrieved of different oocyte grades in goats

Grades	No. of ovaries	Regression equation	Regression coefficient (r^2)	P value
A	52	y =-0.06x+2.70	0.07	0.06
B	52	y =-0.05x+2.17	0.11	0.02*
C	52	y =-0.09x+4.08	0.17	0.00**
D	52	y =0.01x+0.41	0.00	0.67
E	52	y =0.04x–0.56	0.14	0.01*

y: factor and x: number of oocytes retrieved
*Significant at P<0.05; **significant at P<0.01

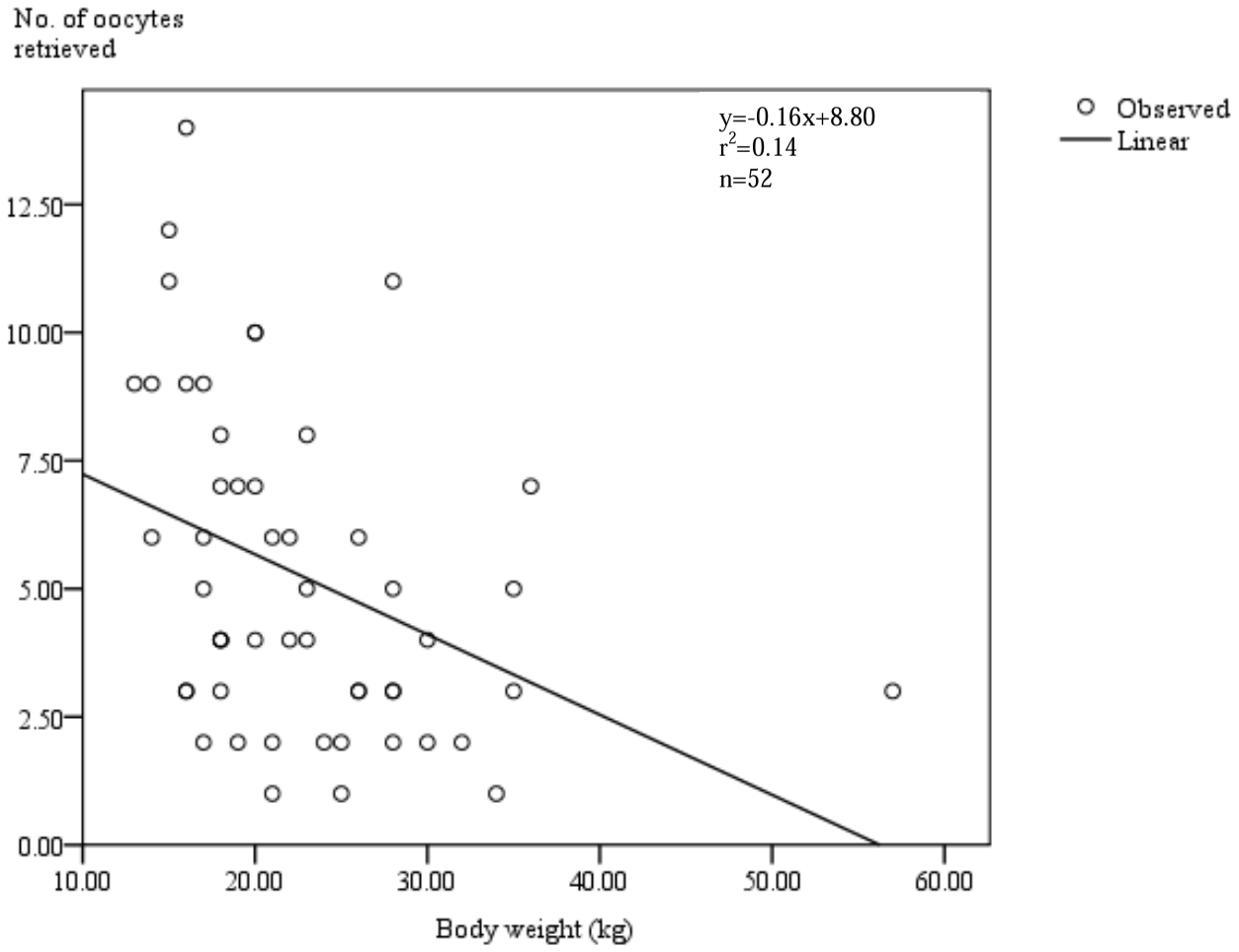

Figure 4.1: Relationship between the number of oocytes retrieved and the body weight (kg) of goats. n= number of ovaries.

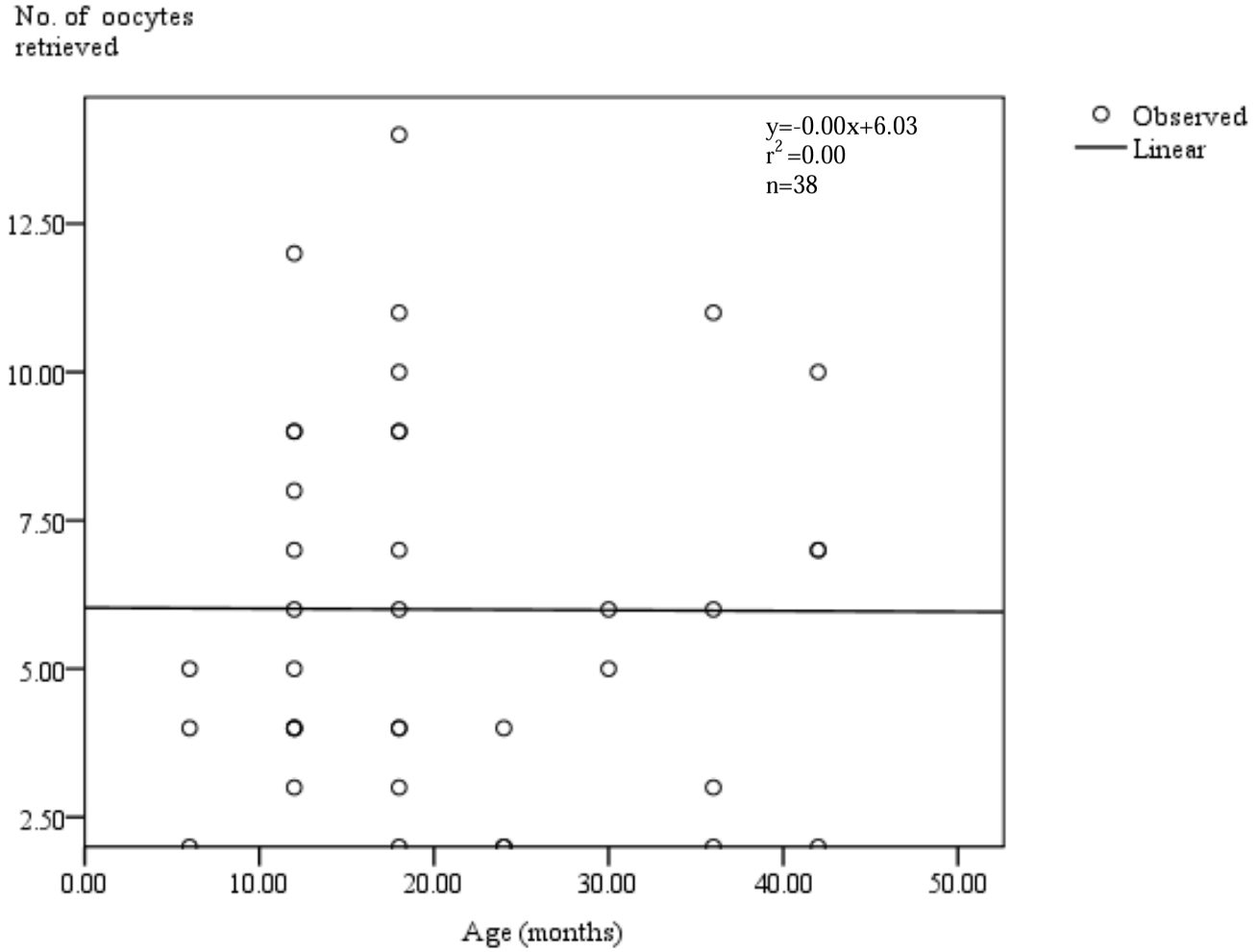

Figure 4.2: Relationship between the number of oocytes retrieved and the age (months) of goats. n= number of ovaries.

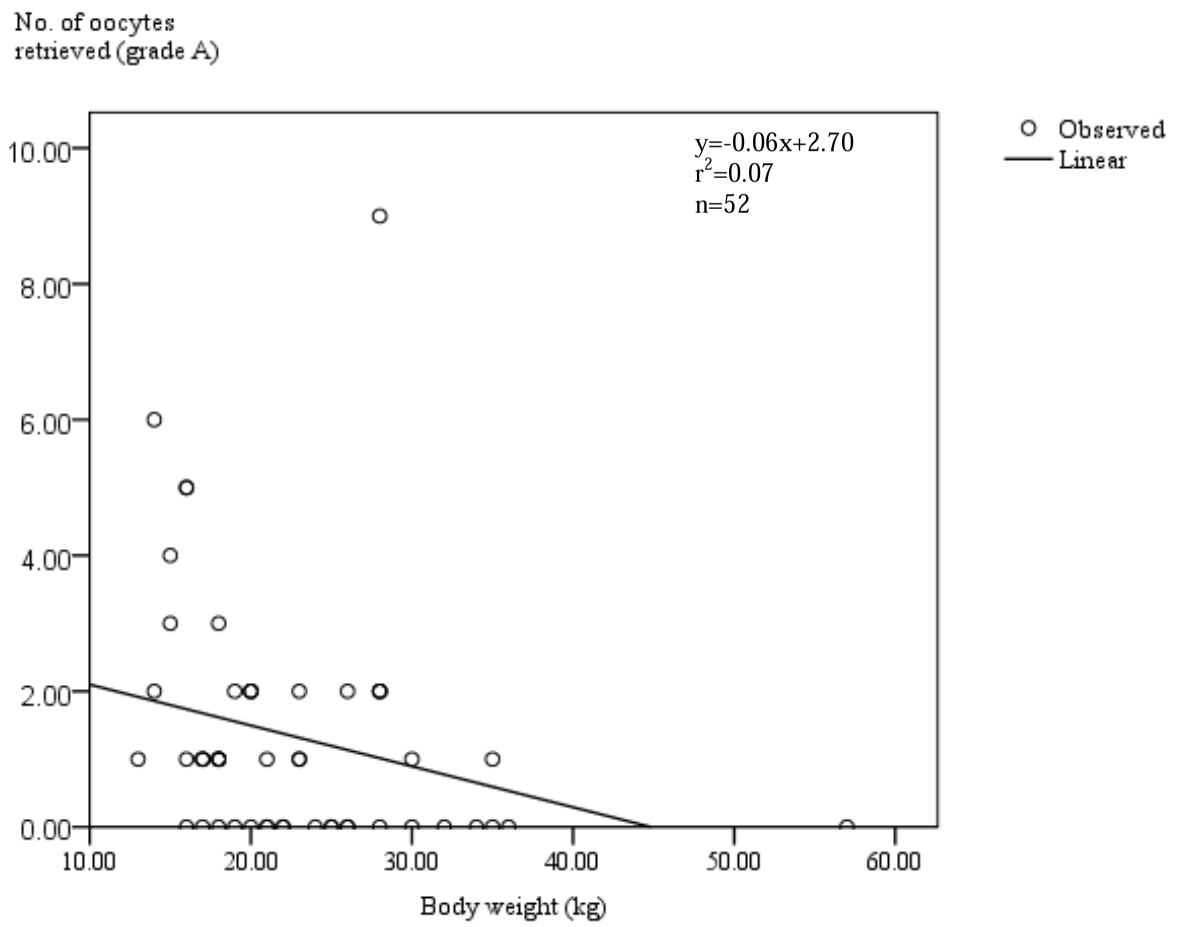

Figure 4.3: Relationship between the Grade A oocytes retrieved and the body weight (kg) of goats. n= number of ovaries.

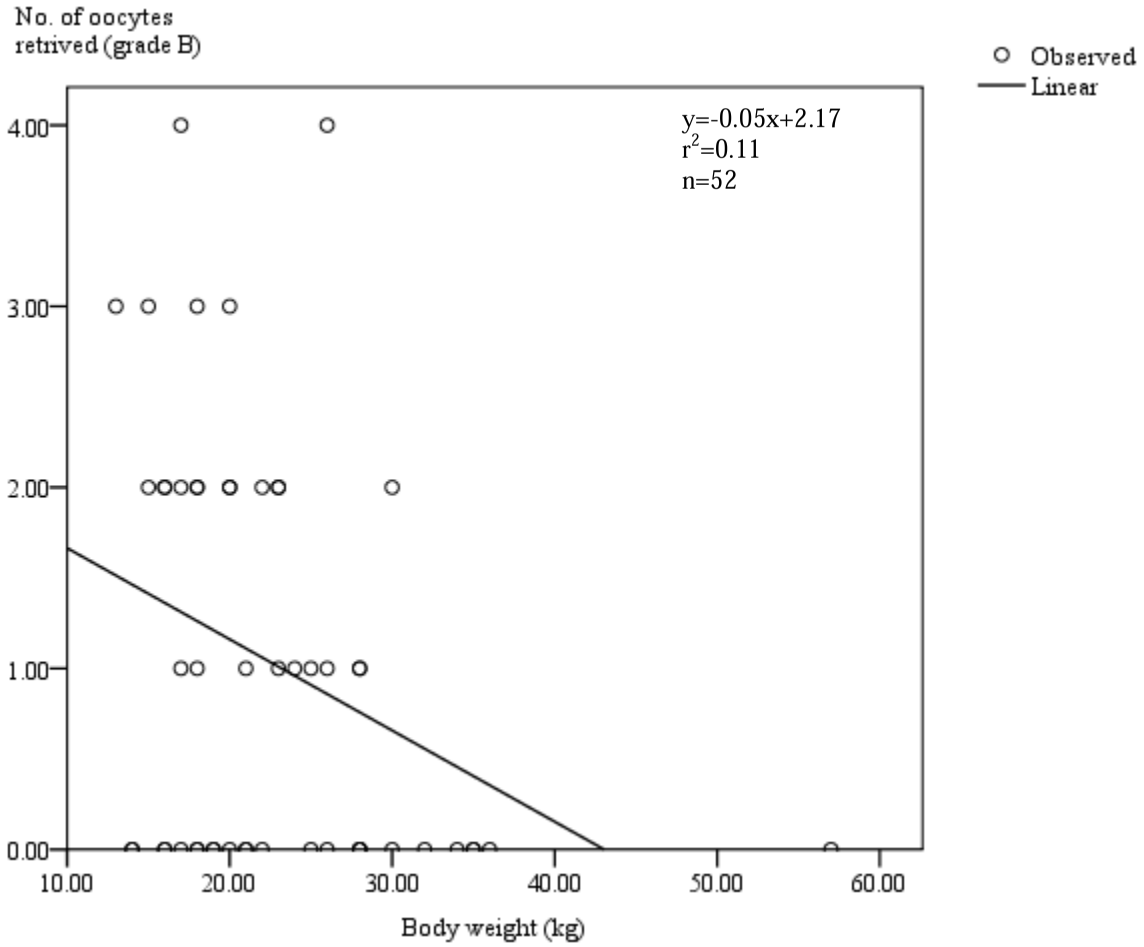

Figure 4.4: Relationship between the Grade B oocytes retrieved and the body weight (kg) of goats. n= number of ovaries.

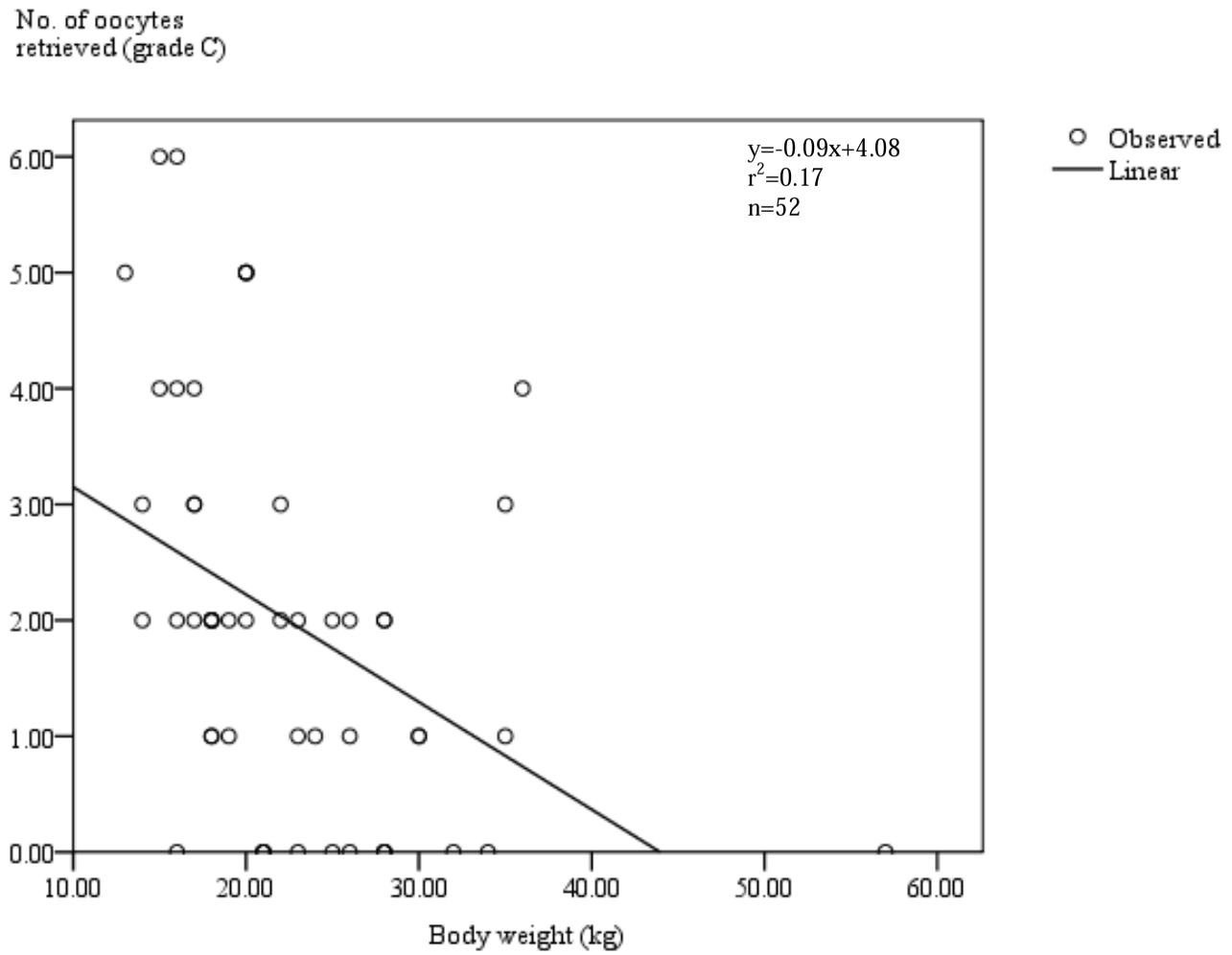

Figure 4.5: Relationship between the Grade C oocytes retrieved and the body weight (kg) of goats. n= number of ovaries.

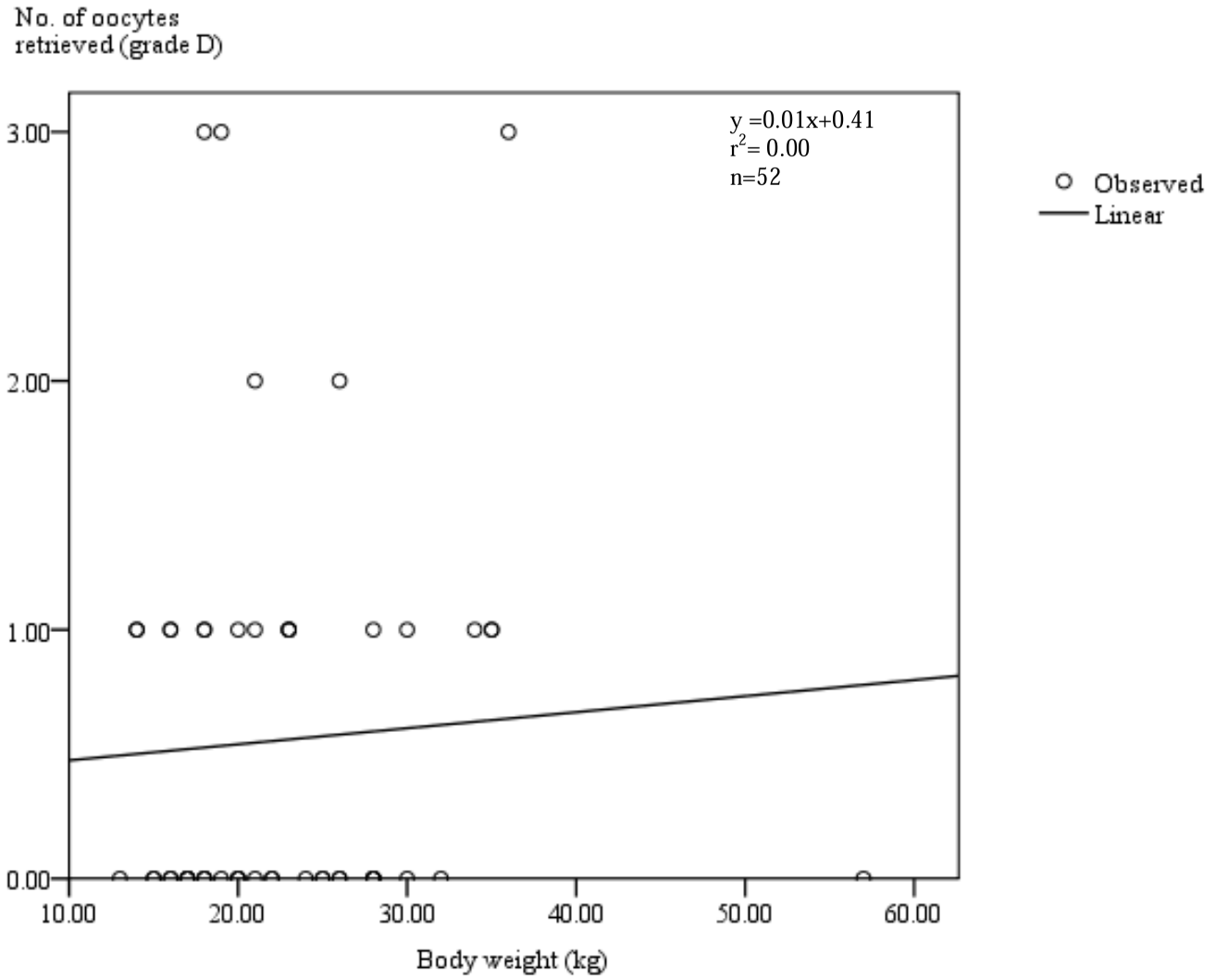

Figure 4.6: Relationship between the Grade D oocytes retrieved and the body weight (kg) of goats. n= number of ovaries.

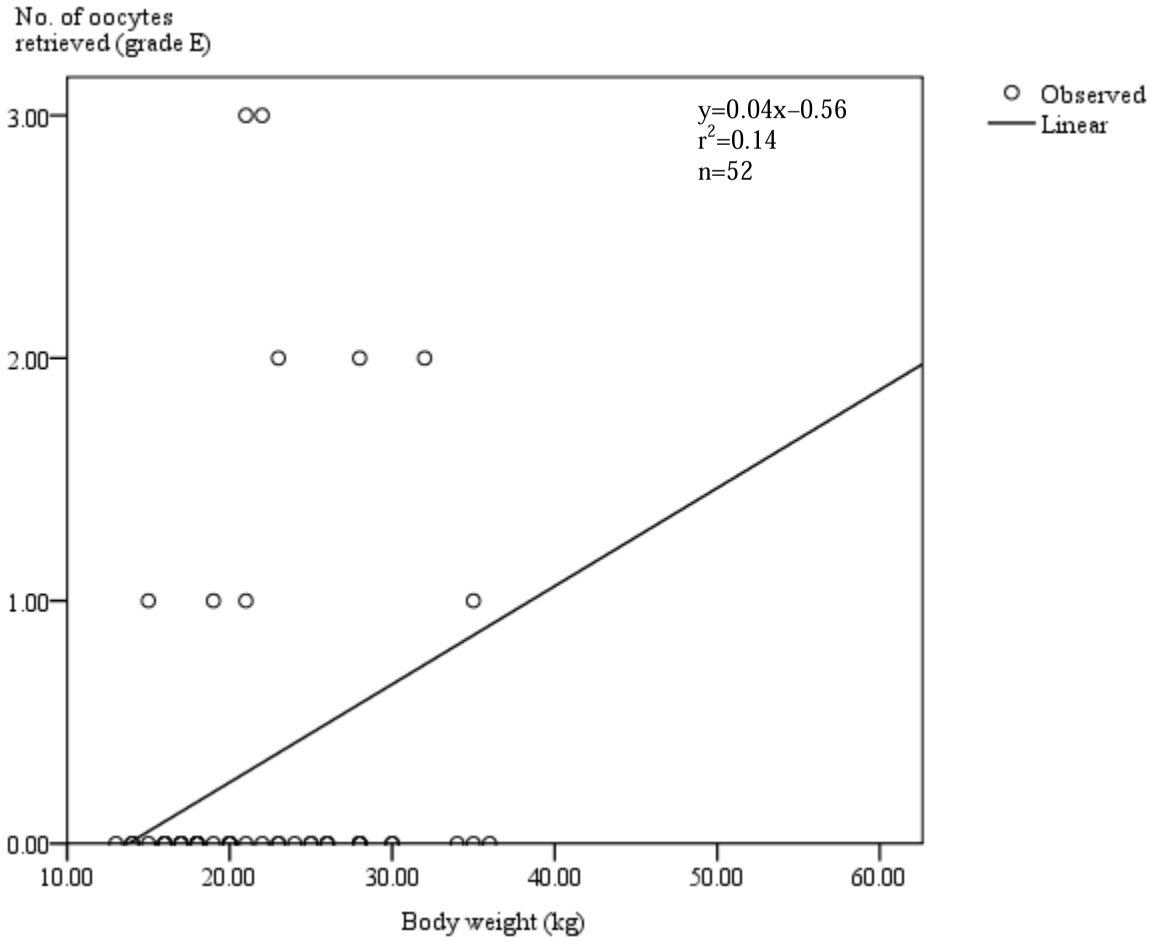

Figure 4.7: Relationship between the Grade E oocytes retrieved and the body weight (kg) of goats. n= number of ovaries.

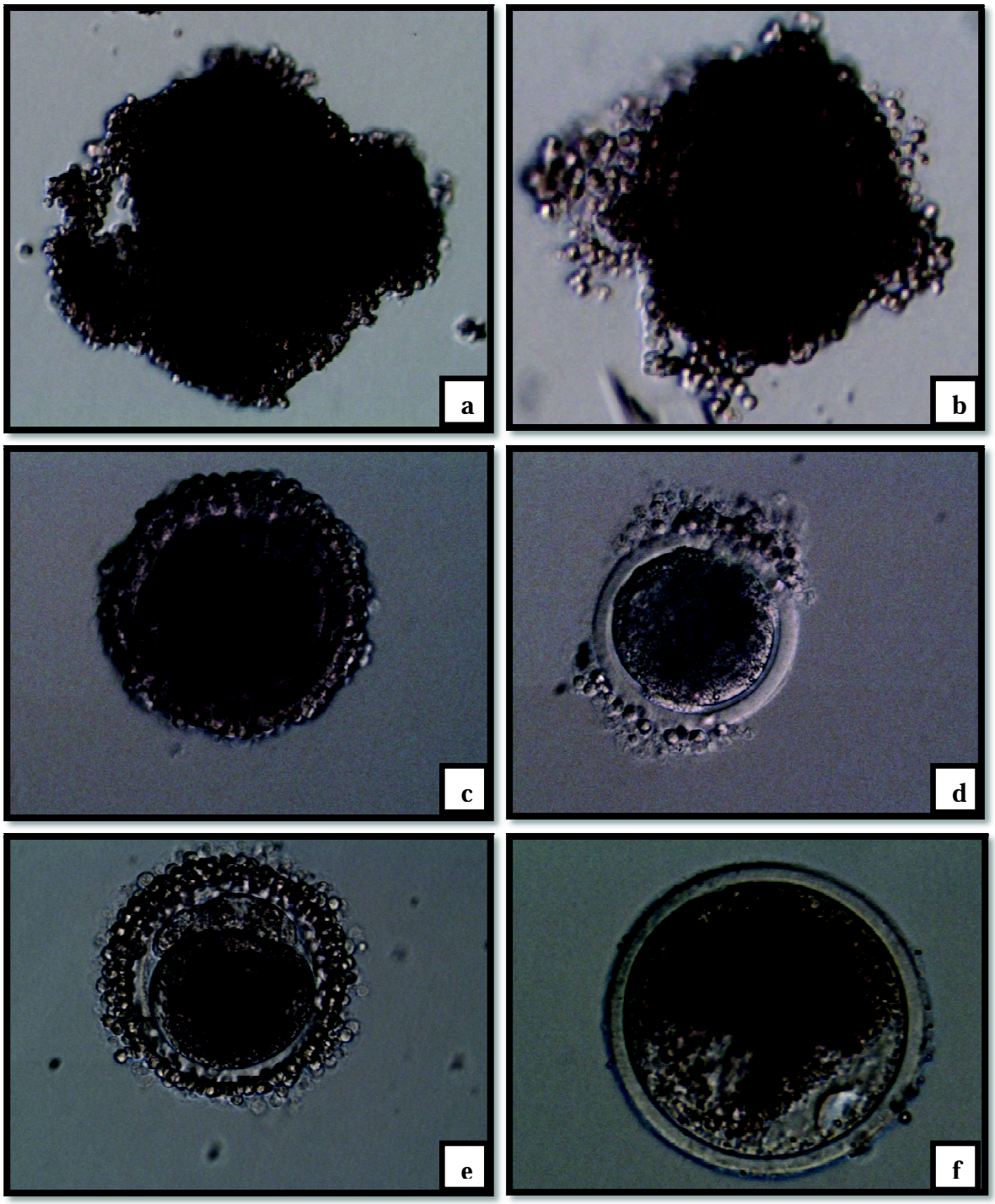

Figure 4.8: Photomicrographs of immature oocyte at different grades retrieved from superovulated-donor goat. (a) Grade A oocyte with more than 5 multilayered compact cumulus cells with evenly granulated cytoplasm; (b) Grade B with 3 to 5 layers of cumulus cells with evenly granulated cytoplasm; (c) Grade C oocyte with 1 to 2 layers of cumulus cells with evenly granulated cytoplasm; (d) Grade D oocyte with incomplete investment of 1 to 2 layers of cumulus cells with evenly granulated cytoplasm and (e-f) Grade E oocyte with abnormal, size, shape and heterogeneous ooplasm as well as denuded.

4.2 EFFECT OF PMSG DOSAGE ON QUANTITY AND QUALITY OF OOCYTE, IVM RATE AND SUBSEQUENT EMBRYO DEVELOPMENT FOLLOWING ICSI IN GOAT (EXPERIMENT 2)

A total of 416 oocytes were retrieved from 82 ovaries with an average of 5.07±0.32 oocytes retrieved per ovary for the PMSG-stimulated dosage groups (Tables 4.14 and 4.15). No significant difference ($P>0.05$) was observed in number of follicles aspirated between the two PMSG dosage groups. However, a significant difference ($P<0.05$) was observed in the number of oocytes retrieved per ovary between 1200 IU (6.21±0.66) and 1500 IU (4.48±0.33) PMSG dosages. For Grades A and B oocytes, there was a significant difference ($P<0.05$) in the number of oocytes retrieved per ovary between 1200 IU (1.93±0.39 and 1.39±0.24, respectively) and 1500 IU (1.07±0.17 and 0.80±0.15, respectively) PMSG dosages. No significant differences ($P>0.05$) were observed in the percentage and number of Grades C (37.91±3.14% and 1.93±0.17, respectively) and D (8.98±1.82% and 0.43±0.08, respectively) oocytes retrieved per ovary between 1200 and 1500 IU PMSG dosages. There were also significant differences ($P<0.05$) in the percentage and number of oocytes retrieved per ovary among the oocyte grades within each PMSG dosage group. For the two PMSG groups, average percentages for oocytes of Grades A, B, C, D and E retrieved per ovary were 24.09±2.99%, 19.58±2.64%, 37.91±3.14%, 8.98±1.82% and 9.43±2.54%, respectively. Correspondingly, the average numbers were 1.37±0.18, 1.00±0.13, 1.93±0.17, 0.43±0.08 and 0.35±0.09, respectively.

The results of IVM oocytes retrieved at different PMSG dosages are depicted in Tables 4.16 and 4.17. A total of 239 oocytes were matured from 69 ovaries with an average of 3.46±0.26 oocytes matured per ovary. Out of the total, 112 oocytes were matured from 27 ovaries for 1200 IU PMSG dosage and 127 oocytes were matured from 42 ovaries for 1500

IU PMSG dosage. A significant difference ($P<0.05$) was observed in the number of oocytes matured per ovary between 1200 IU (4.15±0.47) and 1500 IU (3.02±0.29) PMSG dosages. No significant differences ($P>0.05$) were observed in the percentage and number of oocytes matured per ovary between the two PMSG dosages in the respective grades. However, there were significant differences ($P<0.05$) in the percentage and number of oocytes matured per ovary among the oocyte grades within each PMSG dosage. Combination of Grades A, B and C (96.86% and 3.36, respectively) yielded higher percentage and number of oocytes matured per ovary compared to combination of Grades D and E (3.14% and 0.10, respectively). The average percentages for oocytes of Grades A, B, C, D and E matured per ovary were 33.48±4.00%, 27.25±3.71%, 36.13±4.16%, 1.45±0.89% and 1.69±1.47%, respectively. Correspondingly, the average numbers were 1.26±0.17, 0.90±0.12, 1.20±0.14, 0.06±0.04 and 0.04±0.03, respectively.

The overall maturation rate of different grades oocyte at two PMSG dosages is depicted in Table 4.18. A total of 174 and 242 immature oocytes were used in IVM for 1200 IU and 1500 IU PMSG dosages, respectively, out of which 54 and 58, 39 and 43, 61 and 97, 15 and 20 as well as 5 and 24 for Grades A, B, C, D and E, respectively. Higher maturation rates ($P<0.05$) were observed at the 1200 IU PMSG dosage (65.67±3.86%) compared to the 1500 IU PMSG dosage (48.11±4.43%). No significant differences ($P>0.05$) were observed in maturation rates between the two PMSG dosages groups for oocytes in the respective grades studied. However, maturation rates of oocytes for Grades A, B and C were apparently higher ($P>0.05$) at 1200 IU PMSG dosage compared to 1500 IU PMSG dosage. In both PMSG dosages, maturation rates for Grades A, B and C (75.42±5.01%, 74.85±5.16% and 48.23±4.52%, respectively) oocytes were significant higher ($P<0.05$) than Grades D and E (9.52±5.38% and 5.88±4.27%, respectively) oocytes. In general, maturation rates of better

quality oocytes (Grades A, B and C) at 1200 and 1500 IU PMSG dosages were 74.05% and 61.06%, respectively. In both PMSG dosages, the combined average maturation rate of Grades A, B and C oocytes was approximately 66.17%.

In the present study, only oocytes of Grades A, B and C were used in ICSI experiments and oocytes of Grades D and E were omitted due to the low number of maturation obtained. The results of matured oocytes cleaved at different PMSG dosage is depicted in Tables 4.19 and 4.20. A total of 73 oocytes were cleaved from 124 matured oocytes with an average of 2.43±0.29 oocytes cleaved per ovary. A total of 36 oocytes were cleaved from 58 matured oocytes at 1200 IU PMSG dosage and 37 oocytes were cleaved from 66 matured oocytes at 1500 IU PMSG dosage. An average of 2.57±0.45 and 2.31±0.38 oocytes were cleaved per ovary at 1200 and 1500 IU PMSG dosage, respectively. No significant differences ($P>0.05$) were observed in the percentage and number of oocytes cleaved per ovary between the two PMSG dosages, in the respective grades. However, even though insignificant, percent of oocytes cleaved per ovary from Grade A were apparently higher ($P>0.05$) at 1200 IU PMSG dosage compared to 1500 IU PMSG dosage. For the all two PMSG dosages, average percentages for oocytes of Grades A, B, C, D and E cleaved per ovary were 41.45±6.70%, 30.39±6.87% and 28.17±6.44%, respectively. Correspondingly, the average numbers were 1.07±0.18, 0.60±0.12 and 0.77±0.18, respectively.

The cleavage rate of different grades oocyte at two PMSG dosages is depicted in Table 4.21. A total of 58 and 66 matured oocytes were used for the ICSI experiments derived from 1200 and 1500 IU PMSG dosage, respectively. Matured oocytes of Grades A (25 and 22), B (14 and 15) and C (19 and 29) used were obtained from 1200 and 1500 IU PMSG dosages, respectively. No significant differences ($P>0.05$) were observed in the cleavage rates of matured oocytes in the respective grades derived from the two PMSG dosages. However, the cleavage rates of oocytes from Grade A, even though insignificant, apparently higher

(P>0.05) at 1200 IU (77.27±8.19%) compared to 1500 IU (55.56±13.19%) PMSG dosage. In both PMSG dosages, cleavage rates for Grades A, B and C were 65.94±8.08%, 62.96±9.86% and 42.65±8.43%, respectively. In general, cleavage rates of Grades A, B and C at 1200 and 1500 IU PMSG dosage were 60.20% and 54.21%, respectively. In both PMSG dosages, the combined average cleavage rate of Grades A, B and C oocytes was approximately 57.18%.

The developmental rates of different grades oocyte at two PMSG dosages is depicted in Table 4.22. A total of 34 and 34 two-cell stage embryos were obtained from ICSI experiments derived from 1200 and 1500 IU PMSG dosage, respectively. The 2-cell stage embryos yielded from oocytes of Grades A (17 and 13), B (8 and 9) and C (9 and 12) were obtained from 1200 and 1500 IU PMSG dosage, respectively. In both PMSG dosages, the averages of developmental rates at 4-, 8-cell and morula in oocytes of Grade A were 64.86±8.25%, 51.81±8.83% and 22.83±7.60%, respectively; Grade B were 54.63±10.68%, 46.30±11.08% and 24.07±10.01%, respectively; and Grades C were 24.14±6.88%, 21.54±6.19% and 1.85±1.85%, respectively. In the respective oocyte grades, no significant differences (P>0.05) were observed in developmental rates between two PMSG dosages. However, the developmental rates of Grades A, B and C oocytes were apparently more competent in developing up to the morula stage (P>0.05) at 1200 IU compared to 1500 IU PMSG dosage.

The developmental rate of different grades of oocyte at 1200 and 1500 IU PMSG dosage is depicted in Tables 4.23 and 4.24, respectively. PMSG dosage at 1200 IU apparently yielded higher (P>0.05) developmental rates at 4-, 8-cell and morula in oocytes of Grade A were 75.00±9.20%, 65.91±11.04% and 15.91±9.70%, respectively; Grade B were 62.50±15.67%, 56.25±17.52% and 25.00±16.37%, respectively; and Grade C were 30.83±11.66%, 30.83±11.66% and 5.00±5.00%, respectively; compared to 1500 IU in oocytes of Grade A were 55.56±13.19%, 38.89±12.87% and 29.17±11.63%, respectively;

Grade B were 48.33±15.00%, 38.33±14.50% and 23.33±13.19%, respectively; and Grade C were 20.20±8.63%, 16.08±7.00% and 0.00±0.00%, respectively.

Regardless of the oocyte grades (Table 4.25), the cleavage rates were apparently higher ($P>0.05$) at 1200 IU (65.04±7.36%) compared to 1500 IU (47.87±8.06%) PMSG dosage. Higher developmental rates at 4- and 8-cell ($P<0.05$) were observed at 1200 IU (63.04±7.72% and 58.60±7.65%, respectively) compared to 1500 IU (36.51±7.14% and 28.89±6.64%, respectively) PMSG dosage. No significant difference ($P>0.05$) were observed in development rate at morula stage between the two PMSG dosages. In both PMSG dosages, the averages of developmental rates at 4-, 8-cell and morula were 47.26±5.64%, 40.93±5.52% and 15.39±4.16%, respectively.

Table 4.14: Percentage (%, mean±SEM) of oocytes retrieved from different PMSG dosage groups

PMSG dosage	No. of ovaries	No. of follicles aspirated	No. of oocytes retrieved per ovary	Percent of oocytes retrieved per ovary				
				Grade A	Grade B	Grade C	Grade D	Grade E
1200 IU	28	9.43±0.85^{a} (n=264)	6.21±0.66^{b} (n=174)	29.43±5.55^{az} (n=54)	25.80±4.79^{az} (n=39)	32.45±4.60^{az} (n=61)	9.88±2.84^{ay} (n=15)	2.44±1.82^{ay} (n=5)
1500 IU	54	8.56±3.58^{a} (n=462)	4.48±0.33^{a} (n=242)	21.33±3.50^{ay} (n=58)	16.35±3.10^{axy} (n=43)	40.75±4.10^{az} (n=97)	8.52±2.35^{ax} (n=20)	13.06±3.65^{bxy} (n=24)
Average	41 (n=82)	8.85±0.43 (n=726)	5.07±0.32 (n=416)	24.09±2.99^{y} (n=112)	19.58±2.64^{y} (n=82)	37.91±3.14^{z} (n=158)	8.98±1.82^{x} (n=35)	9.43±2.54^{x} (n=29)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.15: Number (n, mean±SEM) of oocytes retrieved from different PMSG dosage groups

PMSG dosage	No. of ovaries	No. of follicles aspirated	No. of oocytes retrieved per ovary	No. of oocytes retrieved per ovary				
				Grade A	Grade B	Grade C	Grade D	Grade E
1200 IU	28	9.43±0.85^{a} (n=264)	6.21±0.66^{b} (n=174)	1.93±0.39^{bz} (n=54)	1.39±0.24^{bz} (n=39)	2.18±0.34^{az} (n=61)	0.54±0.16^{ay} (n=15)	0.18±0.12^{ay} (n=5)
1500 IU	54	8.56±3.58^{a} (n=462)	4.48±0.33^{a} (n=242)	1.07±0.17^{ay} (n=58)	0.80±0.15^{axy} (n=43)	1.80±0.19^{az} (n=97)	0.37±0.09^{ax} (n=20)	0.44±0.12^{ax} (n=24)
Average	41 (n=82)	8.85±0.43 (n=726)	5.07±0.32 (n=416)	1.37±0.18^{y} (n=112)	1.00±0.13^{y} (n=82)	1.93±0.17^{z} (n=158)	0.43±0.08^{x} (n=35)	0.35±0.09^{x} (n=29)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.16: Percentage (%, mean±SEM) of oocytes matured from different PMSG dosage groups

PMSG dosage	No. of ovaries	No. of oocytes matured per ovary	Percent of oocytes matured per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
1200 IU	27	4.15±0.47^{b} (n=112)	36.16±6.04^{az} (n=44)	32.73±5.81^{az} (n=32)	29.26±4.89^{az} (n=34)	1.85±1.85^{ay} (n=2)	0.00±0.00^{ay} (n=0)
1500 IU	42	3.02±0.29^{a} (n=127)	31.75±5.34^{ayz} (n=43)	23.72±4.79^{ay} (n=30)	40.56±6.01^{az} (n=49)	1.19±0.88^{ax} (n=2)	2.78±2.40^{ax} (n=3)
Average	34.50 (n=69)	3.46±0.26 (n=239)	33.48±4.00^{z} (n=87)	27.25±3.71^{z} (n=62)	36.13±4.16^{z} (n=83)	1.45±0.89^{y} (n=4)	1.69±1.47^{y} (n=3)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.17: Number (n, mean±SEM) of oocytes matured from different PMSG dosage groups

PMSG dosage	No. of ovaries	No. of oocytes matured per ovary	No. of oocytes matured per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
1200 IU	27	4.15±0.47^{b} (n=112)	1.63±0.32^{az} (n=44)	1.19±0.21^{az} (n=32)	1.26±0.24^{az} (n=34)	0.07±0.07^{ay} (n=2)	0.00±0.00^{ay} (n=0)
1500 IU	42	3.02±0.29^{a} (n=127)	1.02±0.18^{ayz} (n=43)	0.71±0.14^{ay} (n=30)	1.17±0.18^{az} (n=49)	0.05±0.03^{ax} (n=2)	0.07±0.05^{ax} (n=3)
Average	34.50 (n=69)	3.46±0.26 (n=239)	1.26±0.17^{z} (n=87)	0.90±0.12^{y} (n=62)	1.20±0.14^{yz} (n=83)	0.06±0.04^{x} (n=4)	0.04±0.03^{x} (n=3)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.18: Maturation rate (%, mean±SEM) of different grades of oocyte at two PMSG dosage groups

PMSG dosage	Percent MII oocytes*	Percent maturation**				
		Grade A	Grade B	Grade C	Grade D	Grade E
1200 IU	65.67±3.86^{b} (112/174)***	83.56±5.59^{az} (44/54)	84.26±4.95^{az} (32/39)	54.32±6.37^{ay} (34/61)	6.06±6.06^{ax} (2/15)	0.00±0.00^{ax} (0/5)
1500 IU	48.11±4.43^{a} (127/242)	70.00±7.39^{az} (43/58)	68.07±7.94^{az} (30/97)	45.12±5.99^{ay} (49/97)	11.77±8.06^{ax} (2/20)	7.14±5.16^{ax} (3/24)
Average	54.10±3.32 (239/416)	75.42±5.01^{z} (87/112)	74.85±5.16^{z} (62/136)	48.23±4.52^{y} (83/158)	9.52±5.38^{x} (4/35)	5.88±4.27^{x} (3/29)

* Mean percentage of MII oocytes was based on total oocytes from all grades used for IVM, in the respective PMSG dosage groups
** Mean percentage of maturation rate was based on oocytes used for IVM, in the respective grades
*** Mean total of matured oocytes per total of oocytes used for IVM
^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.19: Percentage (%, mean±SEM) of oocytes cleaved from different PMSG dosage groups

PMSG dosage	No. of oocytes injected per ovary	No. of oocytes cleaved per ovary	Percent of oocytes cleaved per ovary		
			Grade A	Grade B	Grade C
1200 IU	3.87±0.71^{a} (n=58)	2.57±0.45^{a} (n=36)	53.57±9.91^{az} (n=18)	25.00±8.87^{ay} (n=8)	21.43±8.09^{ay} (n=10)
1500 IU	3.00±0.34^{a} (n=66)	2.31±0.38^{a} (n=37)	30.83±8.49^{az} (n=14)	35.10±10.41^{az} (n=10)	34.06±9.79^{az} (n=13)
Average	3.35±0.35 (n=124)	2.43±0.29 (n=73)	41.45±6.70^{z} (n=32)	30.39±6.87^{z} (n=18)	28.17±6.44^{z} (n=23)

^{a} Mean values within a column with same superscript were not significantly different (P>0.05)
^{yz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.20: Number (n, mean±SEM) of oocytes cleaved from different PMSG dosage groups

PMSG dosage	No. of oocytes injected per ovary	No. of oocytes cleaved per ovary	No. of oocytes cleaved per ovary		
			Grade A	Grade B	Grade C
1200 IU	3.87±0.71[a] (n=58)	2.57±0.45[a] (n=36)	1.29±0.24[az] (n=18)	0.57±0.20[az] (n=8)	0.71±0.27[az] (n=10)
1500 IU	3.00±0.34[a] (n=66)	2.31±0.38[a] (n=37)	0.88±0.26[az] (n=14)	0.63±0.16[az] (n=10)	0.81±0.26[az] (n=13)
Average	3.35±0.35 (n=124)	2.43±0.29 (n=73)	1.07±0.18[z] (n=32)	0.60±0.12[z] (n=18)	0.77±0.18[z] (n=23)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Table 4.21: Cleavage rate (%, mean±SEM) of different grades of oocyte at two PMSG dosage groups

PMSG dosage	Percent cleavage[*]		
	Grade A	Grade B	Grade C
1200 IU	77.27±8.19[az] (17/25)[**]	62.50±15.67[az] (8/14)	40.83±12.94[az] (9/19)
1500 IU	55.56±13.19[az] (13/22)	63.33±13.33[az] (9/15)	43.73±11.30[az] (12/29)
Average	65.94±8.08[z] (30/47)	62.96±9.86[z] (17/29)	42.65±8.43[z] (21/48)

[*] Mean percentage of cleavage was based on oocytes used for ICSI, in the respective grades
[**] Mean total of cleaved embryos per total of oocytes used for ICSI
[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Table 4.22: Developmental rate (%, mean±SEM) of different grades of oocyte at two PMSG dosage groups

Oocyte grade	PMSG dosage	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
				4-cell	8-cell	Morula
Grade A	1200 IU	2.27±0.33[a] (n=25)	77.27±8.19[a] (n=17)	75.00±9.20[a] (n=16)	65.91±11.05[a] (n=15)	15.91±9.70[a] (n=4)
	1500 IU	1.83±0.24[a] (n=22)	55.56±13.19[a] (n=13)	55.56±13.19[a] (n=13)	38.89±12.87[a] (n=11)	29.17±11.63[a] (n=8)
	Average	2.04±0.20 (n=47)	65.94±8.08 (n=30)	64.86±8.25 (n=29)	51.81±8.83 (n=26)	22.83±7.60 (n=12)
Grade B	1200 IU	1.75±0.37[a] (n=14)	62.50±15.67[a] (n=8)	62.50±15.67[a] (n=8)	56.25±17.52[a] (n=7)	25.00±16.37[a] (n=3)
	1500 IU	1.50±0.22[a] (n=15)	63.33±13.33[a] (n=9)	48.33±15.00[a] (n=7)	38.33±14.50[a] (n=6)	23.33±13.19[a] (n=3)
	Average	1.61±0.20 (n=29)	62.96±9.86 (n=17)	54.63±10.68 (n=15)	46.30±11.08 (n=13)	24.07±10.01 (n=6)
Grade C	1200 IU	1.90±0.41[a] (n=19)	40.83±12.94[a] (n=9)	30.83±11.66[a] (n=8)	30.83±11.66[a] (n=8)	5.00±5.00[a] (n=2)
	1500 IU	1.71±0.25[a] (n=29)	43.73±11.30[a] (n=12)	20.20±8.63[a] (n=8)	16.08±7.00[a] (n=6)	0.00±0.00[a] (n=0)
	Average	1.77±0.22 (n=48)	42.65±8.43 (n=21)	24.14±6.88 (n=16)	21.54±6.19 (n=14)	1.85±1.85 (n=2)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

Table 4.23: Developmental rate (%, mean±SEM) of different grades of oocyte at 1200 IU PMSG dosage

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	2.27±0.33[a] (n=25)	77.27±8.19[a] (n=17)	75.00±9.20[bz] (n=16)	65.91±11.04[az] (n=15)	15.91±9.70[ay] (n=4)
Grade B	1.75±0.37[a] (n=14)	62.50±15.67[a] (n=8)	62.50±15.67[abz] (n=8)	56.25±17.52[az] (n=7)	25.00±16.37[az] (n=3)
Grade C	1.90±0.41[a] (n=19)	40.83±12.94[a] (n=9)	30.83±11.66[az] (n=8)	30.83±11.66[az] (n=8)	5.00±5.00[az] (n=2)
Average	2.00±0.21 (n=58)	60.63±7.29 (n=34)	56.32±7.52[z] (n=32)	51.15±7.81[z] (n=30)	14.66±6.02[y] (n=9)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)

[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.24: Developmental rate (%, mean±SEM) of different grades of oocyte at 1500 IU PMSG dosage

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	1.83±0.24^{a} (n=22)	55.56±13.19^{a} (n=13)	55.56±13.19^{az} (n=13)	38.89±12.87^{az} (n=11)	29.17±11.63^{bz} (n=8)
Grade B	1.50±0.22^{a} (n=15)	63.33±13.33^{a} (n=9)	48.33±15.00^{az} (n=7)	38.33±14.50^{az} (n=6)	23.33±13.19^{abz} (n=3)
Grade C	1.71±0.25^{a} (n=29)	43.73±11.30^{a} (n=12)	20.20±8.63^{az} (n=8)	16.08±7.00^{ayz} (n=6)	0.00±0.00^{ay} (n=0)
Average	1.69±0.14 (n=66)	52.39±7.17 (n=34)	38.29±7.05^{z} (n=28)	28.80±6.31^{yz} (n=23)	14.96±5.22^{y} (n=11)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)

^{yz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.25: Developmental competence (%, mean±SEM) of *in vitro* produced goat embryos at two PMSG dosages regardless of the oocyte grades

PMSG dosage	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
1200 IU	3.87±0.71[a] (n=58)	65.04±7.36[a] (n=34)	63.04±7.72[b] (n=32)	58.60±7.65[b] (n=30)	14.41±5.61[a] (n=9)
1500 IU	3.00±0.34[a] (n=66)	47.87±8.06[a] (n=34)	36.51±7.14[a] (n=28)	28.89±6.64[a] (n=23)	16.06±5.96[a] (n=11)
Average	3.35±0.35 (n=124)	54.83±5.75 (n=68)	47.26±5.64 (n=60)	40.93±5.52 (n=53)	15.39±4.16 (n=20)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)

4.3 EFFECT OF OOCYTE QUALITY AND DURATION OF IVM ON THE RATES OF MATURATION, CLEAVAGE AND DEVELOPMENTAL COMPETENCE OF *IN VITRO* PRODUCED GOAT EMBRYOS BY ICSI TECHNIQUE (EXPERIMENT 3)

The results of IVM oocytes retrieved at different IVM durations are depicted in Tables 4.26 and 4.27. A total of 239 oocytes were matured from 69 ovaries with an average of 3.46±0.26 oocytes matured per ovary. A total of 148 oocytes were matured from 35 ovaries in 22 to 25 hours and 91 oocytes were matured from 34 ovaries in 26 to 29 hours IVM. A significant difference ($P<0.05$) was observed in the number of oocytes matured per ovary between 22 to 25 hours (4.23±0.38) and 26 to 29 hours (2.68±0.31) IVM. No significant differences ($P>0.05$) were observed in the percentage and number of oocytes matured per ovary between the two IVM durations, in the respective grades. However, there were significant differences ($P<0.05$) in the percentage and number of oocytes matured per ovary among the oocyte grades within each IVM durations. Grades A, B and C (96.86%, average 3.36 oocytes per ovary) yielded higher number of oocytes matured compared to Grades D and E (3.14%, average of 0.10 oocytes per ovary). The average percentages for oocytes of Grades A, B, C, D and E matured per ovary were 33.48±4.00%, 27.25±3.71%, 36.13±4.16%, 1.45±0.89% and 1.69±1.47%, respectively. Correspondingly, the average numbers were 1.26±0.17, 0.90±0.12, 1.20±0.14, 0.06±0.04 and 0.04±0.03, respectively.

The maturation rate of different grades oocyte at two PMSG dosages is depicted in Table 4.28. A total of 208 and 212 immature oocytes were used for 22 to 25 hours and 26 to 29 hours of IVM duration, respectively. Immature oocytes for Grades A (63 and 49), B (47 and 36), C (85 and 73), D (10 and 27) and E (3 and 27) were used for durations of 22 to 25 hours and 26 to 29 hours IVM, respectively. Higher maturation rates ($P<0.05$) were observed in the 22 to 25 hours (71.59±3.44%) compared to the 26 to 29 hours (38.74±4.30%) IVM. No

significant differences (P>0.05) were observed in maturation rates between the two IVM durations for oocytes of Grades C, D and E. However, maturation rates for Grades A and B oocytes were significantly different (P<0.05) between 22 to 25 hours (90.56±4.18% and 91.32±3.23%, respectively) and 26 to 29 hours (59.03±8.27% and 51.33±8.94%, respectively) IVM. In both IVM durations, maturation rates for Grades A, B and C (75.42±5.01%, 73.14±5.32% and 48.23±4.52%, respectively) oocytes were significant higher (P<0.05) than Grades D and E (9.20±5.20% and 5.56±4.04%, respectively) oocytes. In general, maturation rates of better quality oocytes (Grades A, B and C) in 22 to 25 and 26 to 29 hours IVM were 79.30% and 50.90%, respectively. In both IVM durations, the combined average maturation rate of Grades A, B and C oocytes was approximately 65.60%.

In the present study, only oocytes of Grades A, B and C were used in ICSI experiments and oocytes of Grades D and E were omitted due to the low number of maturation obtained. The results of matured oocytes cleaved at different PMSG dosages are depicted in Tables 4.29 and 4.30. A total of 73 oocytes were cleaved from 124 matured oocytes with an average of 2.43±0.29 oocytes cleaved per ovary. A total of 62 oocytes were cleaved from 94 matured oocytes in 22 to 25 hours and 11 oocytes were cleaved from 30 matured oocytes in 26 to 29 hours IVM. An average of 2.70±0.35 and 1.57±0.30 oocytes were cleaved per ovary in 22-25 and 26 to 29 hours IVM, respectively. No significant differences (P>0.05) were observed in the percentage and number of oocytes cleaved per ovary between the two IVM durations, in the respective grades. However, percent of oocytes cleaved per ovary from Grade A were apparently higher (P>0.05) in 22 to 25 hours compared with 26 to 29 hours IVM. For the all two IVM durations, average percentages for oocytes of Grades A, B, C, D and E cleaved per ovary were 41.45±6.70%, 30.39±6.87% and 28.17±6.44%, respectively. Correspondingly, the average numbers were 1.07±0.18, 0.60±0.12 and 0.77±0.18, respectively.

The cleavage rate of different grades oocyte at two IVM durations is depicted in Table 4.31. A total of 94 and 30 matured oocytes were used for the ICSI experiments derived from 22 to 25 and 26 to 29 hours IVM, respectively. Matured oocytes of Grades A (37 and 10), B (23 and 6) and C (34 and 14) used were obtained from 22 to 25 and 26 to 29 hours IVM, respectively. No significant differences ($P>0.05$) were observed in cleavage rates between the two IVM durations for oocytes of Grades B and C. However, cleavage rates for Grade A oocytes were significant differences ($P<0.05$) between 22 to 25 hours (78.92±7.36%) and 26 to 29 hours (38.89±20.03%) IVM. In both IVM durations, cleavage rates for Grades A, B and C were 68.48±8.18%, 68.52±9.32% and 44.63±8.55%, respectively. In general, cleavage rates of Grades A, B and C in 22 to 25 and 26 to 29 hours IVM were 67.30% and 42.96%, respectively. In both IVM durations, the combined average cleavage rate of Grades A, B and C oocytes was approximately 60.54%.

The developmental rates of different grades oocyte at two IVM durations is depicted in Table 4.32. A total of 62 and 11 two-cell stage embryos were obtained from ICSI experiments derived from 22 to 25 and 26 to 29 hours IVM, respectively. The 2-cell stage embryos yielded from oocytes of Grades A (28 and 4), B (15 and 3) and C (19 and 4) were obtained from 22 to 25 and 26 to 29 hours IVM, respectively. In both IVM durations, the averages of developmental rates at 4-, 8-cell and morula in oocytes of Grade A were 67.39±8.37%, 54.35±9.10% and 22.83±7.60%, respectively; Grade B were 60.19±10.45%, 51.85±11.10% and 29.63±10.74%, respectively; and Grades C were 26.11±7.23%, 22.78±6.40% and 1.85±1.85%, respectively. No significant differences ($P>0.05$) were observed in developmental rates of Grades B and C oocytes between two IVM durations. However, a significant difference ($P<0.05$) was observed in the early embryo development stages (4- and 8-cell) of Grade A oocytes between 22 to 25 hours (77.45±7.85% and 65.69±9.66%, respectively) and 26 to 29 hours (38.89±20.03% and 22.22±16.48%,

respectively) IVM. Also, the developmental rates of Grades A, B and C oocytes were apparently more competent in developing up to the morula stage (P>0.05) in 22 to 25 hours compared to 26 to 29 hours IVM.

The developmental rate of different grades of oocyte at 22 to 25 and 26 to 29 hours IVM is depicted in Tables 4.33 and 4.34, respectively. For Grades A and B oocytes, a significant difference (P<0.05) was observed in all embryo development stages (2-cell to morula) compared to Grade C oocytes with 22 to 25 hours IVM. Also, 22 to 25 hours IVM apparently yielded higher (P>0.05) developmental rates at 4-, 8-cell and morula in oocytes of Grade A were 77.45±7.85%, 65.69±9.66% and 27.94±9.67%, respectively; Grade B were 67.95±10.92%, 64.10±12.06% and 41.03±13.69%, respectively; and Grade C were 35.59±9.31%, 30.29±7.95% and 2.94±2.94%, respectively; compared to 26-29 hours IVM in oocytes of Grade A were 38.89±20.03%, 22.22±16.48% and 8.33±8.33%, respectively; Grade B were 40.00±24.50%, 20.00±20.00% and 0.00±0.00%, respectively; and Grade C were 10.00±10.00%, 10.00±10.00% and 0.00±0.00%, respectively.

Regardless of the oocyte grades (Table 4.35), the cleavage rates were significant higher (P<0.05) in 22 to 25 hours (70.06±5.18%) compared to 26 to 29 hours (35.71±11.17%) IVM. Higher developmental rates at 4-, 8-cell and morula (P<0.05) were observed in 22 to 25 hours (66.58±5.32%, 59.40±5.27% and 24.55±5.90%, respectively) compared to 26 to 29 hours (21.43±8.81%, 15.48±8.64% and 2.38±2.38%, respectively) IVM. In both IVM durations, the averages of developmental rates at 2-, 4-, 8-cell and morula were 57.07±5.91%, 49.50±5.89%, 42.78±5.77% and 16.16±4.15%, respectively.

Table 4.26: Percentage (%, mean±SEM) of oocytes matured from different IVM durations

IVM duration (h)	No. of ovaries	No. of oocytes matured per ovary	Percent of oocytes matured per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
22 to 25	35	4.23±0.38^{b} (n=148)	36.22±5.29^{az} (n=55)	30.54±4.88^{az} (n=41)	33.24±4.91^{az} (n=52)	0.00±0.00^{ay} (n=0)	0.00±0.00^{ay} (n=0)
26 to 29	34	2.68±0.31^{a} (n=91)	30.65±6.06^{ayz} (n=32)	23.87±5.62^{ay} (n=21)	39.11±6.80^{az} (n=31)	2.94±1.79^{ax} (n=4)	3.43±2.97^{ax} (n=3)
Average	34.50 (n=69)	3.46±0.26 (n=239)	33.48±4.00^{z} (n=87)	27.25±3.71^{z} (n=62)	36.13±4.16^{z} (n=83)	1.45±0.89^{y} (n=4)	1.69±1.47^{y} (n=3)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.27: Number (n, mean±SEM) of oocytes matured from different IVM durations

IVM duration (h)	No. of ovaries	No. of oocytes matured per ovary	No. of oocytes matured per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
22 to 25	35	4.23±0.38^{b} (n=148)	1.57±0.26^{az} (n=55)	1.17±0.17^{az} (n=41)	1.49±0.24^{az} (n=52)	0.00±0.00^{ay} (n=0)	0.00±0.00^{ay} (n=0)
26 to 29	34	2.68±0.31^{a} (n=91)	0.94±0.20^{az} (n=32)	0.62±0.15^{az} (n=21)	0.91±0.14^{az} (n=31)	0.12±0.07^{ay} (n=4)	0.09±0.07^{ay} (n=3)
Average	34.50 (n=69)	3.46±0.26 (n=239)	1.26±0.17^{z} (n=87)	0.90±0.12^{y} (n=62)	1.20±0.14^{yz} (n=83)	0.06±0.04^{x} (n=4)	0.04±0.03^{x} (n=3)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.28: Maturation rate (%, mean±SEM) of different grades of oocyte at two IVM durations

IVM duration (h)	Percent MII oocytes*	Percent maturation**				
		Grade A	Grade B	Grade C	Grade D	Grade E
22 to 25	71.59±3.44^{b} (148/208)***	90.56±4.18^{bz} (55/63)	91.32±3.23^{bz} (41/47)	56.01±5.54^{ay} (52/85)	0.00±0.00^{ax} (0/10)	0.00±0.00^{ax} (0/3)
26 to 29	38.74±4.30^{a} (91/212)	59.03±8.27^{az} (32/49)	51.33±8.94^{az} (21/36)	42.34±6.64^{az} (31/73)	12.70±7.08^{ay} (4/27)	6.67±4.83^{ay} (3/27)
Average	52.82±3.36 (239/420)	75.42±5.01^{z} (87/112)	73.14±5.32^{z} (62/83)	48.23±4.52^{y} (83/158)	9.20±5.20^{x} (4/37)	5.56±4.04^{x} (3/30)

* Mean percentage of MII oocytes was based on total oocytes from all grades used for IVM, in the respective IVM duration groups
** Mean percentage of maturation was based on oocytes used for IVM, in the respective grades
*** Mean total of matured oocytes per total of oocytes used for IVM
^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.29: Percentage (%, mean±SEM) of oocytes cleaved from different IVM durations

IVM duration (h)	No. of oocytes injected per ovary	No. of oocytes cleaved per ovary	Percent of oocytes cleaved per ovary		
			Grade A	Grade B	Grade C
22 to 25	4.09±0.49^{b} (n=94)	2.70±0.35^{a} (n=62)	46.81±7.79^{az} (n=28)	26.59±6.67^{az} (n=15)	26.59±7.38^{az} (n=19)
26 to 29	2.14±0.23^{a} (n=30)	1.57±0.30^{a} (n=11)	23.80±11.42^{az} (n=4)	42.86±20.20^{az} (n=3)	33.33±14.09^{az} (n=4)
Average	3.35±0.35 (n=124)	2.43±0.29 (n=73)	41.47±6.70^{z} (n=32)	30.39±6.87^{z} (n=18)	28.17±6.44^{z} (n=23)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{z} Mean values within a row with same superscripts were not significantly different (P>0.05)

Table 4.30: Number (n, mean±SEM) of oocytes cleaved from different IVM durations

IVM duration (h)	No. of oocytes injected per ovary	No. of oocytes cleaved per ovary	No. of oocytes cleaved per ovary		
			Grade A	Grade B	Grade C
22 to 25	4.09±0.49[b] (n=94)	2.70±0.35[a] (n=62)	1.22±0.21[az] (n=28)	0.65±0.15[az] (n=15)	0.83±0.23[az] (n=19)
26 to 29	2.14±0.23[a] (n=30)	1.57±0.30[a] (n=11)	0.57±0.30[az] (n=4)	0.43±0.20[az] (n=3)	0.57±0.20[az] (n=4)
Average	3.35±0.35 (n=124)	2.43±0.29 (n=73)	1.07±0.18[z] (n=32)	0.60±0.12[z] (n=18)	0.77±0.18[z] (n=23)

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)
[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Table 4.31: Cleavage rate (%, mean±SEM) of different grades of oocyte at two IVM durations

IVM duration (h)	Percent cleavage[*]		
	Grade A	Grade B	Grade C
22 to 25	78.92±7.36[bz] (28/37)[**]	75.64±9.55[az] (15/23)	47.35±9.96[ay] (19/34)
26 to 29	38.89±20.03[az] (4/10)	50.00±22.36[az] (3/6)	40.00±16.33[az] (4/14)
Average	68.48±8.18[z] (32/124)	68.52±9.32[z] (18/29)	44.63±8.55[z] (23/48)

[*] Mean percentage of cleavage was based on oocytes used for ICSI, in the respective grades
[**] Mean total of cleaved oocytes per total of oocytes used for ICSI
[ab] Mean values within a column with different superscripts were significantly different (P<0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.32: Developmental rate (%, mean±SEM) of different grades of oocyte at two IVM duration

Oocyte grade	IVM duration (h)	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
				4-cell	8-cell	Morula
Grade A	22 to 25	2.18±0.25[a] (n=37)	78.92±7.36[b] (n=28)	77.45±7.85[b] (n=27)	65.69±9.66[b] (n=25)	27.94±9.67[a] (n=11)
	26 to 29	1.67±0.33[a] (n=10)	38.89±20.03[a] (n=4)	38.89±20.03[a] (n=4)	22.22±16.48[a] (n=3)	8.33±8.33[a] (n=1)
	Average	2.04±0.20 (n=47)	68.48±8.18 (n=32)	67.39±8.37 (n=31)	54.35±9.10 (n=28)	22.83±7.60 (n=12)
Grade B	22 to 25	1.77±0.26[a] (n=23)	75.64±9.55[a] (n=15)	67.95±10.92[a] (n=14)	64.10±12.06[a] (n=13)	41.03±13.69[a] (n=8)
	26 to 29	1.20±0.20[a] (n=6)	50.00±22.36[a] (n=3)	40.00±24.50[a] (n=2)	20.00±20.00[a] (n=1)	0.00±0.00[a] (n=0)
	Average	1.61±0.20 (n=29)	68.52±9.32 (n=18)	60.19±10.45 (n=16)	51.85±11.10 (n=14)	29.63±10.74 (n=8)
Grade C	22 to 25	2.00±0.32[a] (n=34)	47.35±9.96[a] (n=19)	35.59±9.31[a] (n=17)	30.29±7.95[a] (n=14)	2.94±2.94[a] (n=2)
	26 to 29	1.40±0.16[a] (n=14)	40.00±16.33[a] (n=4)	10.00±10.00[a] (n=1)	10.00±10.00[a] (n=1)	0.00±0.00[a] (n=0)
	Average	1.78±0.22 (n=48)	44.63±8.55 (n=23)	26.11±7.23 (n=18)	22.78±6.40 (n=15)	1.85±1.85 (n=2)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)

Table 4.33: Developmental rate (%, mean±SEM) of different grades of oocyte at 22 to 25 hours of IVM duration

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	2.18±0.25^{a} (n=37)	78.92±7.36^{b} (n=28)	77.45±7.85^{bz} (n=27)	65.69±9.66^{bz} (n=25)	27.94±9.67^{aby} (n=11)
Grade B	1.77±0.26^{a} (n=23)	75.64±9.55^{b} (n=15)	67.95±10.92^{bz} (n=14)	64.10±12.06^{bz} (n=13)	41.03±13.69^{bz} (n=8)
Grade C	2.00±0.32^{a} (n=34)	47.35±9.96^{a} (n=19)	35.59±9.31^{az} (n=17)	30.29±7.95^{az} (n=14)	2.94±2.94^{ay} (n=2)
Average	2.00±0.16 (n=94)	66.60±5.52 (n=62)	59.68±5.89^{z} (n=58)	52.45±6.02^{z} (n=52)	22.52±5.63^{y} (n=21)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)

^{yz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.34: Developmental rate (%, mean±SEM) of different grades of oocyte at 26 to 29 hours of IVM duration

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	1.67±0.33[a] (n=10)	38.89±20.03[a] (n=4)	38.89±20.03[az] (n=4)	22.22±16.48[az] (n=3)	8.33±8.33[az] (n=1)
Grade B	1.20±0.20[a] (n=6)	50.00±22.36[a] (n=3)	40.00±24.50[az] (n=2)	20.00±20.00[az] (n=1)	0.00±0.00[az] (n=0)
Grade C	1.40±0.16[a] (n=14)	40.00±16.33[a] (n=4)	10.00±10.00[az] (n=1)	10.00±10.00[az] (n=1)	0.00±0.00[az] (n=0)
Average	1.43±0.13 (n=30)	42.06±10.52 (n=11)	25.40±9.46[z] (n=7)	15.87±7.84[yz] (n=5)	2.38±2.38[y] (n=1)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.35: Developmental competence (%, mean±SEM) of *in vitro* produced goat embryos at two IVM duration regardless of the oocyte grades

IVM duration (h)	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
22 to 25	4.09±0.49[b] (n=94)	70.06±5.18[b] (n=62)	66.58±5.32[b] (n=58)	59.40±5.27[b] (n=52)	24.55±5.90[b] (n=21)
26 to 29	2.14±0.23[a] (n=30)	35.71±11.17[a] (n=11)	21.43±8.81[a] (n=7)	15.48±8.64[a] (n=5)	2.38±2.38[a] (n=1)
Average	3.35±0.35 (n=124)	57.07±5.91 (n=73)	49.50±5.89 (n=65)	42.78±5.77 (n=57)	16.16±4.15 (n=22)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)

4.4 EFFECT OF CHEMICAL TREATMENT ON THE DEVELOPMENTAL COMPETENCE OF *IN VITRO* PRODUCED GOAT EMBRYOS FERTILSIED BY ICSI TECHNIQUE (EXPERIMENT 4)

In the present study, only oocytes of Grades A, B and C were used in ICSI experiments and oocytes of Grades D and E were omitted due to the low number of maturation obtained. The results of sham injection with different activation regimens are depicted in Tables 4.36 and 4.37. A total of 29 oocytes were cleaved from 108 matured oocytes with an average of 2.23±0.51 oocytes cleaved per ovary. A total of 9 oocytes were cleaved from 38 matured oocytes in treatment Ca^{2+} ionophore and 20 oocytes were cleaved from 39 matured oocytes in treatment Ca^{2+} ionophore and 6-DMAP following sham injection. No embryos cleaved for oocytes of Grades A, B and C in the sham control group. An average of 1.50±0.34 and 2.86±0.86 oocytes were cleaved per ovary in treatment Ca^{2+} ionophore and treatment Ca^{2+} ionophore and 6-DMAP following sham injection, respectively. No significant differences (P>0.05) were observed in the percentage and number of oocytes cleaved per ovary between the two treatments following sham injection, in the respective grades. However, percent of oocytes cleaved per ovary from Grades B and C were apparently higher (P>0.05) in treatment Ca^{2+} ionophore compared to treatment Ca^{2+} ionophore and 6-DMAP following sham injection. For the all two treatments following sham injection, average percentages for oocytes of Grades A, B and C cleaved per ovary were 47.07±11.84%, 18.22±8.30% and 34.71±11.44%, respectively. Correspondingly, the average numbers were 1.00±0.25, 0.46±0.18 and 0.77±0.26, respectively.

The cleavage rate of different grades oocyte after sham control and two chemical treatments following sham injection is depicted in Table 4.38. A total of 31, 38 and 39 matured oocytes were used for the sham control, treatment Ca^{2+} ionophore; and Ca^{2+} ionophore and 6-DMAP following sham injection, respectively. Matured oocytes of Grades

A (10, 12 and 18), B (11, 15 and 7) and C (10, 11 and 14) used were used for sham control, treatment Ca^{2+} ionophore; and Ca^{2+} ionophore and 6-DMAP following sham injection, respectively. No significant differences (P>0.05) were observed in cleavage rates between the three groups following sham injection, in the respective oocyte grades. In three groups sham injection, cleavage rates for Grades A, B and C were 44.42±11.22%, 26.92±10.77% and 37.82±11.81%, respectively. In general, the cleavage rates of Grades A, B and C in treatment Ca^{2+} ionophore; and Ca^{2+} ionophore and 6-DMAP following sham injection were 25.46% and 49.76%, respectively. In both chemical treatments following sham injection, the combined average cleavage rate of Grades A, B and C oocytes was approximately 36.39%.

The developmental rates of different grades oocyte at two chemical treatments following sham injection is depicted in Table 4.39. A total of 9 and 20 two-cell stage embryos were obtained from treatment Ca^{2+} ionophore; and Ca^{2+} ionophore and 6-DMAP following sham injection, respectively. The 2-cell stage embryos yielded from oocytes of Grades A (5 and 8), B (2 and 4) and C (2 and 8) were obtained from treatment Ca^{2+} ionophore; and Ca^{2+} ionophore and 6-DMAP following sham injection, respectively. In both chemical treatments, the averages of developmental rates at 4-, 8-cell and morula in oocytes of Grade A were 37.28±10.76%, 25.54±10.02% and 12.14±7.72%, respectively; Grade B were 26.92±10.77%, 15.39±8.74% and 0.00±0.00%, respectively; and Grades C were 26.28±9.43%, 14.74±8.02% and 0.00%, respectively. No significant differences (P>0.05) were observed in developmental rates of Grades A and B oocytes between two chemical treatment following sham injection. However, a significant difference (P<0.05) was observed in the early embryo development stages (4-cell) of Grade C oocytes between treatment Ca^{2+} ionophore (5.56±5.56%) and; Ca^{2+} ionophore and 6-DMAP (44.05±13.93%) following sham injection. Also, the developmental rates of Grades A, B and C oocytes were apparently more competent in developing up to the 8-cell stage (P>0.05) in treatment Ca^{2+} ionophore and 6-

DMAP compared to Ca^{2+} ionophore following sham injection.

The developmental rate of different grades of oocyte in treatment Ca^{2+} ionophore and; Ca^{2+} ionophore and 6-DMAP is depicted in Tables 4.40 and 4.41, respectively. Developmental rates at 4-, 8-cell and morula embryo development stage following sham injection using Ca^{2+} ionophore and 6-DMAP treatment apparently yielded higher ($P>0.05$) in oocytes of Grade A were 48.09±18.74%, 45.72±19.38% and 20.00±16.33%, respectively; Grade B were 50.00±22.36%, 30.00±20.00% and 0.00±0.00%, respectively; and Grade C were 44.05±13.93%, 22.62±13.93% and 0.00±0.00%, respectively; compared to Ca^{2+} ionophore treatment in oocytes of Grade A were 29.17±12.89%, 10.42±7.00% and 6.25±6.25%, respectively; Grade B were 12.50±8.18%, 6.25±6.25% and 0.00±0.00%, respectively; and Grade C were 5.56±5.56%, 5.56±5.56% and 0.00±0.00%, respectively.

Regardless of the oocyte grades (Table 4.42), the cleavage rates were significant higher ($P<0.05$) in the Ca^{2+} ionophore and 6-DMAP treatment (42.81±11.04%) compared to the Ca^{2+} ionophore treatment (17.92±6.65%) following sham injection. Higher developmental rates at 4-, 8-cell and morula ($P<0.05$) were observed in the Ca^{2+} ionophore and 6-DMAP treatment (40.31±10.08%, 25.83±9.81% and 5.21±3.50%, respectively) compared to the Ca^{2+} ionophore treatment (11.86±4.68%, 5.72±4.13% and 1.30±1.30%, respectively) following sham injection. However, there is no oocytes were cleaved and no embryos were developed in sham control group. In both chemical treatment following sham injection, the averages of developmental rates at 2-, 4-, 8-cell and morula were 17.41±4.69%, 14.61±4.15%, 8.69±3.38% and 1.81±1.04%, respectively.

Table 4.36: Percentage (%, mean±SEM) of oocytes cleaved following sham injection using different activation regimens

Treatment	No. of oocytes injected per ovary	No. of oocytes cleaved per ovary	Percent of oocytes cleaved per ovary		
			Grade A	Grade B	Grade C
Control	2.58±0.50[a] (n=31)	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)
Ca^{2+} ionophore	3.46±0.68[ab] (n=38)	1.50±0.34[a] (n=9)	63.89±17.44[az] (n=5)	13.89±9.04[ay] (n=2)	22.22±16.48[ayz] (n=2)
Ca^{2+} ionophore + 6-DMAP	4.88±0.85[b] (n=39)	2.86±0.86[a] (n=20)	32.65±15.12[az] (n=11)	21.94±13.86[az] (n=4)	45.41±15.83[az] (n=9)
Average	3.48±0.40 (n=108)	2.23±0.51 (n=29)	47.07±11.84[z] (n=16)	18.22±8.30[z] (n=6)	34.71±11.44[z] (n=11)

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.37: Number (n, mean±SEM) of oocytes cleaved following sham injection using different activation regimens

Treatment	No. of oocytes injected per ovary	No. of oocytes cleaved per ovary	No. of oocytes cleaved per ovary		
			Grade A	Grade B	Grade C
Control	2.58±0.50[a] (n=31)	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)
Ca^{2+} ionophore	3.46±0.68[ab] (n=38)	1.50±0.34[a] (n=9)	0.83±0.17[az] (n=5)	0.33±0.21[az] (n=2)	0.33±0.21[az] (n=2)
Ca^{2+} ionophore + 6-DMAP	4.88±0.85[b] (n=39)	2.86±0.86[a] (n=20)	1.14±0.46[az] (n=8)	0.57±0.30[az] (n=4)	1.14±0.40[az] (n=8)
Average	3.48±0.40 (n=108)	2.23±0.51 (n=29)	1.00± 0.25[z] (n=13)	0.46±0.18[z] (n=6)	0.77±0.26[z] (n=10)

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)

[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Table 4.38: Cleavage rate (%, mean±SEM) of *in vitro* produced goat embryos following sham injection using different activation regimens

Treatment	Percent cleavage[*]		
	Grade A	Grade B	Grade C
Control	0.00 (0/10) [**]	0.00 (0/11)	0.00 (0/10)
Ca^{2+} ionophore	41.67±14.77[az] (5/12)	12.50±8.18[az] (2/15)	22.22±16.48[az] (2/11)
Ca^{2+} ionophore + 6-DMAP	48.10±18.74[az] (8/18)	50.00±22.36[az] (4/7)	51.19±16.11[az] (8/14)
Average	44.42±11.22[z] (13/40)	26.92±10.77[z] (6/33)	37.82±11.81[z] (10/35)

[*] Mean percentage of cleavage was based on oocytes used for sham injection, in the respective grades
[**] Mean total of cleaved oocytes per total of oocytes used for sham injection
[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Table 4.39: Developmental rate (%, mean±SEM) of *in vitro* produced goat embryos following sham injection using different activation regimens

Oocyte grade	Treatment	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
				4-cell	8-cell	Morula
Grade A	Control	1.67±0.33[a] (n=10)	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)
	Ca^{2+} ionophore	1.50±0.27[a] (n=12)	41.67±14.77[a] (n=5)	29.17±12.89[a] (n=4)	10.42±7.00[a] (n=2)	6.25±6.25[a] (n=1)
	Ca^{2+} ionophore+ 6-DMAP	3.00±1.00[a] (n=18)	48.10±18.74[a] (n=8)	48.10±18.74[a] (n=8)	45.72±19.38[a] (n=7)	20.00±16.33[a] (n=3)
	Average	2.00±0.35 (n=40)	44.42±11.22 (n=13)	37.28±10.76 (n=12)	25.54±10.02 (n=9)	12.14±7.72 (n=4)
Grade B	Control	1.57±0.30[a] (n=11)	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)
	Ca^{2+} ionophore	1.88±0.23[a] (n=15)	12.50±8.18[a] (n=2)	12.50±8.18[a] (n=2)	6.25±6.25[a] (n=1)	0.00±0.00[a] (n=0)
	Ca^{2+} ionophore+ 6-DMAP	1.40±0.25[a] (n=7)	50.00±22.36[a] (n=4)	50.00±22.36[a] (n=4)	30.00±20.00[a] (n=3)	0.00±0.00[a] (n=0)
	Average	1.65±0.15 (n=33)	26.92±10.77 (n=6)	26.92±10.77 (n=6)	15.39±8.74 (n=4)	0.00±0.00 (n=0)

(continued)

(continued)

Oocyte grade	Treatment	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
				4-cell	8-cell	Morula
Grade C	Control	1.43±0.30[a] (n=10)	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)
	Ca^{2+} ionophore	1.83±0.31[a] (n=11)	22.22±16.48[a] (n=2)	5.56±5.56[a] (n=1)	5.56±5.56[a] (n=1)	0.00 (n=0)
	Ca^{2+} ionophore+ 6-DMAP	2.00±0.44[a] (n=14)	51.19±16.11[a] (n=8)	44.05±13.93[b] (n=7)	22.62±13.93[a] (n=3)	0.00 (n=0)
	Average	1.75±0.20 (n=35)	37.82±11.81 (n=10)	26.28±9.43 (n=8)	14.74±8.02 (n=4)	0.00 (n=0)

* Mean percentage of development was based on oocytes used for sham injection, in the respective grades

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)

Table 4.40: Developmental rate (%, mean±SEM) of different grades of oocyte following sham injection using Ca^{2+} ionophore activation regimen

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	1.50±0.27[a] (n=12)	41.67±14.77[a] (n=5)	29.17±12.89[az] (n=4)	10.42±7.00[az] (n=2)	6.25±6.25[az] (n=1)
Grade B	1.88±0.23[a] (n=15)	12.50±8.18[a] (n=2)	12.50±8.18[az] (n=2)	6.25±6.25[az] (n=1)	0.00±0.00[az] (n=0)
Grade C	1.83±0.31[a] (n=11)	22.22±16.48[a] (n=2)	5.56±5.56[az] (n=1)	5.56±5.56[az] (n=1)	0.00±0.00[az] (n=0)
Average	1.73±0.15 (n=38)	25.76±7.73 (n=9)	16.67±5.91[z] (n=7)	7.58±3.59[yz] (n=4)	2.27±2.27[y] (n=1)

* Mean percentage of development was based on oocytes used for sham injection, in the respective grades

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.41: Developmental rate (%, mean±SEM) of different grades of oocyte following sham injection using Ca^{2+} ionophore and 6-DMAP activation regimens

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	3.00±1.00[a] (n=18)	48.10±18.74[a] (n=8)	48.09±18.74[az] (n=8)	45.72±19.38[az] (n=7)	20.00±16.33[az] (n=3)
Grade B	1.40±0.25[a] (n=7)	50.00±22.36[a] (n=4)	50.00±22.36[az] (n=4)	30.00±20.00[az] (n=3)	0.00±0.00[az] (n=0)
Grade C	2.00±0.44[a] (n=14)	51.19±16.11[a] (n=8)	44.05±13.93[az] (n=7)	22.62±13.93[ayz] (n=3)	0.00±0.00[ay] (n=0)
Average	2.17±0.39 (n=39)	49.83±10.14 (n=20)	47.05±9.70[z] (n=19)	32.37±9.77[z] (n=13)	6.67±5.60[y] (n=3)

* Mean percentage of development was based on oocytes used for sham injection, in the respective grades

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.42: Developmental competence (%, mean±SEM) of *in vitro* produced goat embryos following sham injection using different activation regimens regardless of the oocyte grades

Treatment	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Control	2.58±0.50[a] (n=31)	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)
Ca^{2+} ionophore	3.46±0.68[ab] (n=38)	17.92±6.65[a] (n=9)	11.86±4.68[a] (n=7)	5.72±4.13[a] (n=4)	1.30±1.30[a] (n=1)
Ca^{2+} ionophore + 6-DMAP	4.88±0.85[b] (n=39)	42.81±11.04[b] (n=20)	40.31±10.08[b] (n=19)	25.83±9.81[b] (n=13)	5.21±3.50[b] (n=3)
Average	3.48±0.40 (n=108)	17.41±4.69 (n=29)	14.61±4.15 (n=26)	8.69±3.38 (n=17)	1.81±1.04 (n=4)

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)

The results of ICSI with different activation regimens are depicted in Tables 4.43 and 4.44. A total of 73 oocytes were cleaved from 124 matured oocytes with an average of 2.43±0.29 oocytes cleaved per ovary. A total of 12 oocytes were cleaved from 30 matured oocytes in ICSI control group, a total of 18 oocytes were cleaved from 37 matured oocytes in treatment Ca^{2+} ionophore and 43 oocytes were cleaved from 57 matured oocytes in treatment Ca^{2+} ionophore and 6-DMAP following ICSI. An average of 2.40±0.51, 1.64±0.20 and 3.07±0.53 oocytes were cleaved per ovary in ICSI control group, treatment Ca^{2+} ionophore and treatment Ca^{2+} ionophore and 6-DMAP following ICSI, respectively. No significant differences ($P>0.05$) were observed in the percentage and number of oocytes cleaved per ovary between the three groups following ICSI, in the respective grades. However, percentage of oocytes cleaved per ovary from Grades B and C were apparently higher ($P>0.05$) in ICSI control group compared to treatment Ca^{2+} ionophore; and treatment Ca^{2+} ionophore and 6-DMAP following ICSI. For the all three groups following ICSI, average percentages for oocytes of Grades A, B and C cleaved per ovary were 41.45±6.70%, 30.39±6.87% and 28.17±6.44%, respectively. Correspondingly, the average numbers were 1.06±0.18, 0.60±0.12 and 0.77±0.18, respectively.

The cleavage rates of different grades oocyte of three groups of ICSI is depicted in Table 4.45. A total of 30, 37 and 57 matured oocytes were used for the ICSI control, treatment Ca^{2+} ionophore; and Ca^{2+} ionophore and 6-DMAP following ICSI, respectively. Matured oocytes of Grades A (7, 16 and 24), B (11, 8 and 10) and C (12, 13 and 23) used were used for ICSI, treatment Ca^{2+} ionophore; and Ca^{2+} ionophore and 6-DMAP following ICSI, respectively. No significant differences ($P>0.05$) were observed in cleavage rates of Grades A and C oocytes between the three groups following ICSI. However, cleavage rates of Grade B oocytes were significant differences ($P<0.05$) between treatment Ca^{2+} ionophore and 6-DMAP (100.00±0.00%); and treatment Ca^{2+} ionophore (56.67±19.44%) following

ICSI as well as ICSI control (30.00±12.25%). In three groups ICSI, cleavage rates for Grades A, B and C were 68.48±8.18%, 68.52±9.32% and 44.63±8.55%, respectively. In general, the cleavage rates of Grades A, B and C in ICSI control, treatment Ca^{2+} ionophore; and Ca^{2+} ionophore and 6-DMAP following ICSI were 35.79%, 51.10% and 79.52%, respectively. In the three ICSI groups, the combined average cleavage rate of Grades A, B and C oocytes was approximately 60.54%.

The developmental rates of different grades oocyte of three groups of ICSI is depicted in Table 4.46. A total of 12, 18 and 43 two-cell stage embryos were obtained from ICSI control, treatment Ca^{2+} ionophore; and Ca^{2+} ionophore and 6-DMAP following ICSI, respectively. The 2-cell stage embryos yielded from oocytes of Grades A (3, 10 and 19), B (4, 4 and 10) and C (5, 4 and 14) were obtained from ICSI group, treatment Ca^{2+} ionophore; and Ca^{2+} ionophore and 6-DMAP following ICSI, respectively. In the three ICSI groups, the averages of developmental rates at 4-, 8-cell and morula in oocytes of Grade A were 67.39±8.37%, 54.35±9.10% and 22.83±7.60%, respectively; Grade B were 60.19±10.45%, 51.85±11.10% and 29.63±10.74%, respectively; and Grades C were 26.11±7.23%, 22.78±6.40% and 1.85±1.85%, respectively. No significant differences ($P>0.05$) were observed in developmental rates of Grades A and C oocytes among the three ICSI groups. However, a significant difference ($P<0.05$) was observed in the 4- and 8-cell embryo development stages of Grade B oocytes between ICSI control (20.00±12.25% and 10.00±10.00%, respectively) and; treatment Ca^{2+} ionophore and 6-DMAP (87.50±12.50% and 75.00±16.37%, respectively) following ICSI. Also, the developmental rates of Grades A, B and C oocytes were apparently more competent in developing up to the morula stage ($P>0.05$) in treatment Ca^{2+} ionophore and 6-DMAP compared to treatment Ca^{2+} ionophore following ICSI and ICSI control.

The developmental rate of different grades of oocyte of ICSI control, treatment Ca^{2+}

ionophore and treatment Ca^{2+} ionophore and 6-DMAP following ICSI is depicted in Tables 4.47, 4.48 and 4.49, respectively. Developmental rates at 4-, 8-cell and morula embryo development stage following ICSI using Ca^{2+} ionophore apparently yielded higher ($P>0.05$) in oocytes of Grade A were 65.15±13.57%, 37.88±13.87% and 9.09±9.09%, respectively; Grade B were 56.67±19.44%, 56.67±19.44% and 26.67±19.44%, respectively; and Grade C were 20.37±11.71%, 20.37±11.71% and 0.00±0.00%, respectively; compared to ICSI control in oocytes of Grade A were 29.17±4.17%, 29.17±4.17% and 0.00±0.00%, respectively; Grade B were 20.00±12.25%, 10.00±10.00% and 0.00±0.00%, respectively; and Grade C were 21.43±14.87%, 14.29±9.22% and 0.00±0.00%, respectively. However, a significant higher ($P<0.05$) developmental rates were observed at the 4-, 8-cell and morula embryo development stages using Ca^{2+} ionophore and 6-DMAP following ICSI of Grades A (77.50±11.12%, 77.50±11.12% and 42.50±12.07%, respectively) and B (87.50±12.50%, 75.00±16.37% and 50.00±18.90%, respectively) oocytes compared to Grade C (33.79±12.22%, 30.15±11.35% and 4.55±4.55%, respectively) oocytes.

Regardless of the oocyte grades (Table 4.50), the cleavage rates were significant higher ($P<0.05$) in the Ca^{2+} ionophore and 6-DMAP treatment (73.21±8.31%) following ICSI compared to ICSI control group (38.09±13.15%). Higher developmental rates at 4- and 8-cell ($P<0.05$) were observed in the Ca^{2+} ionophore and 6-DMAP treatment (61.76±8.72% and 56.85±9.35%, respectively) following ICSI compared to ICSI control group (29.05±13.54% and 19.52±7.88%, respectively). Also, a significant differences ($P<0.05$) were observed in the rates of development to the morula stage between the Ca^{2+} ionophore and 6-DMAP treatment (29.87±7.34%) following ICSI versus both ICSI control (0.00±0.00%) and Ca^{2+} ionophore treatment (8.57±4.90%) following ICSI. In three ICSI groups, the averages of developmental rates at 2-, 4-, 8-cell and morula were 57.07±5.91%, 49.50±5.89%, 42.78±5.77% and 16.16±4.15%, respectively.

Table 4.43: Percentage (%, mean±SEM) of oocytes cleaved following ICSI using different activation regimens

Treatment	No. of oocytes injected per ovary	No. of oocytes cleaved per ovary	Percent of oocytes cleaved per ovary		
			Grade A	Grade B	Grade C
Control	4.29±1.13[a] (n=30)	2.40±0.51[a] (n=12)	20.00±12.25[az] (n=3)	43.33±19.44[az] (n=4)	36.67±18.56[az] (n=5)
Ca^{2+} ionophore	2.64±0.31[a] (n=37)	1.64±0.20[a] (n=18)	51.52±11.78[az] (n=10)	22.73±10.37[az] (n=4)	25.76±12.18[az] (n=4)
Ca^{2+} ionophore + 6-DMAP	3.56±0.58[a] (n=57)	3.07±0.53[a] (n=43)	41.19±9.76[az] (n=19)	31.79±10.54[az] (n=10)	27.02±8.28[az] (n=14)
Average	3.35±0.35 (n=124)	2.43±0.29 (n=73)	41.45±6.70[z] (n=32)	30.39±6.87[z] (n=18)	28.17±6.44[z] (n=23)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Table 4.44: Number (n, mean±SEM) of oocytes cleaved following ICSI using different activation regimens

Treatment	No. of oocytes injected per ovary	No. of oocytes cleaved per ovary	No. of oocytes cleaved per ovary		
			Grade A	Grade B	Grade C
Control	4.29±1.13[a] (n=30)	2.40±0.51[a] (n=12)	0.60±0.40[az] (n=3)	0.80±0.37[az] (n=4)	1.00±0.45[az] (n=5)
Ca^{2+} ionophore	2.64±0.31[a] (n=37)	1.64±0.20[a] (n=18)	0.91±0.21[az] (n=10)	0.36±0.15[ay] (n=4)	0.36±0.15[ay] (n=4)
Ca^{2+} ionophore + 6-DMAP	3.56±0.58[a] (n=57)	3.07±0.53[a] (n=43)	1.35±0.31[az] (n=19)	0.71±0.19[az] (n=10)	1.00±0.33[az] (n=14)
Average	3.35±0.35 (n=124)	2.43±0.29 (n=73)	1.06±0.18[z] (n=32)	0.60±0.12[z] (n=18)	0.77±0.18[z] (n=23)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Table 4.45: Cleavage rate (%, mean±SEM) of *in vitro* produced goat embryos following ICSI using different activation regimens

Treatment	Percent cleavage*		
	Grade A	Grade B	Grade C
Control	41.65±8.34^{az} (3/7) **	30.00±12.25^{az} (4/11)	35.71±17.98^{az} (5/12)
Ca^{2+} ionophore	65.15±13.57^{az} (10/16)	56.67±19.44^{az} (4/8)	31.48±14.29^{az} (4/13)
Ca^{2+} ionophore + 6-DMAP	77.50±11.12^{ayz} (19/24)	100.00±0.00^{bz} (10/10)	61.06±12.78^{ay} (14/23)
Average	68.48±8.18^{z} (32/47)	68.52±9.32^{z} (18/29)	44.63±8.55^{z} (23/48)

* Mean percentage of cleavage was based on oocytes used for ICSI, in the respective grades
** Mean total of cleaved oocytes per total of oocytes used for ICSI
^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{yz} Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.46: Developmental rate (%, mean±SEM) of *in vitro* produced goat embryos following ICSI using different activation regimens

Oocyte grade	Treatment	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
				4-cell	8-cell	Morula
Grade A	Control	3.50±0.50[b] (n=7)	41.65±8.34[a] (n=3)	29.17±4.17[a] (n=2)	29.17±4.17[a] (n=2)	0.00±0.00[a] (n=0)
	Ca^{2+} ionophore	1.46±0.21[a] (n=16)	65.15±13.57[a] (n=10)	65.15±13.57[a] (n=10)	37.88±13.87[a] (n=7)	9.09±9.09[a] (n=2)
	Ca^{2+} ionophore+ 6-DMAP	2.40±0.27[a] (n=24)	77.50±11.12[a] (n=19)	77.50±11.12[a] (n=19)	77.50±11.12[a] (n=19)	42.50±12.07[a] (n=10)
	Average	2.04±0.20 (n=47)	68.48±8.18 (n=32)	67.39±8.37 (n=31)	54.35±9.10 (n=28)	22.83±7.60 (n=12)
Grade B	Control	2.20±0.49[a] (n=11)	30.00±12.25[a] (n=4)	20.00±12.25[a] (n=3)	10.00±10.00[a] (n=2)	0.00±0.00[a] (n=0)
	Ca^{2+} ionophore	1.60±0.40[a] (n=8)	56.67±19.44[a] (n=4)	56.67±19.44[ab] (n=4)	56.67±19.44[ab] (n=4)	26.67±19.44[a] (n=2)
	Ca^{2+} ionophore+ 6-DMAP	1.25±0.16[a] (n=10)	100.00±0.00[b] (n=10)	87.50±12.50[b] (n=9)	75.00±16.37[b] (n=8)	50.00±18.90[a] (n=6)
	Average	1.61±0.20 (n=29)	68.52±9.32 (n=18)	60.19±10.45 (n=16)	51.85±11.10 (n=14)	29.63±10.74 (n=8)

(continued)

(continued)

Oocyte grade	Treatment	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
				4-cell	8-cell	Morula
Grade C	Control	1.71±0.42[a] (n=12)	35.71±17.98[a] (n=5)	21.43±14.87[a] (n=4)	14.29±9.22[a] (n=3)	0.00±0.00[a] (n=0)
	Ca^{2+} ionophore	1.44±0.24[a] (n=13)	31.48±14.29[a] (n=4)	20.37±11.71[a] (n=3)	20.37±11.71[a] (n=3)	0.00±0.00[a] (n=0)
	Ca^{2+} ionophore+ 6-DMAP	2.09±0.42[a] (n=23)	61.06±12.78[a] (n=14)	33.79±12.22[a] (n=11)	30.15±11.35[a] (n=9)	4.55±4.55[a] (n=2)
	Average	1.78±0.22 (n=48)	44.63±8.55 (n=23)	26.11±7.23 (n=18)	22.78±6.40 (n=15)	1.85±1.85 (n=2)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)

Table 4.47: Developmental rate (%, mean±SEM) of different grades of oocyte following ICSI without activation regimen

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	3.50±0.50[a] (n=7)	41.67±8.34[a] (n=3)	29.17±4.17[az] (n=2)	29.17±4.17[az] (n=2)	0.00±0.00[y] (n=0)
Grade B	2.20±0.49[a] (n=11)	30.00±12.25[a] (n=4)	20.00±12.25[az] (n=3)	10.00±10.00[az] (n=2)	0.00±0.00[z] (n=0)
Grade C	1.71±0.42[a] (n=12)	35.71±17.98[a] (n=5)	21.43±14.87[az] (n=4)	14.29±9.22[az] (n=3)	0.00±0.00[z] (n=0)
Average	2.14±0.31 (n=30)	34.52±9.64 (n=12)	22.02±8.27[z] (n=9)	14.88±5.81[yz] (n=7)	0.00±0.00[y] (n=0)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.48: Developmental rate (%, mean±SEM) of different grades of oocyte following ICSI using Ca^{2+} ionophore activation regimen

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	1.46±0.21[a] (n=16)	65.15±13.57[a] (n=10)	65.15±13.57[az] (n=10)	37.88±13.87[ayz] (n=7)	9.09±9.09[ay] (n=2)
Grade B	1.60±0.40[a] (n=8)	56.67±19.44[a] (n=4)	56.67±19.44[az] (n=4)	56.67±19.44[az] (n=4)	26.67±19.44[az] (n=2)
Grade C	1.44±0.24[a] (n=13)	31.48±14.29[a] (n=4)	20.37±11.71[az] (n=3)	20.37±11.71[az] (n=3)	0.00±0.00[az] (n=0)
Average	1.48±0.14 (n=37)	51.33±8.97 (n=18)	47.33±8.96[z] (n=17)	35.33±8.46[z] (n=14)	9.33±5.62[y] (n=4)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Table 4.49: Developmental rate (%, mean±SEM) of different grades of oocyte following ICSI using Ca^{2+} ionophore and 6-DMAP activation regimens

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	2.40±0.27^{b} (n=24)	77.50±11.12^{ab} (n=19)	77.50±11.12^{bz} (n=19)	77.50±11.12^{bz} (n=19)	42.50±12.07^{bz} (n=10)
Grade B	1.25±0.16^{a} (n=10)	100.00±0.00^{b} (n=10)	87.50±12.50^{bz} (n=9)	75.00±16.37^{bz} (n=8)	50.00±18.90^{bz} (n=6)
Grade C	2.09±0.42^{ab} (n=23)	61.06±12.78^{a} (n=14)	33.79±12.22^{az} (n=11)	30.15±11.35^{az} (n=9)	4.55±4.55^{az} (n=2)
Average	1.97±0.20 (n=57)	77.47±6.67 (n=43)	63.68±8.05^{z} (n=39)	58.85±8.22^{z} (n=36)	30.17±7.63^{y} (n=18)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

^{ab} Mean values within a column with different superscripts were significantly different ($P<0.05$)

^{yz} Mean values within a row with different superscripts were significantly different ($P<0.05$)

Table 4.50: Development competence (%, mean±SEM) of *in vitro* produced goat embryos following ICSI using different activation regimens regardless of the oocyte grades

Treatment	No. of oocytes injected	Percent cleavage (2-cell)	Percent development		
			4-cell	8-cell	Morula
Control	4.29±1.13[a] (n=30)	38.09±13.15[a] (n=12)	29.05±13.54[a] (n=9)	19.52±7.88[a] (n=7)	0.00±0.00[a] (n=0)
Ca^{2+} ionophore	2.64±0.31[a] (n=37)	48.10±9.05[ab] (n=18)	45.71±8.99[ab] (n=17)	38.33±8.64[ab] (n=14)	8.57±4.90[a] (n=4)
Ca^{2+} ionophore + 6-DMAP	3.56±0.58[a] (n=57)	73.21±8.31[b] (n=43)	61.76±8.72[b] (n=39)	56.85±9.35[b] (n=36)	29.87±7.34[b] (n=18)
Average	3.35±0.35 (n=124)	57.07±5.91 (n=73)	49.50±5.89 (n=65)	42.78±5.77 (n=57)	16.16±4.15 (n=22)

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)

The number of oocytes injected and cleavage as well as developmental rates between sham injection and ICSI in each of the two chemical treatments were also compared. The comparative results of sham injection and ICSI without any chemical treatment (sham control versus ICSI control) is depicted in Table 4.51. An average of 2.58±0.50 and 4.29±1.13 oocytes were injected for the sham and ICSI control group, respectively. No significant difference ($P>0.05$) was observed in the number of oocytes injected between sham injection and ICSI groups. There is no oocytes were cleaved and no embryos were developed in sham control group which is significantly lower ($P<0.05$) lower than ICSI control group. A few embryos from ICSI control group developed to 8-cell stage (19.52±7.88%) and it is still significant difference ($P<0.05$) with the sham control group. In the two control groups, the averages of developmental rates at 2-, 4- and 8-cell were 14.04±6.32%, 10.70±5.78% and 7.19±3.54%, respectively.

The comparative results of Ca^{2+} ionophore treatment following sham injection and ICSI is depicted in Table 4.52. An average of 3.46±0.68 and 2.64±0.31 oocytes were injected for the sham and ICSI treatment group, respectively. No significant difference ($P>0.05$) was observed in the number of oocytes injected between sham injection and ICSI treatment groups. However, treatment Ca^{2+} ionophore following ICSI group (48.10±9.05%) yielded significant higher ($P<0.05$) rates of cleavage compared to treatment following sham injection group (17.92±6.65%). No significant differences ($P>0.05$) were observed in the rates of development of morula stage embryos between sham and ICSI treatment groups. Significantly higher ($P<0.05$) developmental rates were observed at the 4- and 8-cell stages in the treatment Ca^{2+} ionophore following ICSI group (45.71±8.99% and 38.33±8.64%, respectively) compared to treatment following sham injection group (11.86±4.68% and

5.72±4.13%, respectively). In the two Ca^{2+} ionophore treatment groups, the averages of developmental rates at 2-, 4-, 8-cell and morula stages were 34.82±6.50%, 30.82±6.35%, 23.98±6.06% and 5.37±2.85%, respectively.

The comparative results of Ca^{2+} ionophore and 6-DMAP treatment following sham injection and ICSI is depicted in Table 4.53. An average of 4.87±0.85 and 3.56±0.58 oocytes were injected for the sham and ICSI treatment group, respectively. No significant difference ($P>0.05$) was observed in the number of oocytes injected between sham injection and ICSI treatment groups. However, treatment Ca^{2+} ionophore and 6-DMAP following ICSI group (73.21±8.31%) yielded significant higher ($P<0.05$) rates of cleavage compared to treatment following sham injection group (42.81±11.04%). Also, significantly higher ($P<0.05$) developmental rates were observed at the 4-, 8-cell and morula stages in the treatment Ca^{2+} ionophore and 6-DMAP treatment following ICSI group (61.76±8.71%, 56.85±9.35% and 29.87±7.34%, respectively) compared to treatment following sham injection group (40.31±10.08%, 25.83±9.81% and 5.21±3.50%, respectively). In the two Ca^{2+} ionophore and 6-DMAP treatment groups, the averages of developmental rates at 2-, 4-, 8-cell and morula stages were 63.08±7.16%, 54.11±6.92%, 46.51±7.56% and 21.65±5.53%, respectively.

Table 4.51: Comparative development competence (%, mean±SEM) of *in vitro* produced goat embryos between sham injection and ICSI without using activation regimens

Treatment	No. of oocytes injected	Percent cleavage (2-cell)	Percent development		
			4-cell	8-cell	Morula
Sham control	2.58±0.50[a] (n=31)	0.00±0.00[a] (n=0)	0.00±0.00[a] (n=0)	0.00±0.00[a] (n=0)	0.00 (n=0)
ICSI control	4.29±1.13[a] (n=30)	38.09±13.15[b] (n=12)	29.05±13.54[b] (n=9)	19.52±7.88[b] (n=7)	0.00 (n=0)
Average	3.21±0.54 (n=61)	14.04±6.32 (n=12)	10.70±5.78 (n=9)	7.19±3.54 (n=7)	0.00 (n=0)

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)

Table 4.52: Comparative development competence (%, mean±SEM) of *in vitro* produced goat embryos between sham injection and ICSI using Ca^{2+} ionophore activation regimen

Treatment	No. of oocytes injected	Percent cleavage (2-cell)	Percent development		
			4-cell	8-cell	Morula
Sham + Ca^{2+} ionophore	3.46±0.68[a] (n=38)	17.92±6.65[a] (n=9)	11.86±4.68[a] (n=7)	5.72±4.13[a] (n=4)	1.30±1.30[a] (n=1)
ICSI + Ca^{2+} ionophore	2.64±0.31[a] (n=37)	48.10±9.05[b] (n=18)	45.71±8.99[b] (n=17)	38.33±8.64[b] (n=14)	8.57±4.90[a] (n=4)
Average	3.00±0.35 (n=75)	34.82±6.50 (n=27)	30.82±6.35 (n=24)	23.98±6.06 (n=18)	5.37±2.85 (n=5)

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)

Table 4.53: Comparative development competence (%, mean±SEM) of *in vitro* produced goat embryos between sham injection and ICSI using Ca^{2+} ionophore and 6-DMAP activation regimens

Treatment	No. of oocytes injected	Percent cleavage (2-cell)	Percent development		
			4-cell	8-cell	Morula
Sham + Ca^{2+} ionophore + 6-DMAP	4.87±0.85[a] (n=39)	42.81±11.04[a] (n=20)	40.31±10.08[a] (n=19)	25.83±9.81[a] (n=13)	5.21±3.50[a] (n=3)
ICSI + Ca^{2+} ionophore + 6-DMAP	3.56±0.58[a] (n=57)	73.21±8.31[b] (n=43)	61.76±8.71[a] (n=39)	56.85±9.35[a] (n=36)	29.87±7.34[b] (n=18)
Average	4.00±0.49 (n=96)	63.08±7.16 (n=63)	54.11±6.92 (n=58)	46.51±7.56 (n=49)	21.65±5.53 (n=21)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

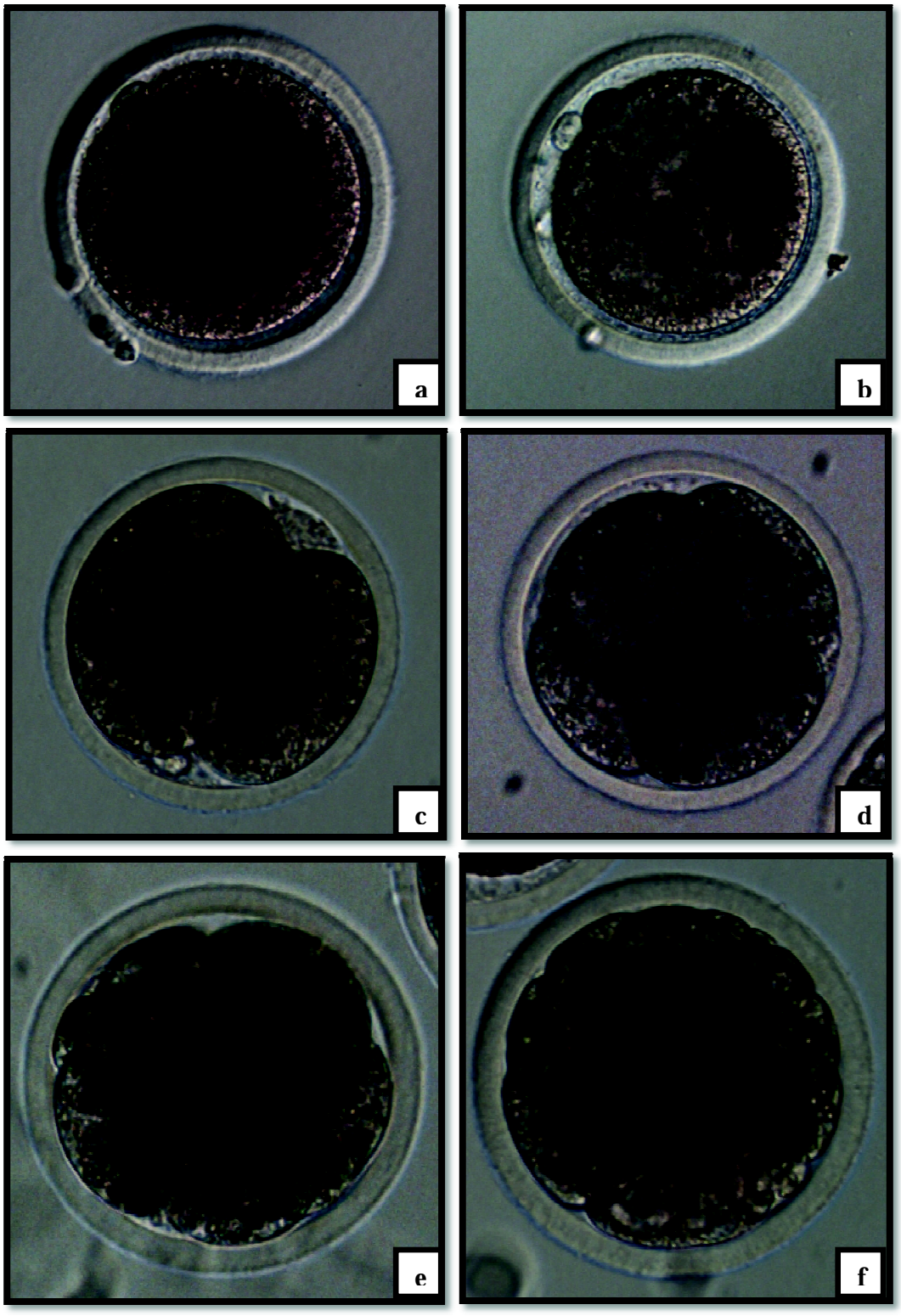

Figure 4.9: Photomicrographs of goat embryo at different developmental stages produced by ICSI. (a-b) Mature oocyte prior to ICSI with the first polar body; (c) 2-cell stage embryo; (d) 4-cell stage embryo; (e) 8-cell stage embryo and (f) morula stage embryo.

Chapter 5

5.0 DISCUSSION

Chapter 5

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5.1 EFFECT OF GOAT GENOTYPE, BODY WEIGHT, AGE, OOCYTE RETRIEVAL CYCLE AND OVARIAN STIMULATION RESPONSE AFTER SUPEROVULATION ON QUANTITY AND QUALITY OF OOCYTES (EXPERIMENT 1)

For the goat genotypes, no significant differences were observed in the percentage and number of oocytes retrieved per ovary between Jamnapari and Boer crossbred goats, in the respective grades. An average of 5.0 oocytes per ovary was retrieved from both breeds. The number of oocytes retrieved in the present experiment (10.0 oocytes per goat) was found to be lower than that of Younis *et al.* (1991) who used Angora, Alpine, Nubian and Toggenburg ovarietomised superovulated-donor goats (29.5 oocytes per goat; large follicles greater than 7 mm were aspirated), Baldassarre *et al.* (2002) who used Nigerian Dwarf, Alpine and Saanens superovulated-donor goats (12.4 oocytes per goat; LOPU-derived oocytes), Baldassarre *et al.* (2003) who used Nigerian Dwarf, Saanen and the Angora superovulated-donor goats (13.4 oocytes per goat; LOPU-derived oocytes), Pierson *et al.* (2004) who used Nigerian Dwarf (Breed Early Lactate Early, BELE®) superovulated-donor goats (12.9 oocytes per goat; LOPU-derived oocytes), Baldassarre *et al.* (2007) who used Saanen and Toggenburg superovulated-donor goats (15.7 oocytes per goat; LOPU-derived oocytes) and Abdullah *et al.* (2008) who used local mixed-breed and Boer crossbred superovulated-donor goats (16.8 oocytes per goat; LOPU-derived oocytes). On the other hand, a slightly higher number of oocytes were obtained in the present experiment compared to that of Gibbons *et al.* (2007) who used Criolla superovulated-donor goats (6.6 oocytes per goat; LOPU-derived oocytes). All the previous studies above, except Abdullah *et al.* (2008) (70 mg Ovagen™), used Folltropin® (range from 60 mg to 133 mg) for superovulation prior to oocyte retrieval. In the

present study, Folligon® was used (1200 IU to 1500 IU) for superovulation, and the number of oocytes obtained per goat was probably not conclusive. Therefore, further studies are needed in the future to elucidate the interaction between different sources of gonadotrophin and different breeds of goat.

Genotype is widely recognised as a major factor of variation (Torres *et al.*, 1987) for ovum pick-up (OPU) (Darwash *et al.*, 1997; Weigel *et al.*, 2000; Merton *et al.*, 2009) in regards to their ability to produce a viable cohort of follicles, thereby affecting the number of COCs collection and subsequent IVP (Machado *et al.*, 2006) as well as embryonic development (Berthelot *et al.*, 2000). Lehloenya *et al.* (2005) indicated that female Boer goats tended to demonstrate a high oestrus response, a shorter time interval from CIDR removal to onset of oestrus as well as exhibiting a shorter induced oestrus period. These characteristics may be ascribed to the Boer goat higher sensitivity to exogenous hormones and high fertility especially during the natural oestrous cycle when the ovaries are very sensitive to hormonal stimulation. Most of the differences in superovulatory response were related to the different prolificacies of the breeds used in multiple ovulation and embryo transfer (MOET) technique (Cahill and Dufour, 1979), with highly prolific breeds having a greater response to exogenous stimulation (Bindon *et al.*, 1971; Smith *et al.*, 1976; Piper *et al.*, 1982). Also, Picazo *et al.* (1996) described the interaction between the types of gonadotrophin used and the breed when comparing non-prolific breeds. Difference in superovulatory responses between breeds may be related to a differential follicular dynamics and function in response to different types of hormones (Ammoun *et al.*, 2006). It has been reported that a high individual variability within the breeds in regards to the superovulatory response that might be related to expression or sensitivity of FSH receptors in the ovary (Driancourt *et al.*, 1986; Abdennebi *et al.*, 1999; Dufour *et al.*, 2000).

To the best of our knowledge, this is the first attempt to study the effect of common goat genotypes (for examples, Jamnapari and Boer crossbred) in Malaysia in regards to the quantity and quality of oocytes retrieved from the oestrus synchronisation and superovulation treatments, particularly with a combination of Folligon® (PMSG) and Ovidrel® (hCG) through LOPU technique. In a nutshell, the present results suggest that the two goat breeds studied, that is Jamnapari and Boer crossbred goats, show no difference in the terms of quantity and quality of oocytes retrieved after superovulation using PMSG-hCG treatment.

For the body weight group, present results show that the number of oocytes retrieved was significantly higher in the light compared with that of heavy body weight goats (6.5 versus 3.4 oocytes per ovary, respectively). However, specific research on the effect of body weight on the superovulatory response of goat is limited. Therefore, the reasons for the difference in this phenomenon cannot be explained, and it is beyond the scope of this study. Most of the available information in domestic animals regarding the effect of nutrition on the embryo recovery rate and quality was from superovulated cattle and sheep. Adamiak *et al.* (2005) showed that a detrimental effect of high feeding level on oocytes from animals with moderately high body condition (associated with hyperinsulinaemia for a significant proportion of animals) but not from the low body condition ones. Working with cattle, Freret *et al.* (2005) suggested that a reduction in dietary intake, leading to a lower growth rate and associated metabolic changes, could improve *in vitro* embryo production in superovulated overfed dairy heifers. In addition, it has been shown that there is an enhancement of *in vitro* embryo production after a reduction of dietary intake (McEvoy *et al.*, 1997; Nolan *et al.*, 1998a; Armstrong *et al.*, 2001). Moreover, a detrimental effect of high live weight gain and dietary intake could have been expected on embryo production according to *in vivo* results in sheep (McEvoy *et al.*, 1993; Creed *et al.*, 1994; McEvoy *et al.*, 1995) and in cattle (Mantovani *et al.*, 1993; Negrao *et al.*, 1997; Yaakub *et al.*, 1999), or *in vitro* in cattle with

impaired embryo development associated with high plasma concentrations of both insulin and IGF-I (Armstrong *et al.*, 2001). This could probably be explained that reproductive performance is commonly correlated with body weight changes. For instance, the mean body weight at puberty in the Boer goat varied, depending on the dietary energy level (Greyling, 1988). Severe body weight loss is usually accompanied by anoestrus (Richards *et al.*, 1989) in a various species of domestic animals.

The present results indicate that the competent oocytes (Grades A, B and C) retrieved from light, medium and heavy body weight goats were 87.76%, 76.42% and 43.19%, respectively. This suggests that the number of oocytes retrieved per individual is of better quality (Grades A, B and C), albeit significantly decreasing with goat body weight. This result is in agreement with the significant findings that overfeeding animal has been shown to be detrimental to oocyte quality and embryo development *in vivo* (Mantovani *et al.*, 1993; McEvoy *et al.*, 1995; Negrao *et al.*, 1997) and *in vitro* (Papadopoulos *et al.*, 2001), whereas dietary restriction based on standard feeding could have a positive effect on oocyte quality (Lozano *et al.*, 2003) and blastocyst production *in vitro*(McEvoy *et al.*, 1997; Nolan *et al.*, 1998a; Armstrong *et al.*, 2001). Overfeeding can also lead to reduced pregnancy rates (Parr *et al.*, 1987) and a decrease in the rate of development *in vitro*and the viability of embryos collected on day 2 after fertilisation (Creed *et al.*, 1994). Dietary intake can influence ovarian activity via effects at various levels of the hypothalamus-pituitary-ovarian axis. Changes in the plane of nutrition can affect follicular growth (Gutierrez *et al.*, 1997; Gong *et al.*, 2002; Diskin *et al.*, 2003; Mihm and Bleach, 2003) by inducing changes in plasma metabolites and metabolic hormones such as insulin and insulin-like growth factor-I (IGF-I; Armstrong *et al.*, 2001; Ferguson *et al.*, 2003) and/or in hormones and growth factors in follicular fluid (Landau *et al.*, 2000). Furthermore, dietary intake can affect oocyte morphology (O'Callaghan *et al.*, 2000), oocyte developmental capacity and embryo production. Nutrition

is considered to be an important factor, affecting reproductive function in domestic ruminants, influencing the onset of ovarian cyclicity in post-partum sheep (Tchamitchian *et al.*, 1973; Restall and Starr, 1977), cattle (Robinson, 1996; O'Callaghan and Boland, 1999) and goats (Walkden-Brown *et al.*, 1994). Therefore, the levels of feeding seem to be important that determine the quantity and quality of oocytes obtained, and it could be related to the specific body weight of goats. Further studies are needed to explain this situation.

For the age group, no significant differences were observed in the number of oocytes retrieved per ovary among the three age groups, in the respective oocyte grades. An average of 6.0 oocytes per ovary was retrieved from three different age groups. Results obtained compare well to Donaldson (1984a), who also observed no effect of age on the total number of embryos recovered but a cut-off point, beyond nine years of age was suggested after which there was a decline in the response in terms of the percentage of transferable embryos recovered. The decline in superovulatory response may be due to a reduction in the numbers of follicles capable of responding to gonadotrophin treatment in older animals (Lerner *et al.*, 1986). This result is in agreement with Katska and Smorg (1984); and Wani *et al.* (1999) who indicated that age of the animal had no significant effect on the quantity and quality of oocytes recovered. In the present experiment, the insignificant results obtained among the age groups studied could be due to small difference in the range of age. This could not be avoided due to relatively low number of donor goats used in the present study. Hence, the results of age groups obtained from previous studies cannot be compared with the present results since different classification of age criteria were used.

Age could contribute greatly to the variation in ovarian response to superovulation between animals within the same group as animals of different ages have been shown to have different physiological needs and respond differently (Jainudeen *et al.*, 2000). In the present experiment, the number of oocytes retrieved in aged less than 18 months old goats (11.8

oocytes per goat) was found to be lower than those reported by Baldassarre *et al.* (2002) (28.4 oocytes per goat; aged less than 3 months old) and Koeman *et al.* (2003) (25.0 oocytes per goat; aged 3 months old). Coincidently, the number of oocytes retrieved in aged more than 24 months old goats (11.8 oocytes per goat) in the present study was also found to be lower than that of Koeman *et al.* (2003) (16.0 oocytes per goat; aged 24 to 48 months old), Katska-Ksiazkiewicz *et al.* (2004) (24.5 oocytes per goat; aged 36 to 48 months) and Baldassarre *et al.* (2007) (15.7 oocytes per goat; aged 84 to 96 months old). However, this result is in agreement with by Baldassarre *et al.* (2002) (10.9 oocytes per goat; aged 3 to 5 months).

The present results indicate that the number of better quality oocytes (Grades A, B and C) retrieved from Groups 1, 2 and 3 donor goats were 85.69%, 89.76% and 79.72%, respectively. The donor goats aged 18 to 24 months old are apparently to yield slightly higher even though statistically insignificant in the number of oocytes compared to the other 2 groups studied. This could probably be explained by Wani *et al.* (1999) who showed that low number of visible follicles present in small ovaries (aged less than 12 months old), whereas the large sized ovaries (aged more than 36 months old) have a good pool of visible follicles for aspiration. Hence, the larger sized ovary in goats yielded a higher number of oocytes compared with small ovaries goats. In one study, young goats (aged 12 to 24 months old) recorded a longer time interval from CIDR removal to the onset of oestrus when evaluating the effect of age on oestrus response following superovulation (Lehloenya and Greyling, 2010). Similar results have been reported in cattle (Drion *et al.*, 2001a) and goats (Baril *et al.*, 2000). The young goats (aged 12 to 24 months old) may have responded slower and were less sensitive to the exogenous hormones due to high sensitivity to the negative effects of steroids (Baril *et al.*, 2000). It could imply that the hormonal threshold level is higher and a greater stimulus is needed to elicit an ovarian response. Baldassarre *et al.* (2007) also found that the older goats (aged 84 to 96 months old) had ovaries which appeared flatter (less rounded) and

the follicles appeared to be smaller in diameter and greyish in colour (less vascularised) under laparoscopic observation if compared with younger goat. However, present results from the three age groups studied indicate that oocytes from donor goats less than 18 months old could be retrieved even at a rate equivalent with the older donor goats studied. Age is not a constraint and the oocytes can be recovered during prepuberty, puberty and above (Salykbaev *et al.*, 1986; Armstrong *et al.*, 1994a; Baldassarre *et al.*, 2007).

For the OR cycle group, no significant differences were observed in the number of oocytes retrieved per PMSG-induced ovary between the two OR cycles, in the respective grades for an interval of 3 months between OR surgeries. The present results are in agreement with Stangl *et al.* (1999), Baldassarre *et al.* (2003), Pierson *et al.* (2004), Valasi *et al.* (2006) and Gibbon *et al.* (2007) that repeated gonadotrophin stimulation and LOPU have no important effect on follicular response and oocyte yield; yet, a high variability in the number of recovered follicles and oocytes between animals and replicates was confirmed (Cognie *et al.*, 2004). Therefore, these results indicate that hormonal treatment and LOPU can be repeated at least 2 times in the same donor goats with minimal surgical adhesions and no significant changes in overall response, in other words, without any OR cycle effects on the quantity and quality of oocytes retrieved.

Even though insignificant, the present results indicate that the number of oocytes retrieved in the first OR was the highest and this number steadily declined for the subsequent consecutive OR cycle. This could be explained that the repeated follicular punctured might alter endocrine profiles slightly and thus cause minor morphological changes in the ovaries (Petyim *et al.*, 2001). On the other hand, Roy *et al.* (1999) and Drion *et al.* (2001a) who reported that repeated use of eCG treatment for induction of ovulation is generally followed by decreasing fertility in goats and it have linked to the presence of anti-eCG antibodies developed as an immune response to previous treatments, indicating the importance of

gonadotrophin choice used for superovulation. Nonetheless, it is noteworthy that oocytes in better quality (Grades A, B and C) were found to be majority (80.04%) in the two repeated OR cycles. These findings are consistent and in agreement with Alberio *et al.* (2002), Baldassarre *et al.* (2003) and Gibbons *et al.* (2007) that good quality oocytes remained unaffected between OR cycles. Although some donor animals in which oviducts and follicles had been manipulated 2 to 3 times developed slight severe adhesions between the ovary and infundibulum, they still became pregnant and kid after insemination or natural mating (Stangl *et al.*, 1999).

Although the incidence of time interval between gonadotrophin stimulation and LOPU was not analysed in the present experiment, any possible negative intervals effect between repeated hormonal treatment and LOPU on oocyte retrieval is likely to be insignificant, since the number of oocytes retrieved and viability did not differ statistically between the first two OR cycles. This is in agreement with Pierson *et al.* (2004) and Rahman *et al.* (2007b) who reported that LOPU can be repeated up to 5 times in the goats at different intervals and in different seasons with little or no important changes in overall response. The present results also indicate that no significant decline in the quality of oocytes retrieved between the two OR cycles. Similar results have been reported by Gibbons *et al.* (2007) that the interval between ovarian stimulation and oocyte recoveries can be reduced to periods as short as 4 days without affecting follicular development and oocyte quality. The reiteration of the protocols had no effect on the number of follicles present in the successive LOPU as described when longer intervals were applied either with (Alberio *et al.*, 2002; Baldassarre *et al.*, 2003) or without hormonal treatment (Kuhholzer *et al.*, 1997). Other studies also reported that the ovary enables to recover from LOPU in less than 5 weeks due to LOPU is less traumatic to the ovary which only the theca is perforated during aspiration.

For the stimulation response group, present results show that the ovarian stimulation response from hyperstimulated donor goats in the retrieval of oocytes significantly affected the quantity and quality of oocytes retrieved, where the quantity was higher in excellent (more than 10 follicles) (6.6 oocytes per ovary) compared to the satisfactory (less than 5 follicles) (3.4 oocytes per ovary) ovarian stimulation response. The present results are in accordance with the findings of Ward *et al.* (2006) and Mossa *et al.* (2007) who found that animals (i.e. cattle and sheep) with the highest number of follicles produced more embryos after superovulation than those with fewer follicles. The reason for this variability in the number of follicles is still unknown. It could be caused by intrinsic factors such as the number of primordial follicles at birth (Erickson, 1966), genetic mechanisms (Spearow and Bradfoed, 1983), different hormonal levels; or by external factors like nutrition (Lucy *et al.*, 1991), and/or environmental conditions (Kafi and McGowan, 1997). While factors that regulate the numbers of ovarian follicles in mammals are not clear. It has recently been shown that the numbers of follicles in cattle are highly variable among animals but highly repeatable within individuals (Burns *et al.*, 2005; Ward *et al.*, 2006).

As shown in the present results, competent oocytes (Grades A, B and C) retrieved from the excellent (5.7 oocytes per ovary) were found to be higher than satisfactory (2.4 oocytes per ovary) ovarian stimulation responses. The results of the present experiment demonstrate that goat with high follicle numbers respond best to superovulation and produce more high quality oocytes. This is in agreement with Cushman *et al.* (1999), Kawamata (1994) and Singh *et al.* (2004) that high numbers of antral follicles at unknown stages of follicular waves are positively associated with an increased responsiveness to gonadotrophin treatments during superovulation. Moreover, Monniaux *et al.* (1983) and Crushman *et al.* (1999) reported that the total number of surface follicles on the ovary was correlated with superovulatory response. However, a direct comparison between the present experiment with

the others on the ovarian stimulation response was not possible. It is believed that this is the first attempt to study the effect of ovarian stimulation response in regards to quantity and quality of oocytes retrieved from superovulation donor goats through LOPU technique.

For the interaction of age and body weight, present results show that a significant positive correlation (r=0.56) between the age and body weight in Boer crossbred goats. This is in accordance with the findings of McGregor and Butler (2010) who reported that body weight of goats increased substantially with age and approached the maximum value at about 5 years of age. In addition, the mature body weight of farm animals especially in goats is positively associated with their potential to grow rapidly when young (Tulloh, 1963; Devendra and Burns, 1970; Gall, 1981; Kempster *et al.*, 1982; McGregor, 1985; Warmington and Kirton, 1990). Kridli *et al.* (2006) also found that age at puberty in sheep was moderately correlated ($P<0.05$) with body weight. The lack of significance in body weight in their study may be due to the fact that heterosis resulted in faster growth rate in the crossbreds (Hunter, 1982). These crossbreds reached the designated weight required to initiate puberty at a younger age.

For the regression coefficients of body weight on the number of oocytes retrieved in goats, present results show that a significant cause and effect between the body weight and the number of oocytes recovered ($r^2=0.14$). A significant linear decline in the number of oocytes recovered ($y=-0.16x+8.80$) was associated with the increasing body weight. This is in agreement with Laster *et al.* (1972), Dyrmunds-son (1973) and Smith (1985) who well established the positive cause and effect between the weight at breeding and fertility or ovulation rate. Hence, according to the present results, body weight could be a predictive factor of the number of oocytes retrieved in goats.

For the regression coefficients of age on the number of oocytes retrieved in goats, present results show that no cause and effect between age and the number of oocytes

recovered. This suggests that age of the goat had no significant cause and effect on the quantity and quality of oocytes retrieved. The present results are in accordance with the findings Katska and Smorg (1984), Salykbaev *et al.* (1986) and Armstrong *et al.* (1994a) that reported age is not a constraint and oocytes can be obtained from even prepubertal animals at a rate equivalent or higher than in pubertal animals.

For the regression coefficients of body weight on the number of oocytes retrieved among the oocyte grades in goats, the association between body weight and Grades B, C and E oocyte retrieved were low but positive. A significant linear decline in the number of Grades B and C oocytes recovered was associated with the increasing body weight. However, a highly significant liner increase in the number of Grade E oocytes recovered was associated with the increasing body weight. It is important to take into account that variation in body weight can have possible divergent effects on follicle growth, oocyte maturation and embryo quality (Freret *et al.*, 2006). The present results suggest that light body weight goats would give better quality oocytes retrieved compared to heavy body weight for the IVP of goat embryos.

The quantity and quality of oocytes recovered per ovary has been an important consideration for *in vitro* production of goat embryos. Despite variations in response to superovulation, LOPU consistently yielded oocytes and may be done repeatedly without ovarian damage or decreased donor fertility. Moreover, LOPU in combination with IVP of embryos increased the number of offspring produced by genetically valuable goats and enabled production of offspring from goats unable to reproduce using AI or multiple ovulation embryo transfer (MOET) such as prepubertal or aged goats.

In summary, the results from this experiment indicate that retrieval of oocytes from the common goat genotypes in Malaysia can be maximised without affecting oocyte quality with gonadotrophin treatment and LOPU. The present results are difficult to make a direct

comparison with other research groups due to multiple variables involved such as breed, age, different OR cycles, interval between OR cycle, body weight, the variety of ovarian stimulation protocols, ovarian stimulation response and the use of different hormones. Hence, further studies on oestrus synchronisation and superovulation focusing the various factors involved are needed to improve the yield of oocyte per goat quantitatively and qualitatively. However, in respect of *in vitro* developmental competence of these oocytes from various qualities obtained from different factors need to be improved and investigated further.

5.2 EFFECT OF PMSG DOSAGE ON QUANTITY AND QUALITY OF OOCYTE, IVM RATE AND SUBSEQUENT EMBRYO DEVELOPMENT FOLLOWING ICSI IN GOAT (EXPERIMENT 2)

This pioneering experiment was undertaken to develop an appropriate ovarian superovulation protocol using two different PMSG (Folligon®) dosages with the combination of hCG (Ovidrel™) and LOPU for oocyte retrieval in goat at the University of Malaya. Previous studies in our laboratory (Phua, 2006; Anna, 2007; Chan, 2008; Rahman, 2008) consisted a combination of FSH (Ovagen™) and hCG (Ovidrel™) treatment was replaced with PMSG since technical problem at the manufacturers level throughout the experiment. Therefore, the present experiments attempts to study the quantity and quality of goat oocytes retrieved as well as developmental competence of these oocytes after IVM and ICSI at two different PMSG dosages through LOPU.

In this experiment, regardless of the factors studied, the average numbers of oocytes retrieval per goat from the LOPU (10.1 oocytes) through PMSG+hCG treatment was found to be lower than previous studies in our laboratory (Chan, 2008; Rahman, 2008) who obtained 17.6 oocytes and 17.2 oocytes, respectively, through FSH+hCG treatment. Lower superovulatory responses with PMSG may be associated with its relatively long circulating

half-life, resulting in excessive follicular development, failure of ovulation, early regression of CL, short or irregular oestrous cycles and potential risk of embryo expulsion (Amstrong *et al.*, 1983b; Amoah and Gelaye, 1990; Pendleton *et al.*, 1992; Siddiqui *et al.*, 2002). PMSG remains active in the blood for 5 to 7 days after administration (Siddiqui *et al.*, 2002). The action of PMSG is more FSH-like than LH-like. The LH component in the preparation causes luteinisation of premature follicles, desensitisation of LH receptors, premature ovulation and drastically decreases the superovulalory response (Boland *et al.*, 1991; Breuel *et al.*, 1991; Herrler *et al.*, 1991). A decreased FSH/LH ratio in PMSG results in fewer ovulations and tends to promote the formation of ovarian cysts (Murphy *et al.*, 1984; Boland *et al.*, 1991).

FSH has surpassed PMSG as the method of choice for goat superovulation because the ovulation response and; number of total and transferable embryos were reported higher in FSH- than in PMSG-treated goats (Amstrong *et al.*, 1983a,b; Tsunoda and Sugie, 1989; Goel and Agrawal, 1990; Mahmood *et al.*, 1991; Pampoukidou *et al.*, 1992; Pendelton *et al.*, 1992). PMSG treatment resulted in the production of unfertilised eggs leading to lowered embryo production (Goel and Agrawal, 2005). Jabbour and Evans (1991) reported similar observations following superovulation with a PMSG preparation. Tsunoda and Sugie (1989) and Rosnina *et al.* (1992) reported that the average number of normal embryos recovered per goat was significantly higher in FSH-treated (9.4 and 6.8, respectively) than PMSG-treated goats (5.7 and 3.0, respectively). Eiamvitayakorn *et al.* (1988) and Pampoukidou *et al.* (1992) recorded an average number of ovulations was higher after FSH-treated (9.6 and 12.5, respectively) than after PMSG-treated (5.7 and 3.9, respectively). However, the present results of PMSG-treated goats which involved oocyte retrieval, therefore, could not be directly compared with the embryo recovered by most of the researchers. Furthermore, most of the embryo recovered from PMSG-treated goats not underwent LOPU procedure.

However, the number of oocytes retrieved per goat (10.1 oocytes) with 60 hours time interval between PMSG+hCG treatment and LOPU in the present experiment was found to be higher than Graff *et al.* (1999) who collected oocytes by laparoscopic aspiration (7.9 oocytes per goat) from genetically superior goats after FSH priming; and Gibbons *et al.* (2007) who retrieved oocytes (6.6 oocytes per goat) from the FSH-stimulated donor goats. Coincidently, the present results indicate that the number of oocytes retrieved from PMSG-stimulated donor goats was seemed better than previous findings in our laboratory (Hisham, 2006; Phua, 2006; Anna, 2007; Rahman *et al.*, 2007a) who obtained less than 7.0 oocytes per goat with usual LOPU after 36 hours post-FSH+hCG treatment. The reason for such variation in the number of oocytes retrieved was probably related to the timing of LOPU which affects oocyte developmental competence as LOPU disrupts the oocyte *in vivo* maturation process. In humans (Thornton *et al.*, 1990; Mansour *et al.*, 1994), monkeys (Ng *et al.*, 2002; Chen *et al.*, 2006) and pigs (Ratky *et al.*, 2003), LOPU was performed 36 hours after hCG injection; the majority of the oocytes collected were at metaphase II and were meiotically and developmentally competent. However, goat oocytes collected 36 hours after FSH+hCG treatment (which is a general practice for retrieval oocytes from live goats) were still at the immature stages and required *in vitro* maturation for 27 hours before reaching meiotic competence (Baldassarre *et al.*, 2007). The present results are in agreement with Abdullah *et al.* (2008) that have suggested that LOPU at 60 or 72 hours post-FSH+hCG treatment can improve OR rate and oocyte quality. The reasons for the different results of maturation stage obtained after oocyte retrieval from gonadotrophin treatment between humans, monkeys and goats could not be explained at the present time and warrant further studies in the future.

The present results show that ovarian superovulation protocol using two different PMSG dosages combined with hCG significantly affected the quantity and quality of oocytes retrieved, where the quantity was higher in 1200 IU (12.4 per goat) compared to the 1500 IU

(9.0 oocytes per goat) PMSG dosage. It has been reported that there is a high incidence of ovulation failure when using high doses of eCG for superovulation with consequent decreases in recovery rate and development rate of embryos (Mehaisen *et al.*, 2005). Moreover, this case is in agreement with several authors who have observed a negative effect of high PMSG dose for superovulation treatment on embryo production (García-Ximénez and Vicente, 1990). Gonzalez *et al.* (1994) reported that the number of total ova/embryos increased but the number and percentage of fertilised and transferable embryos decreased at the high dose of PMSG were in agreement with previous reports (Gordon *et al.*, 1963; Mauleon *et al.*, 1970; Moore, 1975 and Wang *et al.*, 1988). McGowan *et al.* (1985) also showed a similar detrimental effect of gonadotrophin dose on numbers of fertilised ova and transferable embryos with human menopausal gonadotrophin (hMG). However, no such detrimental effects were observed with increasing doses of a highly purified porcine pituitary extract (Ott *et al.*, 1979; Abdul *et al.*, 1989; Graff *et al.*, 1999). The total dose and the source or purification method of eCG are factors that may have contributed to these differences; consequently, the efficacy of eCG varies from batches to batches, resulting in variable superovulation response.

PMSG has been used widely as a means of inducing superovulation in conjunction with embryo transfer technique for the purpose of genetic improvement and increasing large number of goat population. Not much work has been reported on the different dose response of PMSG on the number and quality of oocytes as well as embryo developmental rate through LOPU. However, a different dose of PMSG for superovulation treatments ranging from 700 to 1250 IU (Armstrong *et al.*, 1983a,b; Pendleton *et al.*, 1992; Pintado *et al.*, 1998; Saharrea *et al.*, 1998; Faruk *et al.*, 2004; Goel and Agrawal, 2005; Faruk *et al.*, 2006) had been applied for embryo recovery in goats. Therefore, a direct comparison was not possible as a wide variation in superovulatory regimes were used either in oocyte or embryo recovery

in goats. To our best knowledge, this is the first report on the effect of two different dosages of PMSG on the superovulatory response in goat through LOPU. Taking this into consideration, the present results confirm that donor goats are satisfactorily able to be superovulated through PMSG+hCG treatment for LOPU and produce significantly higher proportion in oocytes of better quality, which are Grades A, B and C (81.6%, average of 4.3 oocytes per ovary) compared to Grades D and E (18.4%, average of 0.8 oocytes per ovary).

As for the maturation rates in the present experiment, it seems that lower dosage of PMSG for superovulatory regime and LOPU has a positive effect on IVM of oocytes as evidenced by significantly higher ($P<0.05$) maturation rate at 1200 IU (65.7%, average of 4.2 oocytes per ovary) compared with 1500 IU (48.1%, average of 3.0 oocytes per ovary) PMSG dosage. The reason for higher maturation rate at 1200 IU PMSG dosage was probably because it could drive the pre-maturation oocyte to the completion of the growth phase. The influence of FSH upon follicle recruitment and development *in vivo* may, therefore, have contributed to an increased likelihood in selection of follicles destined to produce more mature oocytes (Junk *et al.*, 2003). Low dose administration of FSH in luteal phase has been observed to result in efficient maturation and fertilisation of immature human oocytes after IVM (Suikkari *et al.*, 2000).The higher dosage in FSH treatment can induce a quick and abnormal follicular development, resulting in higher number of large follicles growth (Berlinguer *et al.*, 2004). However, this treatment has the consequent asynchrony between growing oocyte and follicular status (Blondin *et al.*, 1996). A plateau period (Sirard *et al.*, 1999) is necessary to complete 'oocyte capacitation' (Hyttel *et al.*, 1997) before further *in vitro* maturation process could competently proceed. The rapid acceleration of follicle development that occurred during the higher dosage in FSH treatment could de-synchronise the oocyte-follicle growth and adversely precede the complete cytoplasmic maturational process of the oocyte (Armstrong and Evans, 1983).

The acquisition of meiotic competence and subsequently developmental competence by the goat oocyte occurs progressively during follicular growth (Crozet *et al.*, 1995). The competence to undergo cytoplasmic maturation is acquired progressively by oocytes during follicular growth. Martino *et al.* (1994) reported that follicles measuring more than 3 mm diameter contained more cumulus cell layers and gave better IVM results. Similarly, a significant improved yield in the number of MII oocytes after IVM of cumulus-enclosed oocytes retrieved from follicles greater than 4 mm in diameter has been observed in humans following FSH priming in the follicular phase (Wynn *et al.*, 1998). Also, it has been reported that as follicle size increased, oocyte completed their growth and achieved meiotic competence, thus giving better *in vitro* embryo production yield (De Smedt *et al.*, 1994; Cognie, 1999; Qian *et al.*, 2001; Han *et al.*, 2006). However, the effect of lack of cytoplasmic factors such as mRNA, proteins and transcription factors, in higher dosage FSH treatment could be the cause of the decrease in maturation rate of oocytes, even though derived from large follicles (Blondin *et al.*, 1997). Becker *et al.* (2002) who found that follicles larger than 5 mm diameter appeared in higher dosage FSH treatment may contain high levels of oestrogen in the follicular fluid, disturbing the subsequent developmental competence of these oocytes. Therefore, the follicular history seems to be vital for the oocyte to be in the right condition to reach complete developmental capacity.

In this experiment, the maturation rate (54.1%) with 60 hours post-PMSG+hCG and LOPU-derived goat oocytes was found comparable with previous studies in our laboratory, where Phua (2006) and Anna (2007) obtained 31 to 55.5% with 36 hours post-FSH+hCG and LOPU-derived oocytes. However, in the present experiment, maturation rate were found lower with Chan (2008) and Rahman (2008) who obtained 64.0% and 88.3%, respectively, with 60 hours post-FSH+hCG and LOPU-derived oocytes. On the other hand, although not statistically different, the present results show high maturation rate of competent oocytes

(Grades A, B and C) at PMSG dosage of 1200 IU (74.1%) compared to 1500 IU (61.1%). The maturation rates of Grades A, B and C oocytes (75.4%, 74.9% and 48.2%, respectively) were significantly higher compared to Grades D and E oocytes (9.5% and 5.9%, respectively), regardless the PMSG dosage. These results indicate that the vital role of cumulus cells (1 to 5 layers) in influencing the *in vitro* maturation of goat oocytes. Oocyte quality is generally assessed by oocyte grades based on cumulus mass investment and cytoplasmic uniformity. The cumulus cells could exert their enhancing effect on oocyte maturation without direct attachment to oocytes and it is important for cytoplasmic maturation (Nagai, 2001; Tanghe *et al.*, 2002). The present results were unable to compare maturation rate of LOPU-derived oocytes with other researchers as no reports mentioned IVM rates of different grades in their studies except for Chan (2008) from our laboratory who reported a higher maturation rate (81.0%) of competent oocytes than the present experiment. Furthermore, the reason for such variations in maturation rate may not be possible to determined, as a direct comparison was not feasible due to various influencing factors such as type of gonadotrophin used in superovulation regimes; and time interval between FSH+hCG treatment and LOPU used in the present experiment.

As for the cleavage and subsequent embryo development rates in the present experiment, only competent oocytes (Grades A, B and C) were used to investigate the effect of PMSG dosage for superovulation in ICSI experiments. Incompetent oocytes (Grades D and E) were eliminated from this experiment because of the low number of oocyte obtained after *in vitro* maturation. Although not statistically significant, the present results show higher cleavage and subsequent embryo development rates of competent oocytes at 1200 IU than 1500 IU PMSG dosage. On the other hand, the present results obtained from Grade C oocytes displayed the lowest cleavage and embryo development rates compared with Grades A and B oocytes though not statistically different. However, how the oocytes quality from both PMSG

dosages could affect cleavage and development is not clearly understood. Nonetheless, it has been reported that oocyte quality is determined by various criteria such as the degree of cumulus expansion, number of cumulus cell layers and texture of the ooplasm, where these criteria were found to have significant effects on the rates of maturation, fertilisation and cleavage *in vitro* (Tanghe *et al.*, 2002). This could be observed in the present experiment, where oocytes with the most cumulus cell layers (Grade A) had the highest cleavage rate up to morula stage. It could be probably explained that the cumulus cell play an important role in cytoplasmic maturation, with increased number of cumulus cell layers and COC compactness pre-IVM correlated with improved developmental outcome (Shioya *et al.*, 1988; Abeydeera, 2002). The presence of cumulus cells during IVM has a positive effect on meiotic maturation, intracellular glutathione (GSH) concentration, sperm penetration, pronuclear formation and histone H1 kinase activity (Yamauchi and Nagai, 1999).

Although not statistically different, the present results show higher cleavage rate at PMSG dosage of 1200 IU (65.0%) compared to 1500 IU (47.9%), regardless of oocyte quality. The reasons for this difference currently could not be understood that could be contributed by complexity of intrinsic and extrinsic factors. It is possible that the range of variation in hormonal concentration in the present experiment is not large enough to detect significant differences and that the lower concentration of 1200 IU is sufficient to induce maturation of oocytes and further embryo development as recommended by the previous researchers with the range of 750 to 1250 IU (Armstrong *et al.*, 1983a,b; Pendleton *et al.*, 1992; Pintado *et al.*, 1998; Saharrea *et al.*, 1998; Faruk *et al.*, 2004; Goel and Agrawal, 2005; Faruk *et al.*, 2006). However, in both PMSG dosages used in this experiment, the average cleavage rate (54.8%) was found higher than that of Jimenez-Macedo *et al.* (2006), who obtained 23.7% after ICSI on oocytes obtained from abattoir-prepubertal goats. Conversely, the average cleavage rate was found to be lower than those reported by Keskintepe *et al.*

(1997) (71.0-90.0%; abattoir-slicing), Wang *et al.* (2003) (74.0-89.0%; superovulated-LOPU), Jimenez-Macedo *et al.* (2005) (73.4%; abattoir-slicing) and Jimenez-Macedo *et al.* (2007) (51.0-68.2%; abattoir-slicing). Similarly, the average of cleavage rate also found to be lower than previous studies in our laboratory, where Rahman (2008) obtained cleavage rate (72.1%) with 60 hours post-FSH+hCG and LOPU-derived goat oocytes. This is well-known fact that superovulatory response of PMSG is lower than that of FSH (Armstrong *et al.*, 1983b; Nuti *et al.*, 1987; Mahmood *et al.*, 1991; Nowshari *et al.*, 1992). The morula rate (15.39%) obtained in the present experiment was higher than that of Jimenez-Macedo *et al.* (2005, 2006, 2007) (3.8 to 11.7%; abattoir-slicing), and comparable with that of Keskintepe *et al.* (1997) (15%; abattoir-slicing) and Wang *et al.* (2003) (41%; superovulated-LOPU). On the other hand, the present results were found to be lower than that of Rahman (2008) (22.3%; superovulated-LOPU). The reasons for such variations in the current experiment may not be possible to be determined presently, as a direct comparison was not feasible due to various factors such as the source of ovaries, type of gonadotrophin used for superovulation, chemical treatment after ICSI as well as culture medium. Great ovarian variation exists and there is no real optimal superovulatory treatment protocol established for goats (Greyling *et al.*, 2002). Further studies comprising larger number of animals are required to determine appropriate dosage of PMSG for a consistent response with special consideration of various factors involved.

In summary, the present results of this experiment are encouraging with reference to the use of PMSG for goat superovulation, although the response of the treatment in terms of oocyte recovery, maturation and cleavage rate was only modest compared to FSH regimes. Nonetheless, it was a necessary to determine the optimal dosage of PMSG to be utilised in the present superovulation protocol, where this is to be of major importance in influencing the *in vitro* production of goat embryos in the University of Malaya. PMSG dosage at 1200 IU with the combination hCG treatment could be the preferred protocol to optimise yields of

good quality oocytes for IVM and ICSI embryos in goat, and thus beneficial economically in goat superovulation programme, especially when needed as an alternative to more superior gonadotrophin such as FSH. The present experiment is the first of its kind which reported successful OR, IVM and ICSI embryo development in goat using different dosages of PMSG with combination of hCG treatment and onset of LOPU.

5.3 EFFECT OF OOCYTE QUALITY AND DURATION OF IVM ON THE RATES OF MATURATION, CLEAVAGE AND DEVELOPMENTAL COMPETENCE OF *IN VITRO* PRODUCED GOAT EMBRYOS BY ICSI TECHNIQUE (EXPERIMENT 3)

The results of the present experiment demonstrate the first attempt to study the influence of oocyte quality and duration of IVM on maturation, cleavage and development *in vitro* through ICSI technique in goat. Previous studies including our laboratory, the ICSI experiments were conducted involving 27 hours of IVM duration and without consideration to oocyte quality (Wang *et al.*, 2003; Jimenez-Macedo *et al.*, 2005, 2006, 2007; Rahman, 2008). The present experiment show significantly higher maturation rate ($P<0.05$) in duration of 22 to 25 hours (71.6%, average 4.2 oocytes per ovary) compared to 26 to 29 hours (38.7%, average 2.7 oocytes per ovary) IVM. These results are in agreement with those reported by Cognie *et al.* (2003) who found that cumulus expansion and extrusion of the first polar body at metaphase II occurs in goat oocytes (indicating maturation) between 16 to 24 hours (21.0 to 72.0%) after the initiation of maturation process. In addition, the maturation rate for 22 to 25 hours IVM duration in the present experiment is comparable to those reported by Pawshe *et al.* (1996) and Kang *et al.* (2004) who obtained 62.6% and 71.3%, respectively, however, this may be attributed to the source of ovaries, in which ovaries had not been superovulated. Using the same serum concentration (10% of OGS), the maturation rate was found lower than

that of Tajik and Esfandabadi (2003) who obtained 86.0% in duration of 24 to 25 hours. The difference between these studies could be explained, in part, by the different sources of serum obtained, where in their studies, OGS was obtained from oestrus goat which had been induced by two injections of prostaglandin $F_{2\alpha}$. Nagar and Purohit (2005) have reported that the use of serum could be controversial because of the variety of substances that the serum obtained from different sources may contain beneficial or harmful effects.

On the other hand, Martino *et al.* (1994) deduced from their results and investigation by other researchers (Song and Iritani, 1987; Le Gal *et al.*, 1992) that the time necessary for goat oocytes to reach metaphase II is about 27 hours, independent of the physiology of the animals. Rho *et al.* (2001) also confirmed that a period of 27 hours (73.0%) for IVM of goat oocytes results in significantly higher percentages of nuclear maturation than did 24 hours (55.5%). In contrast, Younis *et al.* (1991) reported that the 24 hours of IVM duration is the best time for goat oocyte maturation *in vitro*. In a study on the basis of sequential configuration of chromosomes, Sharma *et al.* (1996) and Yadav *et al.* (1997) found that the optimal duration of IVM for goat oocytes was 32 and 30 hours, respectively. The reasons for such variations could be probably due to the follicle size (De Smedt *et al.*, 1992; Crozet *et al.*, 1995; Gall *et al.*, 1996), simultaneous influence of culture media components (Teotia *et al.*, 2001; Bormann *et al.*, 2003; Cognie *et al.*, 2003), culture condition (Baldassarre *et al.*, 1996; Samake *et al.*, 2000) and follicle developmental stage (Yadav *et al.*, 1997; Crozet *et al.*, 2000) as well as age of the donor goat (Izquierdo *et al.*, 2002; Koeman *et al.*, 2003).

Different selection criteria have been used to predict the quality of goat oocytes and to improve the embryo development such as follicle diameter (Crozet *et al.*, 1995; Romaguera *et al.*, 2010), oocyte diameter (Jimenez-Macedo *et al.*, 2006; Anguita *et al.*, 2007) and the brilliant cresyl blue (BCB) test (Rodríguez-González *et al.*, 2002). Crozet *et al.* (1995) found a direct and positive relationship between follicle diameter and embryo development,

concluding that follicles larger than 5 mm contain oocytes which are more competent to develop up to blastocyst stage, in which, oocytes could have enough time to be submitted to pre-maturation processes and to acquire a complete developmental competence. It has been shown that follicle diameters of less than 2 mm or more than 7 mm have a detrimental effect on oocyte developmental competence (Abeydeera, 2002). Anguita *et al.* (2007) observed a higher blastocyst rate in oocytes larger than 125 to 135 µm prior to IVM, whereas oocyte smaller than 125 µm were only able to develop to morula. Immature oocytes stained by the BCB test (BCB+) showed higher rates of maturation, normal fertilisation and development to the morula and blastocyst stages than unstained oocytes (BCB-). Thus, oocytes that have finished their growth phase will show decreased G6PD activity indicating complete oocyte maturation whereby will exhibit blue colouration because BCB will remain unreduced state in the ooplasm. The absence of enzymatic activity of G6PD can be an indirect measure of fully-grown oocytes that have finished their intra-ovarian growth phase (Rodríguez-González *et al.*, 2002).

However, in practical situation, it is very difficult to consider multiple criteria in selecting oocyte during retrieval as it is labourious and time consuming. Furthermore, there are a lot more critical factors needed to be optimised within a limited time and less scope to expose the oocytes longer period of time outside the CO_2 incubator for assessing a number of criteria in an IVF or ICSI laboratory. Therefore, visual assessment of morphology features, particularly on the cellular vestments and cytoplasm uniformity surrounding an oocyte is the most important criteria for selection oocytes before maturation (Shioya *et al.*, 1988; Sirard *et al.*, 1988; Mochizuki *et al.*, 1991; Madison *et al.*, 1992; Lonergan *et al.*, 1994). In the present experiment, oocyte grading based on cumulus mass investment and morphology of the oocyte, in which, cumulus cells were reported to affect post-fertilisation embryonic development (Hashimoto *et al.*, 1998; Sun *et al.*, 2001a; Tanghe *et al.*, 2002).

The different oocyte maturation time-scale recorded among goat studies may be accounted by different conditions of IVM and particularly by oocyte quality. Oocyte quality is an important prognostic factor as the nuclear and ooplasmic maturity of the oocyte may be directly related to the success rate of ICSI (Kahraman *et al.*, 2000). As shown in the present results, significantly higher maturation rate ($P<0.05$) were obtained from competent oocytes (Grades A, B and C) in duration of 22 to 25 hours (79.3%) compared to 26 to 29 hours (50.9%) IVM. The maturation rates of Grades A, B and C oocytes (75.4%, 73.1% and 48.2%, respectively) were significantly higher compared to Grades D and E (9.2% and 5.6%, respectively), regardless of IVM duration. Although a number of researchers reported goat OR by LOPU, however, no researchers mentioned IVM rates in their studies except the previous studies in our laboratory where Phua (2006), Anna (2007), Chan (2008) and Rahman (2008) obtained 31.0 to 73.8% maturation rate with LOPU-derived goat oocytes. The reasons for such variations may not be possible to be determined at the moment; however, this may be attributed to the hormone used for superovulation, longer time interval between FSH+hCG treatment and LOPU; and duration of IVM used in this experiment.

In the present experiment, significantly higher maturation rates were obtained from oocytes of Grades A and B (90.6% and 91.3%, respectively) than Grades C, D and E (56.0%, 0.0% and 0.0%, respectively) at duration of 22 to 25 hours IVM. No maturation or low rate of maturation was obtained when CCs were removed before the oocytes were matured *in vitro* (Rajikin *et al.*, 1994; Shirazi *et al.*, 2007). Similar maturation patterns are observed with CFOs in goat in this present experiment. Oocytes of various qualities were cultured including COCs with less than one complete layer of cumulus cell or cumulus-free oocytes (Grade D) as well as oocytes with abnormal morphology (Grade E) due to limited number of oocytes retrieved from LOPU. These results are comparable with Rahman (2008) who obtained maturation rate of Grades A, B, C and D at 95.2%, 84.6%, 74.9% and 42.6%, respectively,

under the same oocyte grading system with the present experiment. On the other hand, other researchers cultured better quality of oocytes with at least 4 complete layers (Jimenez-Macedo *et al.*, 2005, 2006, 2007), 2 and above complete layers (Keskintepe *et al.*, 1997; Rho *et al.*, 2001) or 1 to 2 complete layers (Martino *et al.*, 1995; Wang *et al.*, 2003) of cumulus cell. Hence, a direct comparison on the rate of maturation was not possible as different oocyte grading systems and different IVM durations were employed.

As for the cleavage and subsequent embryo development rates in the present experiment, only competent oocytes (Grades A, B and C) were used to investigate the oocyte quality and duration of IVM in ICSI experiments as previously described. Incompetent oocytes (Grades D and E) were eliminated from this experiment because of the low number of maturation rate obtained. Although not statistically different, the present results show higher cleavage rates of Grades A and B oocytes (64.5% and 64.5%, respectively) compared to Grade C oocytes (44.6%), in both IVM durations. Additionally, higher cleavage rates ($P<0.05$) of Grades A were observed in IVM duration of 22 to 25 hours (78.9%) compared to 26 to 29 hours (38.9%). This finding identifies the essential role of cumulus cells in promoting normal cytoplasmic maturation of oocyte necessary for cleavage and subsequent development of ICSI embryo. Katska-Ksiazkiewicz *et al.* (2007) also reported that the quality of oocyte is the key factor influencing the efficiency of *in vitro* embryo production in goats. Using the same oocyte grading system, the results of cleavage rate in Grades A, B and C oocytes were found to be lower than that of Rahman (2008) who used FSH-hCG-stimulated goats at rates of 84.0%, 66.7% and 57.8%, respectively. The difference between these experiments could be explained, in part, by comparing the hormone effectiveness whereby FSH was found to be superior (Armstrong *et al.*, 1983a,b; Nuti *et al.*, 1987) than eCG. Moor *et al.* (1985) reported that PMSG is more liable than FSH to disrupt the normal function of the oocyte which in according well with findings indicating that PMSG decreases

fertilisation rate and increase embryonic loss at the pre-implantation stage of development (Miller and Armstrong, 1981; Evans and Armstrong, 1983).

In regards to IVM duration, the present results indicate that significantly higher cleavage and morula rates ($P<0.05$) at 22 to 25 hours (70.1% and 24.6%, respectively) compared to 26 to 29 hours (35.7% and 2.4%, respectively) IVM. Thus, the present results show that oocytes completing IVM early are more likely to develop to the morula stage than those that completed it a later time after the initiation of IVM. The reasons for this are unclear, but the duration of IVM plays a critical role for subsequent development since inappropriate timing of maturation may result in oocyte aging (Hunter, 1989; Hunter and Greve, 1997), abnormal chromatin (Dominko and First, 1997) and thus reduced subsequent developmental competence (Marston and Chang, 1964). It has been reported that oocyte age influences the activity of maturation or M-phase promoting factor (MPF) (Kikuchi *et al.*, 2000) and *in vitro*fertilisability (Grupen *et al.*, 1997a). MPF, which induces the M-phase in eukaryotic cells including oocytes (Kishimoto *et al.*, 1982), increased during the process of oocyte maturation and remained at a high level during meiotic arrest. MPF activity in aged oocytes gradually decreased, while the activation ability or fragmentation frequency gradually increased (Kikuchi *et al.*, 1999, 2000).

In some studies, oocytes that developed fast *in vitro* and extrude the polar body early during IVM are more likely to develop to the blastocyst stage (Dominko and First, 1992, 1997). The quality of these rapidly developing blastocysts is similar to those produced *in vivo* (Lonergan *et al.*, 1999). In addition, prolongation of metaphase II arrest before insemination leads to a gradual loss of successful fertilisation and embryo development (First *et al.*, 1988) and an inappropriate time point of insemination can also lead to impaired development (Marston and Chang, 1964). As no similar studies according to oocyte quality and IVM

duration on cleavage and subsequent embryo development by ICSI technique in goat were reported, therefore, it was not possible to compare the present results with other researchers.

In summary, the present results show a better maturation, cleavage and subsequent development rates are achieved at duration of 22 to 25 hours from LOPU-derived goat oocytes according to grades based on cumulus cell investment through ICSI technique. The oocytes from Grades A and B are apparently competently oocytes, with 22 to 25 hours IVM duration, demonstrating a better developmental competence, whilst Grades D and E oocytes being the least competent. To our best knowledge, this is the first report on the effect of oocyte quality and IVM duration employed in ICSI experiment through PMSG-LOPU protocols. The oocytes from Grades A and B at duration of 22 to 25 hours IVM significantly ($P<0.05$) improved embryo yield.

5.4 EFFECT OF CHEMICAL TREATMENTS ON THE DEVELOPMENTAL COMPETENCE OF *IN VITRO* PRODUCED GOAT EMBRYOS FERTILISED BY ICSI TECHNIQUE (EXPERIMENT 4)

The present experiment was conducted to compare two chemical activation treatments on LOPU-derived oocytes from various qualities (Grades A, B and C) following ICSI and; to investigate the most effective one for *in vitro* produced goat embryos. The two activation treatments were calcium ionophore and calcium ionophore followed by 6-DMAP. Artificial oocyte activation always carries the risk of an induction of parthenogenesis (Jimenez-Macedo *et al.*, 2005). That is why sham groups were necessary to include as control (without chemical activation treatments) and with chemical activation regimes as ICSI. The present results indicate that no cleaved embryos were obtained from sham-injected oocytes, in which, cultured without chemical activation treatment. Thus, this may suggest that such mechanical stimulus alone was insufficient to cause a reduction in the maturation promoting factor (MPF)

activity of oocytes after ICSI. Oocyte activation depends on inactivation of MPF that induces mitotic division of fertilised oocyte (Shojo *et al.*, 2000; Shimada *et al.*, 2001). In the sham-injected oocytes, cleavage and morula rates after treatment with calcium ionophore following 6-DMAP (42.8% and 5.2%, respectively) were significantly ($P<0.05$) higher when compared with those after the calcium ionophore treatment (17.9% and 1.3%, respectively).

ICSI has become an integral part of assisted reproduction and is one of the most powerful tools used to overcome infertility in human. However, the efficiency of this procedure is still far from expectations in goat. In goat ICSI, oocyte activation plays a key role in the technique success. Mechanical sperm injection into the ooplasm is sufficient in certain species to activate the oocyte in rabbit (Keefer, 1989), hamster (Hoshi *et al.*, 1992), human (Tesarik and Sousa, 1995), mouse (Kuretake *et al.*, 1996) and sheep (Gomez *et al.*, 1998) for further embryonic development. However, it has been demonstrated that some species such as cattle (Keefer *et al.*, 1990; Rho *et al.*, 1998a), pigs (Probst and Rath, 2003) and goats (Jimenez-Macedo *et al.*, 2005) need the aid of chemical activation after ICSI to improve the embryo development.

As for the cleavage rates in the present experiment, significantly higher rates ($P<0.05$) were obtained from ICSI-injected with calcium ionophore following 6-DMAP (73.2%) than without chemical activation treatment (38.9%) oocytes. In normal fertilisation, the membrane fusion between sperm and oocyte is essential for incorporation of sperm into the ooplasm. And such incorporation of the sperm head into the ooplasm results in release from meiotic arrest of the oocyte. However, the soluble sperm factors are the only oocyte activation factors except for the physical stimulation of the injection procedure in the case of ICSI (Horiuchi and Numabe, 1999). It is reported that the activation due to the injected sperm alone was sufficient to lower the MPF activity of oocytes after ICSI, however, it was not maintained. Following the elevation of Ca^{2+} concentration in ooplasm, which was induced only by the

presence of the injected sperm, MPF activity of oocytes without activation after ICSI was temporarily raised but could not be maintained at low levels, and a shortage of cyclin B which promoting progression toward the first cleavage was apparently inhibited in the ooplasm by the temporary elevation (Fujinami *et al.*, 2004).

In all mammalian species, calcium plays an important role in the intracellular signaling responsible for the initiation and propagation of oocyte activation. It is known that calcium ionophore is able to mimic fertilisation very closely by elevating oocyte intracellular Ca^{2+} levels (Jones *et al.*, 1995). Moreover, it is a histone kinase inhibitor and prevents the reaccumulation of maturation promoting factor (MPF), and this yields an improvement in the efficiency of oocyte activation (Susko-Parrish *et al.*, 1994). However, significantly higher cleavage rate in the present experiment is that oocyte activation using the combination of calcium ionophore and 6-DMAP (73.2%) was more efficient than calcium ionophore alone (48.1%). This finding is in accordance with the report indicating the same combination activation treatment significantly increased the rates of fertilisation, cleavage and blastocyst development after ICSI (Zhou and Zhang, 2005). The probable explanation is that it leads to an arrest of the MPF level in the activated oocytes, with calcium at minimal levels. It is also known that 6-DMAP is a serine treonine kinase inhibitor which accelerates formation of the interphase network of microtubules, the remodeling of sperm chromatin, pronuclear formation and promoting mitosis (Leal and Liu, 1998). In addition, Liu *et al.* (1998) showed that an optimal parthenogenic development could only be obtained if it is followed by chemical activation with 6-DMAP. Using the same combined activation protocol of calcium ionophore and 6-DMAP, the results of cleavage rate in the present experiment is comparable to those reported by Jimenez-Macedo *et al.* (2005) who obtained 73.4%. The slight discrepancies may be attributed to the various factors such as different culture medium used, differences in oocyte quality and the source of ovaries.

The morula rate (29.9%) in the present experiment was found to be higher than Jimenez-Macedo *et al.* (2005) who obtained the morula development rate only 2.6 to 5.2% from prepubertal goat oocytes using the same combination activation treatment of calcium ionophore and 6-DMAP. The reason for higher morula development rate due to the source of ovaries, where they obtained the oocytes recovered from the abattoir which unable to undergo a correct cytoplasmic maturation and consequently correct fertilisation. On the other hand, the significant results of morula rates were found to be lower than that of Keskintepe *et al.* (1997) and Rahman (2008) who using only calcium ionophore following ICSI at rates of 37.7% and 32.5%, respectively. The reason for this difference could not be understood. However, it has been reported that the combination of calcium ionophore and 6-DMAP greatly increase the incidence of chromosomal abnormalities (De la Fuente and King, 1988; Rho *et al.*, 1998b, Jimenez-Macedo *et al.*, 2005), and the development was arrested at the late stages (Liu *et al.*, 1998; Slimane-Bureau and King, 2002). It is possible that the occurrence of chromosomal abnormality may be due to inhibition of extrusion of the second polar body, with some nuclei presumably re-entering S-phase of the cell cycle without having passed through metaphase by 6-DMAP treatment (Ock *et al.*, 2003). In contrast, Jimenez-Macedo *et al.* (2005) could not identify whether these anomalies were caused by the activation protocol or were due to oocyte quality. Therefore, chromosomal abnormalities of embryos following ICSI must be investigated in detail.

Although calcium ionophore and 6-DMAP treatment combined together with oocyte grading used in the present experiment were able to increase cleavage (77.5%) and subsequent embryo development rates up to morula stage (42.5%) which was comparable to the results of other group researchers (Keskintepe *et al.*, 1997; Wang *et al.*, 2003; Jimenez-Macedo *et al.* 2005, 2006, 2007); even no blastocyst was obtained. The reasons for this could probably be due to the suboptimal culture medium. The current culture medium used for ICSI

(mSOF) seemed to be able to support embryonic development only as far as the morula stage after which there seemed to be a block in development. However, Keskintepe *et al.* (1998) and many others as reviewed by Cognie *et al.* (2003), Baldassarre and Karatzas (2004) and Jimenez-Macedo *et al.* (2006, 2007), had successfully used SOF medium for culturing goat embryos up to blastocyst stage. In particular, the only difference between the SOF medium used by Keskintepe *et al.* (1998) and the one used by the present experiment was that the former supplemented SOF medium with sodium citrate-trisodium (0.5 mM). Therefore, the absence of sodium citrate-trisodium salt supplement may be an attribute to the morula block encountered in the present experiment. Other researchers (Ongeri *et al.*, 2001; Borman *et al.*, 2003; Koeman *et al.*, 2003; Wang *et al.*, 2003; Herrick *et al.*, 2004) had also employed G1-G2 sequential culture medium (Gadner and Lane, 1997) successfully culture IVF or ICSI-derived goat embryo to blastocyst stage. Lane *et al.* (2003) reported that G1-G2 medium contained 8 vitamin subtypes, glucose, EDTA and all 21 amino acids which the first three components not included except only 19 amino acids in the SOF medium, in which, all other common components putting aside between G1-G2 and SOF medium. However, Wang *et al.* (2003) obtained 9 and 35% of blastocyst using G1-G2 and mTALP-mKSOM culture media, respectively. The culture medium has been reported to be critical in determining the ability of an embryo to survive (Martino *et al.*, 1993; Lonergan *et al.*, 2001). The use of different culture systems for embryo production provides the necessary specific conditions for embryo development. Likewise, the morphological and biochemical embryo characteristics are conditioned by the culture system employed (Massip *et al.*, 1995; Ohboshi *et al.*, 1997; Kaidi *et al.*, 1998; Pugh *et al.*, 1998; Yamashita *et al.*, 1999). Therefore, it is important to take into consideration the nutrient requirements with regards ICSI-derived goat embryos in order to improve the current IVC system in our laboratory.

Comparing sperm-injected and sham-injected oocytes with different activation treatments after culturing in mSOF medium regarding cleavage and morula rates confirmed that physical stimulation was not sufficient to induce activation of goat oocytes. As for the cleavage and morula rates in the present experiment, significantly higher rates were obtained from sperm-injected compared to sham-injected oocytes. Therefore, it can be predicted that the higher rates of sperm-injected oocytes, in part, are not due to the chemicals only but also the presence of sperm. This was further supported by the development of significant higher rates in ICSI with combination calcium ionophore and 6-DMAP than ICSI control. However, Wang *et al.* (2003) reported that Piezo-ICSI using tail-cut motile sperm is effective for cleavage and subsequent development without exogenous oocyte activation, and that it resulted in the birth of one female kid in Nigerian Dwarf goats. This shows that oocyte activation factors in goat sperm are sufficient for embryo development. Goat ICSI without additional activation treatment is important for achieving high rates of blastocyst development and for the production of healthy goat kids.

In summary, ICSI with the combination of calcium ionophore and 6-DMAP chemical activation treatment can significantly increase cleavage and subsequent embryo development rates of LOPU-derived goat oocytes. However, further efforts are needed to understand the basic cellular and molecular events after ICSI, improve gonadotrophin superovulation regime yield of oocyte and to refine IVC conditions in order to increase the developmental potential of goat oocytes. In addition, further studies including embryo transfer would be interesting and necessary to evaluate the *in vivo* viability of the goat embryos obtained after ICSI technique.

5.5 GENERAL DISCUSSION

The significant findings from the present study reveal the feasibility of ICSI technique that can be integrated as an alternative IVP procedure to produce goat embryos *in vitro*. In addition, ICSI technique is a useful tool for studying more accurately LOPU-derived oocyte developmental competence for subsequent embryo development both *in vivo* and *in vitro*, particularly when considering the possibility of incorporating PMSG as alternative to more popular FSH for superovulation procedure. However, it is well-known that various extrinsic and intrinsic factors are influencing the performance of *in vitro* production of micromanipulated-embryos. These factors include genotype, age, body weight, OR cycle and stimulation response, which in turn affect the superovulatory response and subsequent embryo development *in vivo* and *in vitro*. The focus of this study was to evaluate the factors such as genotype, age, body weight, PMSG dose, LOPU, oocyte quality, IVM duration and ICSI on the developmental competence of goat oocytes and embryos *in vitro*. The major findings obtained from this study include superovulation with 1200 IU PMSG dosage and maturation duration at 22 to 25 hours using competent oocytes (Grades A, B and C) as well as with the combination chemical activation treatment of Ca^{2+} ionophore and 6-DMAP through ICSI technique. Younger, lighter body weight and excellent ovarian stimulation response of oocyte donor will be value-added consideration to the ICSI performance in goats. The following sections highlight the constraints and suggestions for improvement for future research with special reference to oocyte retrieval, IVM, ICSI and IVC in goat.

5.5.1 Oocyte Retrieval

The present study is the first attempt to investigate various factors such as goat genotype, body weight, age, oocyte retrieval cycle and stimulation response from PMSG-superovulated goats in Malaysia. *In vitro* embryo production (IVEP) systems now allow oocytes from very

young animals to undergo fertilisation and form embryos capable of development to normal offspring, albeit at somewhat reduced efficiencies compared to oocytes from adult animals. However, the interactions between external and internal cues that drive young mammals to puberty are complicated and still not fully understood (Ebling, 2005).

Previous superovulation protocol in our laboratory consisted of recombinant ovine FSH (Ovagen™) and hCG combination was replaced with eCG (PMSG) due to production disrupt of Ovagen™ from the manufacturer throughout the experiment. FSH proved to be more efficacious than eCG (Armstrong *et al.*, 1983b; Nuti *et al.*, 1987; Mahmood *et al.*, 1991; Nowshari *et al.*, 1992), provided it contains an appropriate admixture of luteinizing hormone (LH). It have been reported that repeated use of eCG has been reported to result in poor fertility in goats due to the presence of anti-eCG antibodies developed as an immune response to previous treatments (Baril *et al.*, 1992; Baldassarre and Karatzas, 2004) reported that repeated use of eCG has been reported to result in poor fertility in goats due to the presence of anti-eCG antibodies developed as an immune response to previous treatments.

Hormonal regimes to induce multiple ovulations in goats are developed (Cognie, 1999; Cognie *et al.*, 2003). However, the follicles often ovulate within a wide range of hours resulting in variable stages of development of the embryos collected when using standard superovulation protocols (Ebert *et al.*, 1991; Gootwine *et al.*, 1997; Baldassarre *et al.*, 1999; Echelard *et al.*, 2000). It is noted that there are a variety of other processes occurring within the cytoplasm of the oocyte that are required for complete developmental competence following fertilisation although nuclear maturation may be completed successfully. Successful completion of these events is independent of nuclear maturation and is collectively referred to as cytoplasmic maturation (Sun and Nagai, 2003; Krisher, 2004). An oocyte that has not completed cytoplasmic maturation is of poor quality, and thus unable to successfully complete normal developmental processes (Vassena *et al.*, 2003). Therefore,

Baldassarre *et al.* (2004b) explored the use of GnRH for better controlling the timing of ovulation in superovulated goats. The results obtained in terms of total number of ovulations, oocyte recovered and fertilised embryos were comparable to those reported by others (Ebert *et al.*, 1991; Gootwine *et al.*, 1997; Echelard *et al.*, 2000). Furthermore, the use of GnRH and timing of injection following sponge (or other vaginal pessaries such as CIDR) removal is a key parameter for the success of IVEP programme. The researchers suggested that the injection of GnRH at 36 hours after pessaries removal provided almost 80% fertilised oocytes, of which 90% were at the pronuclear stage of development; whilst GnRH at 24 hours may have promoted a premature ovulation of follicles, resulting in lower developmental capacity of the microinjected embryos in goats.

The presence of a dominant follicle at the onset of superovulation has been reported to inhibit the growth of other ovarian follicles and decrease the ovarian response in small ruminants (Rubianes and Menchaca, 2003a). Several studies have reported that the superovulatory response in goats is closely related to the number of gonadotrophin responsive follicles stimulated and the absence of a dominant follicle on the ovary at the onset of superovulation (Heidari *et al.*, 2010). It has been previously shown that the use of a GnRH antagonist increases the number of the small gonadotrophin-responsive follicles (Gonzalez-Bulnes *et al.*, 2003b). This phenomenon is generally the result of the blockage of the GnRH receptors by the application of an antagonist which then causes a rapid decrease in the circulating LH and FSH levels, thus preventing the LH surge (Gonzalez-Bulnes *et al.*, 2004a). It has been observed that once the numbers of small follicles increase and develop, the response to superovulation is greater (Gonzalez-Bulnes *et al.*, 2004a). However, the long-term use of a GnRH antagonist led to an adverse effect on terminal follicular growth, fertilisation rate and embryo viability in the superovulated goats (Cognie *et al.*, 2003).

To date, there is not a single one fulfills all expectations concerning predictability and reliability of the response from the numerous superovulation protocols in use. The variability in number of ovulations and yield of viable oocytes or embryos remains the main drawback. Both intrinsic and extrinsic factors are responsible for the variability. Therefore, the superovulatory regime is needed to modify and therefore, to increase the developmental competence of these LOPU-derived oocytes, in which, to permit oocytes to complete their final maturation following rapid follicular growth.

LOPU technique is an extremely powerful tool for production and propagation of genetically valuable animals by given its repeatability and reliability as a source of immature oocytes, which always results in more than 5 oocytes aspirated per donor while individual variation in the response to gonadotrophin treatment remains (Baldassarre and Karatzas, 2004). It has been also reported that LOPU is an efficient technique for the retrieval of high quantity and quality oocytes from prepuberal, pubertal or ageing animals; where MOET is not possible (Koeman *et al.*, 2003; Baldassarre *et al.*, 2007). LOPU procedure can be repeated several times without ovarian damage or decrease in the donor fertility since it is less traumatic and results in fewer surgical adhesions than standard surgery (laparotomy) generally used to recovery of *in vivo* matured oocytes and embryos (Baldassarre and Karatzas, 2004), therefore, extends the use of donor animal resources (Pierson *et al.*, 2004). It is possible to generate more offspring from genetically valuable animals using LOPU technique, since it can be repeated many times in the same donor within a short time period, as short as 4 days (Gibbons *et al.*, 2007).

5.5.2 *In Vitro* Maturation (IVM)

The duration of IVM (22 to 25 hours) employed in the present study shows achievable and satisfactory maturation, cleavage and subsequent embryo development rates from LOPU-

derived goat oocytes of superior grades (Grades A, B and C) through ICSI technique. However, Han *et al.* (2006) suggested that the developmental potential of an oocyte is determined by multifactor interactions, and multiple factors must be considered together to accurately predict the quality of an oocyte.

It has been demonstrated that the IVM duration necessary for goat oocytes to reach metaphase II is about 27 hours, independent of the physiology of the animals (Martino *et al.*, 1994; Song and Iritani, 1987; Le Gal *et al.*, 1992; Rho *et al.*, 2001). Furthermore, peak MII of goat oocytes was reported at 27 hours when ovarian follicles were stimulated by gonadotrophin treatment (De Smedt *et al.*, 1992). On the other hand, Younis *et al.* (1991) reported that the 24 hours of IVM duration is the best time for goat oocyte maturation *in vitro*. Besides COCs selection, follicle diameter of ovaries for oocyte retrieval might be good selection candidate to predict the quality of oocytes and to improve the subsequent embryo development. Goat oocytes from less than 0.5 mm follicles were unable to undergo germinal vesicle breakdown (GVBD) when matured *in vitro*. They became GVBD-competent in 0.5 mm follicles, MI-competent in 1 to 1.8 mm follicles and MII-competent in 2 to 5 mm follicles (De Smedt *et al.*, 1994; Crozet *et al.*, 1995; Ma *et al.*, 2003). Han *et al.* (2006) showed that the majority of goat oocytes acquired competence for development up to the 8 to 16-cell stage in follicles larger than 2 mm, but did not gain the ability to form morula or balstocyst until follicles larger than 3 mm in diameter. This could be probably explained that oocytes from follicles of larger than 3 mm could have enough time to be submitted to pre-maturation processes and to acquire a complete developmental competence to blastocyst stage (Hyttel *et al.*, 1997). However, it has been shown that follicle diameters of more than 7 mm have a detrimental effect on oocyte developmental competence (Abeydeera, 2002). Mogas *et al.* (1997a) have suggested that a long maturation period could be the cause of non-extrusion of the second polar body. This occurs during oocyte recovery, since some of the

oocytes could be at a more advanced stage of maturation than others. Such mature oocytes exhibit aging which in turn produce errors in the extrusion mechanisms of the second polar body and cause diploid oocytes.

In adult females, the diameter of follicles has been positively related to oocyte diameter (Fair *et al.*, 1995; Raghu *et al.*, 2002) and to subsequent embryo developmental competence (Furher *et al.*, 1989; Crozet *et al.*, 1995; Marchal *et al.*, 2002). In goat oocytes, meiotic competence is acquired when oocyte diameter is greater than 136 µm, both in adult (De Smedt *et al.*, 1994) and prepubertal females (Martino *et al.*, 1994). Anguita *et al.* (2007) observed that the largest oocytes after *in vitro* maturation presented the highest MPF activity, also had the highest percentage of oocytes at the metaphase II when the maximum level of MPF activity is detected (Naito and Toyoda, 1991; Collas *et al.*, 1993; Dedieu *et al.*, 1996; Ledda *et al.*, 2001). The differences in MPF activity are not only a consequence of different rates in metaphase II oocytes but to different degrees of cytoplasmic maturation in those oocytes, which is reflected in the differences in blastocyst yield between oocyte diameters (Anguita *et al.*, 2007). Therefore, follicle and oocyte diameter may be a criterion to be considered as a predictor, either to shorten or lengthen the IVM duration than the conventional IVM duration at 27 hours, depending on the stages of meiosis of the oocyte concerned.

In all mammalian species, nuclear maturation can be achieved when the oocytes are removed from the antral follicles and are cultured *in vitro* (Thibault *et al.*, 1987). Cytoplasm of the oocyte may play a crucial role in assembling the correct metabolic environment for production of sufficient energy for cellular functions during maturation, cleavage and blastocyst formation. Culture conditions used to support IVM can profoundly alter subsequent embryo development. It has been shown that granulosa cell interactions with cumulus oocyte complexes (COCs) during IVM are involved in imparting developmental

competence to the maturing oocytes (Crister *et al.*, 1986; Barboni and Mattiali, 1996). The possible reason for the improvement of cytoplasmic maturation is that secretions of the granulosa cells may promote the synthesis of oocyte glutathione. Glutathione has important roles to play in male pronuclear formation (Calvin *et al.*, 1986) and early development; in which, the levels increase during maturation and decreases during fertilisation and embryo development (Yoshida *et al.*, 1993). This case is in agreement with Teotia *et al.* (2001) who reported that granulosa cell monolayers support cytoplasmic maturation of growing goat oocytes, which is evident by a better maturation rate, active fertilisation, an improved cleavage rate and subsequent a higher rate of morula formation.

5.5.3 Intracytoplasmic Sperm Injection (ICSI)

The present study has demonstrated that combination of Ca^{2+} ionophore and 6-DMAP following ICSI was able to increase cleavage and subsequent embryo development rates up to morula stage. However, the current ICSI procedure in our laboratory need to be refined and improved in order to produce more development competence ICSI-derived goat embryos. Suttner *et al.* (2000) showed different results in embryo development following ICSI depending on oocyte activation, sperm treatment and injection technique. Various chemicals, such as heparin (Keefer *et al.*, 1990; Chen and Seidel, 1997; Wei and Fukui, 1999), caffeine (Goto, 1990; Iwasaki and Li, 1994) and Ca^{2+} ionophore (Urdaneta *et al.*, 2004) are able to increase sperm membrane permeabilisation, acrosome reaction and sperm head decondensation. Jimenez-Macedo *et al.* (2006) reported that conventional ICSI with sperm combining ionomycin and heparin as capacitators increases embryo development and blastocyst goat oocytes compared to sperm were only capacitated with heparin (Jimenez-Macedo *et al.*, 2005). Thus, results with ICSI not only depend on oocyte activation but also on correct sperm capacitation. Inappropriate capacitation of sperm before injection that does

not allow the release of sperm factors responsible for oocyte activation (Stricker, 1999) and results in block of sperm head decondesation. Furthermore, pre-treatment of sperm with dithiothreitol (DTT) prior to ICSI has been demonstrated to induce the reduction of the protamine disulfide bonds in sperm leading to the decondensation of the sperm head (Calvin and Bedford, 1971; Bedford and Calvin, 1974; Tateno and Kamigushi, 1999) as well as increase embryo development (Galli *et al.*, 2003; Suttner *et al.*, 2000).

On the other hand, a number of technical aspects play an important role in the success of an ICSI experiment. The preparation of the holding, and certainly, of the injection pipette is of crucial importance. A good set-up and the correct use of the equipment facilitates the injection procedure. All aspects of the procedure require a lot of training and dedication (Joris *et al.*, 1998). In addition to the technical parameters, oocyte characteristics also play their role and can influence the ICSI results. Stripping cumulus cells from around the oocytes is an important part of the ICSI procedure. It facilitates handling of the oocytes during microinjection and allows an accurate assessment of cytoplasmic morphology, nuclear maturity and structural abnormalities that may adversely affect the ICSI outcome (Serhal *et al.*, 1997). The main morphological abnormalities of the oocyte cytoplasm were significantly granularity of the cytoplasm (dark centre or homogeneous granularity) and cytoplasmic inclusions (vacuoles of different diameter, refractile bodies). In addition, oocytes with darkened and granular centres often fail to fertilise and have reduced developmental potential (Veeck, 1988, 1991; Bedford and Kim, 1993). However, due to limited number of donor goats available for LOPU and thus all the viable oocytes of heterogenous grades were used, including COCs with less than one complete cumulus cell layer or naked oocytes in the present study. Therefore, the percentage of success in embryo yield may be diluted to a certain extent.

The ICSI technique has not only applied to rescue infertile male strains but also could be used in livestock species in several areas such as biodiversity conservation, transgenic production (Kurome *et al.*, 2007; Hirabayashi, 2008; Hirabayashi *et al.*, 2008) or to solve fertilisation problems in IVF systems. Some research groups have pursued an alternative procedure to pronuclear injection for producing transgenic animals using sperm as vector to introduce genes (sperm-mediated gene transfer; SMGT) (Lavitrano *et al.*, 2006). New or improved technologies in mammalian transgenesis such as the use of lentiviral vectors (Whitelaw 2004) and the 'renaissance' of ICSI approaches have shown that generation of transgenic animals now can be made easier (Moreira *et al.*, 2007).

5.5.4 *In Vitro* Culture (IVC) System

In the present study, it is difficult to determine of the ICSI-derived goat embryos not developing beyond the morula stage due to indirectly to the efficacy of the culture system during the whole process of *in vitro* production of embryos or due to the hormonal stimulation, or whether it is a result of reduced developmental competence of goat oocytes matured *in vitro*. Therefore, the present culture system in our laboratory needs to be refined and studied with better understanding of the cellular and molecular mechanisms that regulate goat embryo development whether produced *in vitro* or *in vivo*, to release ICSI-derived goat embryos from the morula block.

A number of studies have shown that *in vitro* development of pre-implantation stage mammalian embryos is better when cultured in groups (well-in-drop, WID) than cultured singly in microdrops (microdrop individual culture, MIC) (Gardner *et al.*, 1994; Kato and Tsunoda, 1994; Keefer *et al.*, 1994; Moessner and Dodson, 1995). However, embryo culture system in the present experiment cultured in small groups due to small number of oocytes available and the required identification after culture. With MIC systems, some studies

indicated that reduced oocyte developmental capacity was related to apoptotic death of follicular cells even before morphological signs of severe atresia were detected (Jewgenow *et al.*, 1999), but others suggested that even a high degree of atresia was not necessarily detrimental (Hagemann *et al.*, 1999). Therefore, the effect of follicular cell apoptosis which is more accurate, fast and non-invasive markers of oocyte quality on oocyte developmental potential must be studied using an individual culture system that is, as efficient and consistent as group culture. Han *et al.* (2006) showed that while late atresia was detrimental, early atresia was beneficial to the competent oocytes which observed using a WID culture system. Interestingly, they also observed that atresia of all degrees was beneficial to the incompetent goat oocytes. The effect of atresia on incompetent oocytes has not been reported but it is important for utilisation of these oocytes.

Several studies have been carried out to optimise culture conditions and many chemically-defined media have been proposed to support *in vitro* produced goat embryos development (Pawshe *et al.*, 1996; Lee *et al.*, 2000; Urdaneta *et al.*, 2003a). Previous studies have shown that the *in vitro* block to development at the 8- to 16- cell stage can be overcome through the use of co-culture with oviduct cells supplemented with serum (Gandolfi and Moor, 1987). There is still discussion as to whether these culture methods support the development of the embryos by producing growth-promoting factors (Gandolfi, 1994) or simply by removing inhibitory components such as glucose and oxygen (Watson *et al.*, 1994). The cleavage stage embryos have a limited capacity to utilise glucose as an energy substrate (Reiger *et al.*, 1992; Thompson *et al.*, 1996). The presence of glucose in the culture medium was found to have an adverse effect on embryo development (Quinn, 1995), as can be seen by the increase in morula development (22% versus 40%) when cultured in glucose free medium (Lim *et al.*, 1993). However, the granulosa cell monolayers used to support embryo development by utilising the glucose from the culture medium and thus depleting its levels,

while it gives pyruvate in return which the growing embryos utilise (Teotia *et al.*, 2001). This is in accordance with the finding of Teotia *et al.* (2001) who obtained that higher morula rate on goat embryo when cultured over granulosa cell monolayer. Therefore, the results suggested that glucose supplementation in the embryo culture medium is not required until approximately day 3 or 4 of development, at which time the supplementation improves development (Kim *et al.*, 1993a). Nevertheless, a high glucose concentration is also detrimental to the *in vitro* embryo development (Thompson *et al.*, 1992).

5.5.5 Future Directions

The present experiment is the first of its kind which reported OR, IVM and ICSI with PMSG-superovulated goat on embryo development and onset of LOPU, thus, many problems had to be faced to cover the wide aspects of these techniques. The problems encountered in the present study include the limited sample size of oocytes, type of gonadotrophin used for superovulation, PMSG dose, oocyte quality, IVM duration, preparation of ICSI needle, management efficiency in the farm and the experience of helpers in LOPU as well as learning curve period for the author. However, findings from the present study offer a window of opportunity for further development or improvement of procedures relating to ICSI technique in the future. Therefore, when all related technical problems have been overcome, it is possible that such time will come whereby ICSI technique could be used routinely as an integral component of other IVP techniques to produce embryo *in vivo* and *in vitro* in goat. In view of the outcomes of this study, various aspects relating to ICSI in goat had been identified for further refined research. Such factors include breed, age, body weight of the donor goats; the optimal dosage of PMSG to be utilised in the present superovulation protocol; oocyte selection based on multifactor interaction such as follicle and oocyte diameter; appropriate IVM duration from gonadotrophin-superovulated goats; the effect of

sperm pre-treatment using DDT and; improve the present *in vitro* culture system. With the possible development and improvement in IVEP through ICSI technique, it is hoped that, in the long-term goal, the reproductive technologies could transform from agricultural farming to medical pharming as well as bioengineering such as further production of pharmaceutical proteins, tissues, and bioorgans through cloning and stem cell research. In a short-term goal, the reproductive technologies could be incorporated into modern farm management in order to drastically increase goat population in Malaysia in line with the inspiration of 9th Malaysia Plan to promote agriculture as a business, so that Malaysia will achieve the status of developed country in all aspects of life through Malaysia Vision 2020.

Chapter 6

6.0 CONCLUSIONS

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This study evaluates the *in vitro* development of goat embryos produced from *in vitro* matured oocytes retrieved from PMSG-superovulated donors through LOPU technique and subsequent *in vitro* fertilised by ICSI technique. From the findings obtained in this study, it could be concluded that:

a) Goat embryos were produced *in vitro* from immature oocytes after ICSI technique in the local settings of ABEL, University of Malaya, Malaysia.

b) Jamnapari and Boer crossbred goats with ages ranging from 6 to 42 months old were successfully PMSG-superovulated and higher number of competent oocytes (Grades A, B and C) were retrieved via the LOPU technique.

c) The light body weight yielded higher quantity and quality of LOPU-derived oocytes compared with that of heavy body weight, probably relating to age of the donor goats.

d) PMSG-induced hormonal treatment and LOPU can be repeated at least 2 times in the same donor goats.

e) Higher quantity and quality LOPU-derived oocytes after PMSG-superovulation were obtained from excellent ovarian stimulation response compared to the satisfactory ovarian stimulation response.

f) Positive correlation was observed between the age and body weight in Boer crossbred goats, whereby younger and lighter body weigh donor goats produced higher quantity and quality of developmental competence oocytes.

g) The number of LOPU-derived oocytes was found to be influenced by the body weight, whereby the number oocytes retrieved was inversely proportional to the body weight of the donor goats.

h) Recovery, maturation, cleavage and subsequent embryo development rates following ICSI technique could be obtained satisfactorily from the donor PMSG-superovulated goat at a dose of 1200 IU through LOPU technique.

i) High rates of maturation, cleavage and subsequent embryo development were achievable at IVM duration of 22 to 25 hours from LOPU-derived goat oocytes through ICSI technique.

j) Oocytes of Grades A and B at 22 to 25 hours IVM duration were able to develop up to morula stage with higher rates of maturation, cleavage and subsequent embryo development rates compared to the longer conventional IVM duration at 27 to 32 hours.

k) High rates of cleavage and subsequent embryo development following ICSI technique could be obtained with the combination of Ca^{2+} ionophore and 6-DMAP chemical activation treatment after insemination.

l) In summary, a satisfactory *in vitro* production of morula in goat embryos can be achieved after superovulation with 1200 IU PMSG dosage and maturation duration at 22 to 25 hours using competent oocytes (Grades A, B and C) as well as with the combination chemical activation treatment of Ca^{2+} ionophore and 6-DMAP through ICSI technique. Younger, lighter body weight and excellent ovarian stimulation response of oocyte donor will be value-added consideration to the ICSI performance in goats.

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APPENDICES

APPENDICES

APPENDIX 1: LIST OF MATERIALS

Appendix Table 1.1: List of equipment and instruments

Equipment/instrument	Model no.	Manufacturer
Abrasive stone or oilstone	-	Hall's Arkansas Oilstones, USA
Atraumatic grasping forceps	PO951R	Aesculap®, Germany
Autoclave	HA-300MII	Hirayama Hiclave, Japan
Centrifuge	D37520	Heraeus, Germany
CIDR applicator	-	Pharmacia and Upjohn, New Zealand
CO_2 incubator	HeraCell 240	Heraeus, Germany
CO_2 insufflator system	PG001	Aesculap®, Germany
Digital balance	AB104	Mettler Toledo, Switzerland
Digital camera (X-Cam-α)	-	microLAMBDA Sdn Bhd, Malaysia
Dissecting microscope	SZH10	Olympus, Japan
Flushing and aspiration system:		
(a) Aspiration system	KMAR-5100	Cook, Australia
(b) Flushing system	KMAR-4000	Cook, Australia
(c) Test tube heater system	KFTH-1012	Cook, Australia
(d) Pedal	6210-725350B	Herga Electric Ltd., UK
Heating stage (Thermoplate)	HATS-U55R30	Tokai Hit, Japan
Impulse sealer	KF-300H	Khind, Taiwan
Inverted microscope	IX71	Olympus, Japan
Laminar flow cabinet	HLF-120	Gelman Sciences, Australia
Laparoscopic system:		Aesculap®, Germany
(a) Endoscopic camera system	PV431	
(b) CCD camera	PV430	
(c) Pediatric Storz laparoscope (7 mm)	PE688A	
(d) Light probe with fibre optic cable	OP913	
(e) Light system (300 W)	OP927	
Liquid nitrogen tank (small)	SC2/1V	MVE, USA
Microforge	-	Technical Products Internationals, USA
Microgrinder	EG-4	Narishige, Japan
Micropipette dispenser	-	Eppendorf, Germany
Micropipette puller	P-97	Sutter Instrument Co., USA
Narishige hydraulic micromanipulators	ON3-99D	Narishige, Japan
Oocyte pick-up needle	FAS set C2	Gynetics Medical Product, Belgium
Oven	40050-IP20	Memmert GmbH, Germany
pH meter	HI-122	Hanna Instruments, Singapore
Pipette pump	PI-PUMP	Glasfirn, Germany
Refrigerator and freezer	SR-21NME	Samsung Electronics, Korea
Spirit burner	-	Shanghai Machinery, China
Stereomicroscope	SZH10	Olympus Optical, Japan
Surgical set	-	Aesculap®, Germany
Surgical table	-	Syarikat Copens Enterprise, Malaysia
Trocar and canula (5.5 mm & 7.0 mm)	EJ456, EJ457	Aesculap®, Germany
Ultrapure water purification system	Milli-Q PF Plus	Millipore, USA
Vapour pressure osmometer	5520	Vapro Wescor, USA

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Equipment/instrument	Model no.	Manufacturer
Verrus needle	PG3	Cook, Australia
Vortex mixer	VTX-3000L	LMS, Japan
Water bath	GMP-GC-19	Memmert GmbH, Germany

Appendix Table 1.2: List of chemicals, reagents and media

Chemicals, reagents and media	Catalogue no.	Manufacturer
6-dimethylaminopurine (6-DMAP)	D2629	Sigma-Aldrich, USA
70% ethanol	-	Prepared from absolute ethanol
Ethyl alcohol 99.8% (absolute ethanol)	ET150-50	Systerm ChemAR®, Poland
Hibiscrub (antiseptic)	HK-06770	SSL International Plc, UK
BME amino acids solution (50X)	B6766	Sigma-Aldrich, USA
Calcium chloride ($CaCl_2.2H_2O$)	C7902	Sigma-Aldrich, USA
Calcium ionophore (Ca^{2+} ionophore)	I0634	Sigma-Aldrich, USA
Cleaning solution 7X®-PF	-	FlowLab™, Australia
Cloprostenol (Estrumate®)	-	Schering-Plough, Australia
Cysteamine	M9768	Sigma-Aldrich, USA
Dimethyl sulphoxide (DMSO)	D5879	Sigma-Aldrich, USA
Disinfectant Gigasept® FF	-	Schülke& Mary GmbH, Germany
Folligon® (FSH)	-	Intervet International, Holland
Gentamicin sulfate salt	G3632	Sigma-Aldrich, USA
Goat/ sheep pellet feed	-	KMM Berhad, Malaysia
Heparin	H0777	Sigma-Aldrich, USA
HEPES	H7006	Sigma-Aldrich, USA
Hydrochloric acid	HY450-70	Systerm ChemAR®, Poland
Hydrofluoric acid	1301030	HmbG Chemicals, Germany
Hyaluronidase (from bovine testes)	H4272	Sigma-Aldrich, USA
Intravaginal progesterone release device	-	Pharmacia and Upjohn, New Zealand
Ketamil injection (ketamine hydrochloride)	L10077	Troy Laboratories, Australia
K-Y Lubricating Jelly	-	Pharmedica Lab, South Africa
L-glutamine	G3126	Sigma-Aldrich, USA
Liquid nitrogen	-	Mox Gases Berhad, Malaysia
Magnesium chloride hexahydrate	M2393	Sigma-Aldrich, USA
MEM (100x)	M7145	Sigma-Aldrich, USA
Mineral oil	M8410	Sigma-Aldrich, USA
Oestradiol-17β	E4389	Sigma-Aldrich, USA
Oestrus goat serum, heat-inactivated	-	Prepared "in-hourse"
Ovidrel® PreFilled Syringe	-	Laboratories Serono, Switzerland
Oxytetracycline (Tetrasol 20%)	E388	Richter Pharma, Austria
PBS Dulbecco A tablets	BR0014G	Oxoid, England
Phenol red solution (0.5%)	15100-043	Gibco BRL, USA
Potassium chloride	P5405	Sigma-Aldrich, USA
Potassium hydrogen orthophosphate	Prod29068	BDH Laboratory Supplies, England
PVP medium (polyvinylpirrolidone, 10%)	10890001	Medicult, Denmark
Sodium bicarbonate	S5761	Sigma-Aldrich, USA
Sodium chloride (NaCl)	S5886	Sigma-Aldrich, USA
Sodium DL-lactate (60% syrup)	L4263	Sigma-Aldrich, USA

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Chemicals, reagents and media	Catalogue no.	Manufacturer
Sodium pyruvate	P3662	Sigma-Aldrich, USA
TCM 199	M4530	Sigma-Aldrich, USA
Weak iodine solution	-	ICN Biomedicals, USA
Xylazine hydrochloride (Ilium Xylazil-20)	L10600	Troy Laboratories, Australia

Appendix Table 1.3: List of labwares and disposables

Labwares and disposables	Manufacturer
Aluminium foil	Reynolds Consumer Products, USA
Autoclave disposal bag	Megalab supplies, Malaysia
Blades (Super Nacet)	Gillette, USA
Borosilicate glass tubing (Microcaps®)	Drummond Scientific Company, USA
Chromic catgut and other suture materials	Aesculap®, Germany
Culture dish	Nunc, Denmark
Disposable glass Pasteur pipette	Hirschmann® Laborgerete, Germany
Disposable hand tissues	Megalab supplies, Malaysia
Falcon™ conical tube	Becton Dickinson, USA
Falcon™ polystyrene round-bottom test tube	Becton Dickinson, USA
Glassware (beaker, flask, measuring cylinder etc.)	Pyrex®, Japan
Lens cleansing tissue (Kimswipe®EX-L)	Kimberly-Clark, USA
Microcentrifuge tube	Elkay, Costelloe
Micropipette tips without filter	Axygen Scientific, USA
Microscope slide	Sail Brand, China
Microscope glass cover slip	Hirschmann® Laborgerate, Germany
Millex®-GS syringe driven filter	Schleicher and Schuell, Germany
Needle	Terumo Corporation, Japan
Parafilm	Pechiney Plastic Packaging, USA
Schott bottle	Duran, Germany
Serological pipette	LP Italiana SPA, Italy
Sterile glove	Ansell International, Malaysia
Syringe	Terumo Corporation, Japan
Tissue culture flask	Nunc, Denmark
Terumo venojector holder	Terumo Corporation, Japan
Vacutainer® blood collection tubes	Becton Dickinson, USA
Vacutainer needle	Becton Dickinson, USA

APPENDIX 2: STATISTICAL DATA

Appendix Table 2.1: Effect of goat genotype on oocyte retrieval

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_Jamnapari	GradeA	12	19.6533	32.81350	9.47244	-1.1954	40.5020	.00	100.00
	GradeB	12	10.6942	17.22447	4.97228	-.2497	21.6381	.00	50.00
	GradeC	12	34.5833	38.05342	10.98508	10.4053	58.7613	.00	100.00
	GradeD	12	14.9300	21.86518	6.31193	1.0375	28.8225	.00	66.67
	GradeE	12	20.1392	33.98242	9.80988	-1.4522	41.7306	.00	100.00
	Total	60	20.0000	29.94328	3.86566	12.2648	27.7352	.00	100.00
P_Boer	GradeA	72	24.1653	26.13499	3.08004	18.0239	30.3067	.00	100.00
	GradeB	72	20.9782	24.51008	2.88854	15.2186	26.7378	.00	100.00
	GradeC	72	37.4137	27.11972	3.19609	31.0409	43.7866	.00	100.00
	GradeD	72	8.6681	16.65513	1.96283	4.7543	12.5818	.00	100.00
	GradeE	72	8.7747	22.82293	2.68971	3.4116	14.1378	.00	100.00
	Total	360	20.0000	25.93730	1.36702	17.3116	22.6884	.00	100.00
N_Jamnapari	GradeA	12	.7500	1.05529	.30464	.0795	1.4205	.00	3.00
	GradeB	12	.4167	.66856	.19300	-.0081	.8414	.00	2.00
	GradeC	12	1.1667	1.26730	.36584	.3615	1.9719	.00	4.00
	GradeD	12	.5833	.79296	.22891	.0795	1.0872	.00	2.00
	GradeE	12	.6667	1.07309	.30977	-.0151	1.3485	.00	3.00
	Total	60	.7167	.99305	.12820	.4601	.9732	.00	4.00
N_Boer	GradeA	72	1.4306	1.68528	.19861	1.0345	1.8266	.00	9.00
	GradeB	72	1.0833	1.21898	.14366	.7969	1.3698	.00	5.00
	GradeC	72	2.0000	1.59223	.18765	1.6258	2.3742	.00	6.00
	GradeD	72	.4167	.70711	.08333	.2505	.5828	.00	3.00
	GradeE	72	.3056	.72460	.08539	.1353	.4758	.00	3.00
	Total	360	1.0472	1.40055	.07382	.9021	1.1924	.00	9.00

Appendix Table 2.2: Effect of body weight on oocyte retrieval

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_Light	GradeA	21	24.3867	18.14149	3.95880	16.1288	32.6446	.00	66.67
	GradeB	21	18.9938	20.34550	4.43975	9.7326	28.2550	.00	66.67
	GradeC	21	44.3843	21.30748	4.64967	34.6852	54.0833	.00	100.00
	GradeD	21	9.4576	14.45344	3.15400	2.8785	16.0367	.00	42.86
	GradeE	21	2.7776	10.97126	2.39412	-2.2164	7.7717	.00	50.00
	Total	105	20.0000	22.36095	2.18221	15.6726	24.3274	.00	100.00
P_Medium	GradeA	23	23.6665	30.84863	6.43238	10.3266	37.0065	.00	100.00
	GradeB	23	24.8652	26.38224	5.50108	13.4567	36.2738	.00	100.00
	GradeC	23	27.8813	28.12318	5.86409	15.7199	40.0427	.00	100.00
	GradeD	23	10.9057	18.72341	3.90410	2.8090	19.0023	.00	66.67
	GradeE	23	12.6813	27.27897	5.68806	.8850	24.4776	.00	100.00
	Total	115	20.0000	27.00693	2.51841	15.0111	24.9889	.00	100.00
P_Heavy	GradeA	8	8.7500	18.07722	6.39126	-6.3629	23.8629	.00	50.00
	GradeB	8	6.2500	17.67767	6.25000	-8.5289	21.0289	.00	50.00
	GradeC	8	28.1850	26.03948	9.20635	6.4155	49.9545	.00	60.00
	GradeD	8	27.6488	33.50318	11.84516	-.3606	55.6581	.00	100.00
	GradeE	8	29.1662	45.20671	15.98299	-8.6275	66.9600	.00	100.00
	Total	40	20.0000	30.20668	4.77610	10.3394	29.6606	.00	100.00
N_Light	GradeA	21	1.8571	1.79682	.39210	1.0392	2.6750	.00	6.00
	GradeB	21	1.2857	1.30931	.28571	.6897	1.8817	.00	4.00
	GradeC	21	2.7143	1.61688	.35283	1.9783	3.4503	.00	6.00
	GradeD	21	.5714	.92582	.20203	.1500	.9929	.00	3.00
	GradeE	21	.0952	.30079	.06564	-.0417	.2322	.00	1.00
	Total	105	1.3048	1.58189	.15438	.9986	1.6109	.00	6.00
N_Medium	GradeA	23	1.2174	1.92959	.40235	.3830	2.0518	.00	9.00
	GradeB	23	1.0435	1.10693	.23081	.5648	1.5222	.00	4.00
	GradeC	23	1.5217	1.67521	.34931	.7973	2.2462	.00	5.00
	GradeD	23	.4348	.66237	.13811	.1484	.7212	.00	2.00
	GradeE	23	.4783	.99405	.20727	.0484	.9081	.00	3.00
	Total	115	.9391	1.39728	.13030	.6810	1.1972	.00	9.00
N_Heavy	GradeA	8	.2500	.46291	.16366	-.1370	.6370	.00	1.00
	GradeB	8	.2500	.70711	.25000	-.3412	.8412	.00	2.00
	GradeC	8	1.2500	1.48805	.52610	.0060	2.4940	.00	4.00
	GradeD	8	.8750	.99103	.35038	.0465	1.7035	.00	3.00
	GradeE	8	.7500	1.16496	.41188	-.2239	1.7239	.00	3.00
	Total	40	.6750	1.04728	.16559	.3401	1.0099	.00	4.00

Appendix Table 2.3: Effect of age on oocyte retrieval

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_Group1	GradeA	14	26.0286	17.47483	4.67035	15.9389	36.1182	.00	55.56
	GradeB	14	21.5479	20.95476	5.60039	9.4489	33.6468	.00	66.67
	GradeC	14	38.1121	19.93755	5.32853	26.6005	49.6237	.00	60.00
	GradeD	14	10.1450	15.45521	4.13058	1.2214	19.0686	.00	42.86
	GradeE	14	4.1664	13.37725	3.57522	-3.5574	11.8902	.00	50.00
	Total	70	20.0000	20.94917	2.50390	15.0048	24.9952	.00	66.67
P_Group2	GradeA	14	22.9314	21.97209	5.87229	10.2451	35.6177	.00	66.67
	GradeB	14	26.8243	24.06976	6.43291	12.9268	40.7218	.00	66.67
	GradeC	14	40.0114	24.37616	6.51480	25.9371	54.0858	.00	100.00
	GradeD	14	10.2329	15.40129	4.11617	1.3404	19.1253	.00	50.00
	GradeE	14	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	70	20.0000	23.44592	2.80232	14.4095	25.5905	.00	100.00
P_Group3	GradeA	10	32.5160	37.77769	11.94636	5.4915	59.5405	.00	100.00
	GradeB	10	13.5230	14.94177	4.72500	2.8343	24.2117	.00	33.33
	GradeC	10	33.6750	26.78657	8.47066	14.5130	52.8370	.00	71.43
	GradeD	10	5.2860	13.57105	4.29154	-4.4221	14.9941	.00	42.86
	GradeE	10	15.0000	33.74743	10.67187	-9.1415	39.1415	.00	100.00
	Total	50	20.0000	28.38871	4.01477	11.9320	28.0680	.00	100.00
N_Group1	GradeA	14	1.5714	1.28388	.34313	.8301	2.3127	.00	5.00
	GradeB	14	1.1429	1.02711	.27451	.5498	1.7359	.00	3.00
	GradeC	14	2.3571	1.73680	.46418	1.3543	3.3599	.00	6.00
	GradeD	14	.6429	1.08182	.28913	.0182	1.2675	.00	3.00
	GradeE	14	.1429	.36314	.09705	-.0668	.3525	.00	1.00
	Total	70	1.1714	1.38283	.16528	.8417	1.5012	.00	6.00
N_Group2	GradeA	14	1.7143	2.01642	.53891	.5500	2.8785	.00	6.00
	GradeB	14	1.7143	1.54066	.41176	.8247	2.6038	.00	4.00
	GradeC	14	2.3571	1.73680	.46418	1.3543	3.3599	.00	6.00
	GradeD	14	.4286	.51355	.13725	.1321	.7251	.00	1.00
	GradeE	14	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	70	1.2429	1.61905	.19351	.8568	1.6289	.00	6.00
N_Group3	GradeA	10	1.8000	2.69979	.85375	-.1313	3.7313	.00	9.00
	GradeB	10	.8000	.91894	.29059	.1426	1.4574	.00	2.00
	GradeC	10	2.4000	1.95505	.61824	1.0014	3.7986	.00	5.00
	GradeD	10	.4000	.96609	.30551	-.2911	1.0911	.00	3.00
	GradeE	10	.5000	1.08012	.34157	-.2727	1.2727	.00	3.00
	Total	50	1.1800	1.79216	.25345	.6707	1.6893	.00	9.00

Appendix Table 2.4: Effect of OR cycle on oocyte retrieval

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_OR1	GradeA	52	21.6625	24.80824	3.44028	14.7558	28.5692	.00	100.00
	GradeB	52	19.6302	23.36826	3.24059	13.1244	26.1360	.00	100.00
	GradeC	52	34.5927	26.04840	3.61226	27.3408	41.8446	.00	100.00
	GradeD	52	12.8967	20.68829	2.86895	7.1371	18.6564	.00	100.00
	GradeE	52	11.2179	27.00996	3.74561	3.6983	18.7375	.00	100.00
	Total	260	20.0000	25.67507	1.59230	16.8645	23.1355	.00	100.00
P_OR2	GradeA	28	29.1411	31.56230	5.96471	16.9025	41.3797	.00	100.00
	GradeB	28	20.0471	25.78854	4.87358	10.0474	30.0469	.00	100.00
	GradeC	28	38.2136	33.68388	6.36566	25.1523	51.2748	.00	100.00
	GradeD	28	2.9511	5.86463	1.10831	.6770	5.2251	.00	16.67
	GradeE	28	9.6471	22.22816	4.20073	1.0280	18.2663	.00	100.00
	Total	140	20.0000	28.45222	2.40465	15.2456	24.7544	.00	100.00
N_OR1	GradeA	52	1.3269	1.79018	.24825	.8285	1.8253	.00	9.00
	GradeB	52	1.0192	1.17974	.16360	.6908	1.3477	.00	4.00
	GradeC	52	1.9615	1.71455	.23776	1.4842	2.4389	.00	6.00
	GradeD	52	.5577	.82637	.11460	.3276	.7878	.00	3.00
	GradeE	52	.3654	.84084	.11660	.1313	.5995	.00	3.00
	Total	260	1.0462	1.44319	.08950	.8699	1.2224	.00	9.00
N_OR2	GradeA	28	1.4643	1.37389	.25964	.9315	1.9970	.00	4.00
	GradeB	28	.9643	1.23175	.23278	.4867	1.4419	.00	5.00
	GradeC	28	1.5714	1.25988	.23810	1.0829	2.0600	.00	5.00
	GradeD	28	.2143	.41786	.07897	.0523	.3763	.00	1.00
	GradeE	28	.3571	.73102	.13815	.0737	.6406	.00	3.00
	Total	140	.9143	1.19040	.10061	.7154	1.1132	.00	5.00

Appendix Table 2.5: Effect of ovarian stimulation response on oocyte retrieval

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_Excellent	GradeA	11	30.2082	32.55766	9.81650	8.3356	52.0807	.00	100.00
	GradeB	11	18.0518	19.82850	5.97852	4.7308	31.3728	.00	50.00
	GradeC	11	37.3018	18.39109	5.54512	24.9465	49.6571	.00	60.00
	GradeD	11	9.8927	14.02692	4.22928	.4693	19.3161	.00	42.86
	GradeE	11	4.5455	15.07557	4.54545	-5.5824	14.6734	.00	50.00
	Total	55	20.0000	23.72146	3.19860	13.5872	26.4128	.00	100.00
P_Good	GradeA	57	22.3998	22.96656	3.04200	16.3060	28.4937	.00	100.00
	GradeB	57	21.9914	23.57131	3.12210	15.7371	28.2457	.00	100.00
	GradeC	57	38.3651	29.63973	3.92588	30.5006	46.2296	.00	100.00
	GradeD	57	10.1947	19.39736	2.56924	5.0479	15.3415	.00	100.00
	GradeE	57	7.0489	19.55648	2.59032	1.8599	12.2380	.00	100.00
	Total	285	20.0000	25.67103	1.52062	17.0069	22.9931	.00	100.00
P_Satisfactory	GradeA	16	22.9169	36.37028	9.09257	3.5365	42.2972	.00	100.00
	GradeB	16	11.6669	26.58324	6.64581	-2.4983	25.8321	.00	100.00
	GradeC	16	31.9794	31.77036	7.94259	15.0501	48.9086	.00	100.00
	GradeD	16	7.0831	12.10217	3.02554	.6343	13.5319	.00	33.33
	GradeE	16	26.3537	38.50061	9.62515	5.8382	46.8693	.00	100.00
	Total	80	20.0000	31.19405	3.48760	13.0581	26.9419	.00	100.00
N_Excellent	GradeA	11	2.0909	2.50817	.75624	.4059	3.7759	.00	9.00
	GradeB	11	1.0909	1.13618	.34257	.3276	1.8542	.00	3.00
	GradeC	11	2.5455	1.50756	.45455	1.5327	3.5582	.00	5.00
	GradeD	11	.6364	.92442	.27872	.0153	1.2574	.00	3.00
	GradeE	11	.2727	.90453	.27273	-.3349	.8804	.00	3.00
	Total	55	1.3273	1.70027	.22926	.8676	1.7869	.00	9.00
N_Good	GradeA	57	1.3333	1.46791	.19443	.9438	1.7228	.00	6.00
	GradeB	57	1.1228	1.22577	.16236	.7976	1.4480	.00	5.00
	GradeC	57	1.9474	1.64122	.21739	1.5119	2.3828	.00	6.00
	GradeD	57	.4386	.73235	.09700	.2443	.6329	.00	3.00
	GradeE	57	.2807	.72591	.09615	.0881	.4733	.00	3.00
	Total	285	1.0246	1.35422	.08022	.8667	1.1825	.00	6.00
N_Satisfactory	GradeA	16	.8125	1.27639	.31910	.1324	1.4926	.00	4.00
	GradeB	16	.4375	.89209	.22302	-.0379	.9129	.00	3.00
	GradeC	16	1.1875	1.10868	.27717	.5967	1.7783	.00	3.00
	GradeD	16	.3125	.47871	.11968	.0574	.5676	.00	1.00
	GradeE	16	.6875	.87321	.21830	.2222	1.1528	.00	3.00
	Total	80	.6875	.98846	.11051	.4675	.9075	.00	4.00

Appendix Table 2.6: Correlation coefficients between body weight and age in Boer crossbred goats

Correlations

		BodyWeight	Age
BodyWeight	Pearson Correlation	1	.555**
	Sig. (2-tailed)		.000
	N	37	36
Age	Pearson Correlation	.555**	1
	Sig. (2-tailed)	.000	
	N	36	36

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix Table 2.7: Regression coefficients of body weight on the number of oocytes retrieved in goats

Model Summary and Parameter Estimates

Dependent Variable:y

Equation	Model Summary					Parameter Estimates	
	R Square	F	df1	df2	Sig.	Constant	b1
Linear	.144	8.421	1	50	.006	8.799	-.156

The independent variable is x.

Appendix Table 2.8: Regression coefficients of age on the number of oocytes retrieved in goats

Model Summary and Parameter Estimates

Dependent Variable:y

Equation	Model Summary					Parameter Estimates	
	R Square	F	df1	df2	Sig.	Constant	b1
Linear	.000	.001	1	36	.978	6.028	-.001

The independent variable is x.

Appendix Table 2.9: Regression coefficients of body weight on the number of Grade A oocytes retrieved in goats

Model Summary and Parameter Estimates

Dependent Variable:y

Equation	Model Summary					Parameter Estimates	
	R Square	F	df1	df2	Sig.	Constant	b1
Linear	.067	3.610	1	50	.063	2.701	-.060

The independent variable is x.

Appendix Table 2.10: Regression coefficients of body weight on the number of Grade B oocytes retrieved in goats

Model Summary and Parameter Estimates

Dependent Variable:y

Equation	Model Summary					Parameter Estimates	
	R Square	F	df1	df2	Sig.	Constant	b1
Linear	.108	6.080	1	50	.017	2.168	-.050

The independent variable is x.

Appendix Table 2.11: Regression coefficients of body weight on the number of Grade C oocytes retrieved in goats

Model Summary and Parameter Estimates

Dependent Variable:y

Equation	Model Summary					Parameter Estimates	
	R Square	F	df1	df2	Sig.	Constant	b1
Linear	.174	10.537	1	50	.002	4.077	-.093

The independent variable is x.

Appendix Table 2.12: Regression coefficients of body weight on the number of Grade D oocytes retrieved in goats

Model Summary and Parameter Estimates

Dependent Variable:y

Equation	Model Summary					Parameter Estimates	
	R Square	F	df1	df2	Sig.	Constant	b1
Linear	.004	.182	1	50	.671	.411	.006

The independent variable is x.

Appendix Table 2.13: Regression coefficients of body weight on the number of Grade E oocytes retrieved in goats

Model Summary and Parameter Estimates

Dependent Variable:y

Equation	Model Summary					Parameter Estimates	
	R Square	F	df1	df2	Sig.	Constant	b1
Linear	.138	7.972	1	50	.007	-.557	.040

The independent variable is x.

Appendix Table 2.14: Effect of PMSG dosage on oocyte retrieval

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_1200IU	GradeA	28	29.4332	29.34067	5.54486	18.0561	40.8103	.00	100.00
	GradeB	28	25.8000	25.32908	4.78675	15.9784	35.6216	.00	100.00
	GradeC	28	32.4454	24.34627	4.60101	23.0049	41.8859	.00	100.00
	GradeD	28	9.8811	15.02412	2.83929	4.0553	15.7068	.00	50.00
	GradeE	28	2.4404	9.62758	1.81944	-1.2928	6.1735	.00	50.00
	Total	140	20.0000	24.64684	2.08304	15.8815	24.1185	.00	100.00
P_1500IU	GradeA	54	21.3261	25.68315	3.49503	14.3160	28.3363	.00	100.00
	GradeB	54	16.3522	22.76385	3.09777	10.1389	22.5656	.00	100.00
	GradeC	54	40.7467	30.14091	4.10166	32.5198	48.9736	.00	100.00
	GradeD	54	8.5170	17.29220	2.35317	3.7972	13.2369	.00	100.00
	GradeE	54	13.0580	26.85398	3.65436	5.7282	20.3877	.00	100.00
	Total	270	20.0000	27.15923	1.65286	16.7458	23.2542	.00	100.00
N_1200IU	GradeA	28	1.9286	2.07147	.39147	1.1253	2.7318	.00	9.00
	GradeB	28	1.3929	1.25725	.23760	.9053	1.8804	.00	4.00
	GradeC	28	2.1786	1.80644	.34139	1.4781	2.8790	.00	6.00
	GradeD	28	.5357	.83808	.15838	.2107	.8607	.00	3.00
	GradeE	28	.1786	.61183	.11563	-.0587	.4158	.00	3.00
	Total	140	1.2429	1.60875	.13596	.9740	1.5117	.00	9.00
N_1500IU	GradeA	54	1.0741	1.27163	.17305	.7270	1.4212	.00	6.00
	GradeB	54	.7963	1.10538	.15042	.4946	1.0980	.00	5.00
	GradeC	54	1.7963	1.41927	.19314	1.4089	2.1837	.00	5.00
	GradeD	54	.3704	.62333	.08482	.2002	.5405	.00	3.00
	GradeE	54	.4444	.86147	.11723	.2093	.6796	.00	3.00
	Total	270	.8963	1.20269	.07319	.7522	1.0404	.00	6.00

Appendix Table 2.15: Effect of PMSG dosage on oocyte maturation per ovary

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_1200IU	GradeA	27	36.1596	31.38557	6.04016	23.7439	48.5753	.00	100.00
	GradeB	27	32.7333	30.17916	5.80798	20.7949	44.6718	.00	100.00
	GradeC	27	29.2552	25.42977	4.89396	19.1955	39.3149	.00	100.00
	GradeD	27	1.8519	9.62250	1.85185	-1.9547	5.6584	.00	50.00
	GradeE	27	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	135	20.0000	27.58249	2.37392	15.3048	24.6952	.00	100.00
P_1500IU	GradeA	42	31.7514	34.59531	5.33817	20.9708	42.5321	.00	100.00
	GradeB	42	23.7248	31.06302	4.79313	14.0448	33.4047	.00	100.00
	GradeC	42	40.5555	38.96187	6.01195	28.4141	52.6969	.00	100.00
	GradeD	42	1.1905	5.69393	.87859	-.5839	2.9648	.00	33.33
	GradeE	42	2.7779	15.58126	2.40424	-2.0776	7.6333	.00	100.00
	Total	210	20.0000	31.96885	2.20606	15.6510	24.3490	.00	100.00
N_1200IU	GradeA	27	1.6296	1.64429	.31644	.9792	2.2801	.00	7.00
	GradeB	27	1.1852	1.07550	.20698	.7597	1.6106	.00	4.00
	GradeC	27	1.2593	1.22765	.23626	.7736	1.7449	.00	4.00
	GradeD	27	.0741	.38490	.07407	-.0782	.2263	.00	2.00
	GradeE	27	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	135	.8296	1.23107	.10595	.6201	1.0392	.00	7.00
N_1500IU	GradeA	42	1.0238	1.17884	.18190	.6565	1.3912	.00	5.00
	GradeB	42	.7143	.89131	.13753	.4365	.9920	.00	3.00
	GradeC	42	1.1667	1.16696	.18007	.8030	1.5303	.00	5.00
	GradeD	42	.0476	.21554	.03326	-.0195	.1148	.00	1.00
	GradeE	42	.0714	.34165	.05272	-.0350	.1779	.00	2.00
	Total	210	.6048	.97381	.06720	.4723	.7372	.00	5.00

Appendix Table 2.16: Effect of oocyte quality and PMSG dosage on the maturation rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_GradeA	PMSG 1200IU	20	83.5560	24.98147	5.58603	71.8643	95.2477	.00	100.00
	PMSG 1500IU	30	70.0000	40.44773	7.38471	54.8966	85.1034	.00	100.00
	Total	50	75.4224	35.42948	5.01048	65.3535	85.4913	.00	100.00
P_GradeB	PMSG 1200IU	18	84.2600	20.98192	4.94549	73.8259	94.6941	50.00	100.00
	PMSG 1500IU	25	68.0668	39.70866	7.94173	51.6759	84.4577	.00	100.00
	Total	43	74.8453	33.83113	5.15920	64.4337	85.2570	.00	100.00
P_GradeC	PMSG 1200IU	22	54.3186	29.87940	6.37031	41.0709	67.5664	.00	100.00
	PMSG 1500IU	43	45.1163	39.29012	5.99169	33.0246	57.2080	.00	100.00
	Total	65	48.2309	36.40415	4.51538	39.2104	57.2514	.00	100.00
P_GradeD	PMSG 1200IU	11	6.0609	20.10176	6.06091	-7.4436	19.5655	.00	66.67
	PMSG 1500IU	17	11.7647	33.21056	8.05474	-5.3106	28.8400	.00	100.00
	Total	28	9.5239	28.48336	5.38285	-1.5208	20.5686	.00	100.00
P_GradeE	PMSG 1200IU	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	PMSG 1500IU	14	7.1429	19.29825	5.15767	-3.9996	18.2853	.00	66.67
	Total	17	5.8824	17.62020	4.27353	-3.1771	14.9418	.00	66.67

Appendix Table 2.17: Effect of PMSG dosage on oocyte cleavage per ovary

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_1200IU	GradeA	14	53.5714	37.08328	9.91092	32.1602	74.9827	.00	100.00
	GradeB	14	25.0000	33.17295	8.86584	5.8465	44.1535	.00	100.00
	GradeC	14	21.4279	30.26107	8.08761	3.9556	38.9001	.00	100.00
	Total	42	33.3331	35.87860	5.53619	22.1525	44.5137	.00	100.00
P_1500IU	GradeA	16	30.8338	33.93941	8.48485	12.7487	48.9188	.00	100.00
	GradeB	16	35.1038	41.65766	10.41441	12.9060	57.3015	.00	100.00
	GradeC	16	34.0625	39.17203	9.79301	13.1892	54.9358	.00	100.00
	Total	48	33.3333	37.61055	5.42861	22.4124	44.2543	.00	100.00
N_1200IU	GradeA	14	1.2857	.91387	.24424	.7581	1.8134	.00	3.00
	GradeB	14	.5714	.75593	.20203	.1350	1.0079	.00	2.00
	GradeC	14	.7143	.99449	.26579	.1401	1.2885	.00	3.00
	Total	42	.8571	.92582	.14286	.5686	1.1456	.00	3.00
N_1500IU	GradeA	16	.8750	1.02470	.25617	.3290	1.4210	.00	3.00
	GradeB	16	.6250	.61914	.15478	.2951	.9549	.00	2.00
	GradeC	16	.8125	1.04682	.26171	.2547	1.3703	.00	4.00
	Total	48	.7708	.90482	.13060	.5081	1.0336	.00	4.00

Appendix Table 2.18: Effect of oocyte quality and PMSG dosage on the cleavage rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_GradeA	PMSG 1200IU	11	77.2727	27.15504	8.18755	59.0297	95.5157	33.33	100.00
	PMSG 1500IU	12	55.5558	45.68992	13.18954	26.5258	84.5858	.00	100.00
	Total	23	65.9422	38.75560	8.08110	49.1830	82.7014	.00	100.00
P_GradeB	PMSG 1200IU	8	62.5000	44.32026	15.66958	25.4473	99.5527	.00	100.00
	PMSG 1500IU	10	63.3330	42.16397	13.33342	33.1707	93.4953	.00	100.00
	Total	18	62.9628	41.83531	9.86068	42.1586	83.7670	.00	100.00
P_GradeC	PMSG 1200IU	10	40.8330	40.90986	12.93683	11.5678	70.0982	.00	100.00
	PMSG 1500IU	17	43.7253	46.57559	11.29624	19.7783	67.6723	.00	100.00
	Total	27	42.6541	43.77556	8.42461	25.3370	59.9711	.00	100.00

Appendix Table 2.19: Effect of PMSG dosage on the developmental rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	PMSG 1200IU	15	65.0400	28.51207	7.36179	49.2505	80.8295	.00	100.00
	PMSG 1500IU	22	47.8682	37.77988	8.05470	31.1175	64.6188	.00	100.00
	Total	37	54.8297	34.95425	5.74644	43.1754	66.4841	.00	100.00
P_4cell	PMSG 1200IU	15	63.0400	29.90883	7.72243	46.4770	79.6030	.00	100.00
	PMSG 1500IU	22	36.5045	33.49974	7.14217	21.6516	51.3575	.00	100.00
	Total	37	47.2622	34.30683	5.64001	35.8237	58.7006	.00	100.00
P_8cell	PMSG 1200IU	15	58.5960	29.63925	7.65282	42.1823	75.0097	.00	100.00
	PMSG 1500IU	22	28.8855	31.15201	6.64163	15.0734	42.6975	.00	100.00
	Total	37	40.9303	33.56216	5.51759	29.7401	52.1205	.00	100.00
P_Morula	PMSG 1200IU	15	14.4047	21.72898	5.61040	2.3716	26.4378	.00	50.00
	PMSG 1500IU	22	16.0605	27.93873	5.95656	3.6731	28.4478	.00	100.00
	Total	37	15.3892	25.29084	4.15779	6.9568	23.8216	.00	100.00

Appendix Table 2.20: Effect of oocyte quality (Grade A) and PMSG dosage on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	PMSG 1200IU	11	77.2727	27.15504	8.18755	59.0297	95.5157	33.33	100.00
	PMSG 1500IU	12	55.5558	45.68992	13.18954	26.5258	84.5858	.00	100.00
	Total	23	65.9422	38.75560	8.08110	49.1830	82.7014	.00	100.00
P_4cell	PMSG 1200IU	11	75.0000	30.50537	9.19772	54.5062	95.4938	25.00	100.00
	PMSG 1500IU	12	55.5558	45.68992	13.18954	26.5258	84.5858	.00	100.00
	Total	23	64.8552	39.56517	8.24991	47.7460	81.9645	.00	100.00
P_8cell	PMSG 1200IU	11	65.9091	36.60148	11.03576	41.3199	90.4983	.00	100.00
	PMSG 1500IU	12	38.8892	44.57094	12.86652	10.5701	67.2082	.00	100.00
	Total	23	51.8117	42.34005	8.82851	33.5025	70.1209	.00	100.00
P_Morula	PMSG 1200IU	11	15.9091	32.15728	9.69579	-5.6945	37.5126	.00	100.00
	PMSG 1500IU	12	29.1667	40.28021	11.62789	3.5738	54.7595	.00	100.00
	Total	23	22.8261	36.42993	7.59617	7.0726	38.5796	.00	100.00

Appendix Table 2.21: Effect of oocyte quality (Grade B) and PMSG dosage on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	PMSG 1200IU	8	62.5000	44.32026	15.66958	25.4473	99.5527	.00	100.00
	PMSG 1500IU	10	63.3330	42.16397	13.33342	33.1707	93.4953	.00	100.00
	Total	18	62.9628	41.83531	9.86068	42.1586	83.7670	.00	100.00
P_4cell	PMSG 1200IU	8	62.5000	44.32026	15.66958	25.4473	99.5527	.00	100.00
	PMSG 1500IU	10	48.3330	47.43428	15.00004	14.4006	82.2654	.00	100.00
	Total	18	54.6294	45.30428	10.67832	32.1002	77.1587	.00	100.00
P_8cell	PMSG 1200IU	8	56.2500	49.55156	17.51912	14.8239	97.6761	.00	100.00
	PMSG 1500IU	10	38.3330	45.84600	14.49778	5.5367	71.1293	.00	100.00
	Total	18	46.2961	46.98620	11.07475	22.9304	69.6618	.00	100.00
P_Morula	PMSG 1200IU	8	25.0000	46.29100	16.36634	-13.7002	63.7002	.00	100.00
	PMSG 1500IU	10	23.3330	41.72210	13.19369	-6.5132	53.1792	.00	100.00
	Total	18	24.0739	42.48110	10.01289	2.9485	45.1992	.00	100.00

Appendix Table 2.22: Effect of oocyte quality (Grade C) and PMSG dosage on the cleavage and developmental rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	PMSG 1200IU	10	40.8330	40.90986	12.93683	11.5678	70.0982	.00	100.00
	PMSG 1500IU	17	43.7253	46.57559	11.29624	19.7783	67.6723	.00	100.00
	Total	27	42.6541	43.77556	8.42461	25.3370	59.9711	.00	100.00
P_4cell	PMSG 1200IU	10	30.8330	36.86183	11.65673	4.4636	57.2024	.00	100.00
	PMSG 1500IU	17	20.1959	35.59936	8.63411	1.8924	38.4994	.00	100.00
	Total	27	24.1356	35.74407	6.87895	9.9957	38.2754	.00	100.00
P_8cell	PMSG 1200IU	10	30.8330	36.86183	11.65673	4.4636	57.2024	.00	100.00
	PMSG 1500IU	17	16.0782	28.84899	6.99691	1.2455	30.9110	.00	100.00
	Total	27	21.5430	32.17506	6.19209	8.8149	34.2710	.00	100.00
P_Morula	PMSG 1200IU	10	5.0000	15.81139	5.00000	-6.3108	16.3108	.00	50.00
	PMSG 1500IU	17	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	27	1.8519	9.62250	1.85185	-1.9547	5.6584	.00	50.00

Appendix Table 2.23: Effect of maturation duration on oocyte maturation per ovary

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_GradeA	22-25h	35	36.2211	31.30926	5.29223	25.4660	46.9762	.00	100.00
	26-29h	34	30.6509	35.30676	6.05506	18.3318	42.9700	.00	100.00
	Total	69	33.4764	33.21078	3.99811	25.4983	41.4545	.00	100.00
P_GradeB	22-25h	35	30.5374	28.88313	4.88214	20.6157	40.4591	.00	100.00
	26-29h	34	23.8656	32.77275	5.62048	12.4306	35.3005	.00	100.00
	Total	69	27.2499	30.81621	3.70984	19.8470	34.6527	.00	100.00
P_GradeC	22-25h	35	33.2414	29.06954	4.91365	23.2557	43.2272	.00	100.00
	26-29h	34	39.1109	39.62929	6.79637	25.2836	52.9382	.00	100.00
	Total	69	36.1336	34.54564	4.15881	27.8349	44.4324	.00	100.00
P_GradeD	22-25h	35	.0000	.00000	.00000	.0000	.0000	.00	.00
	26-29h	34	2.9412	10.43691	1.78992	-.7004	6.5828	.00	50.00
	Total	69	1.4493	7.42001	.89326	-.3332	3.2318	.00	50.00
P_GradeE	22-25h	35	.0000	.00000	.00000	.0000	.0000	.00	.00
	26-29h	34	3.4315	17.30085	2.96707	-2.6051	9.4680	.00	100.00
	Total	69	1.6909	12.17556	1.46577	-1.2340	4.6158	.00	100.00
N_GradeA	22-25h	35	1.5714	1.53940	.26021	1.0426	2.1002	.00	7.00
	26-29h	34	.9412	1.17914	.20222	.5298	1.3526	.00	5.00
	Total	69	1.2609	1.40013	.16856	.9245	1.5972	.00	7.00
N_GradeB	22-25h	35	1.1714	1.01419	.17143	.8230	1.5198	.00	4.00
	26-29h	34	.6176	.88813	.15231	.3078	.9275	.00	3.00
	Total	69	.8986	.98735	.11886	.6614	1.1357	.00	4.00
N_GradeC	22-25h	35	1.4857	1.40108	.23683	1.0044	1.9670	.00	5.00
	26-29h	34	.9118	.83003	.14235	.6222	1.2014	.00	3.00
	Total	69	1.2029	1.18296	.14241	.9187	1.4871	.00	5.00
N_GradeD	22-25h	35	.0000	.00000	.00000	.0000	.0000	.00	.00
	26-29h	34	.1176	.40934	.07020	-.0252	.2605	.00	2.00
	Total	69	.0580	.29125	.03506	-.0120	.1279	.00	2.00
N_GradeE	22-25h	35	.0000	.00000	.00000	.0000	.0000	.00	.00
	26-29h	34	.0882	.37881	.06496	-.0439	.2204	.00	2.00
	Total	69	.0435	.26760	.03222	-.0208	.1078	.00	2.00

Appendix Table 2.24: Effect of oocyte quality and IVM duration on the maturation rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_GradeA	22-25h	26	90.5558	21.29374	4.17605	81.9550	99.1565	.00	100.00
	26-29h	24	59.0279	40.52180	8.27148	41.9171	76.1388	.00	100.00
	Total	50	75.4224	35.42948	5.01048	65.3535	85.4913	.00	100.00
P_GradeB	22-25h	24	91.3200	15.82560	3.23039	84.6374	98.0026	50.00	100.00
	26-29h	20	51.3335	39.96204	8.93578	32.6307	70.0363	.00	100.00
	Total	44	73.1443	35.28799	5.31987	62.4158	83.8728	.00	100.00
P_GradeC	22-25h	28	56.0121	29.29699	5.53661	44.6520	67.3723	.00	100.00
	26-29h	37	42.3424	40.36777	6.63642	28.8831	55.8017	.00	100.00
	Total	65	48.2309	36.40415	4.51538	39.2104	57.2514	.00	100.00
P_GradeD	22-25h	8	.0000	.00000	.00000	.0000	.0000	.00	.00
	26-29h	21	12.6986	32.44885	7.08092	-2.0720	27.4691	.00	100.00
	Total	29	9.1955	28.02596	5.20429	-1.4650	19.8560	.00	100.00
P_GradeE	22-25h	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	26-29h	15	6.6667	18.68749	4.82509	-3.6821	17.0155	.00	66.67
	Total	18	5.5556	17.15024	4.04235	-2.9731	14.0842	.00	66.67

Appendix Table 2.25: Effect of IVM duration on oocyte cleavage rate per ovary

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P22_25h	GradeA	23	46.8117	37.35571	7.78920	30.6579	62.9656	.00	100.00
	GradeB	23	26.5939	32.00577	6.67366	12.7536	40.4342	.00	100.00
	GradeC	23	26.5939	35.37855	7.37694	11.2951	41.8927	.00	100.00
	Total	69	33.3332	35.77704	4.30705	24.7386	41.9278	.00	100.00
P26_29h	GradeA	7	23.8100	30.21169	11.41894	-4.1312	51.7512	.00	66.67
	GradeB	7	42.8571	53.45225	20.20305	-6.5779	92.2922	.00	100.00
	GradeC	7	33.3329	37.26780	14.08590	-1.1341	67.7998	.00	100.00
	Total	21	33.3333	40.13879	8.75900	15.0624	51.6043	.00	100.00
N22_25h	GradeA	23	1.2174	.99802	.20810	.7858	1.6490	.00	3.00
	GradeB	23	.6522	.71406	.14889	.3434	.9610	.00	2.00
	GradeC	23	.8261	1.11405	.23230	.3443	1.3078	.00	4.00
	Total	69	.8986	.97234	.11706	.6650	1.1321	.00	4.00
N26_29h	GradeA	7	.5714	.78680	.29738	-.1562	1.2991	.00	2.00
	GradeB	7	.4286	.53452	.20203	-.0658	.9229	.00	1.00
	GradeC	7	.5714	.53452	.20203	.0771	1.0658	.00	1.00
	Total	21	.5238	.60159	.13128	.2500	.7976	.00	2.00

Appendix Table 2.26: Effect of oocyte quality and IVM duration on the cleavage rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_GradeA	22-25h	17	78.9218	30.35076	7.36114	63.3168	94.5267	.00	100.00
	26-29h	6	38.8883	49.06541	20.03087	-12.6027	90.3793	.00	100.00
	Total	23	68.4783	39.24474	8.18309	51.5076	85.4490	.00	100.00
P_GradeB	22-25h	13	75.6408	34.43720	9.55116	54.8306	96.4510	.00	100.00
	26-29h	5	50.0000	50.00000	22.36068	-12.0832	112.0832	.00	100.00
	Total	18	68.5183	39.56021	9.32443	48.8455	88.1912	.00	100.00
P_GradeC	22-25h	17	47.3529	41.05002	9.95609	26.2470	68.4589	.00	100.00
	26-29h	10	40.0000	51.63978	16.32993	3.0591	76.9409	.00	100.00
	Total	27	44.6296	44.42023	8.54868	27.0576	62.2017	.00	100.00

Appendix Table 2.27: Effect of IVM duration on the developmental rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	22-25h	23	70.0617	24.85764	5.18318	59.3125	80.8110	25.00	100.00
	26-29h	14	35.7143	41.78575	11.16771	11.5879	59.8407	.00	100.00
	Total	37	57.0654	35.96274	5.91224	45.0748	69.0560	.00	100.00
P_4cell	22-25h	23	66.5835	25.51779	5.32083	55.5488	77.6182	25.00	100.00
	26-29h	14	21.4286	32.96526	8.81034	2.3950	40.4621	.00	100.00
	Total	37	49.4978	35.82255	5.88919	37.5540	61.4417	.00	100.00
P_8cell	22-25h	23	59.3996	25.25095	5.26519	48.4802	70.3189	16.67	100.00
	26-29h	14	15.4764	32.33432	8.64171	-3.1929	34.1457	.00	100.00
	Total	37	42.7800	35.12227	5.77407	31.0697	54.4903	.00	100.00
P_Morula	22-25h	23	24.5496	28.31066	5.90318	12.3071	36.7920	.00	100.00
	26-29h	14	2.3807	8.90782	2.38071	-2.7625	7.5239	.00	33.33
	Total	37	16.1614	25.24403	4.15009	7.7446	24.5781	.00	100.00

Appendix Table 2.28: Effect of oocyte quality (Grade A) and IVM duration on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	22-25h	17	78.9218	30.35076	7.36114	63.3168	94.5267	.00	100.00
	26-29h	6	38.8883	49.06541	20.03087	-12.6027	90.3793	.00	100.00
	Total	23	68.4783	39.24474	8.18309	51.5076	85.4490	.00	100.00
P_4cell	22-25h	17	77.4512	32.37768	7.85274	60.8041	94.0982	.00	100.00
	26-29h	6	38.8883	49.06541	20.03087	-12.6027	90.3793	.00	100.00
	Total	23	67.3913	40.11633	8.36483	50.0437	84.7389	.00	100.00
P_8cell	22-25h	17	65.6865	39.84388	9.66356	45.2006	86.1723	.00	100.00
	26-29h	6	22.2217	40.36849	16.48037	-20.1425	64.5858	.00	100.00
	Total	23	54.3478	43.65504	9.10270	35.4700	73.2257	.00	100.00
P_Morula	22-25h	17	27.9412	39.85028	9.66511	7.4521	48.4303	.00	100.00
	26-29h	6	8.3333	20.41241	8.33333	-13.0882	29.7548	.00	50.00
	Total	23	22.8261	36.42993	7.59617	7.0726	38.5796	.00	100.00

Appendix Table 2.29: Effect of oocyte quality (Grade B) and IVM duration on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	22-25h	13	75.6408	34.43720	9.55116	54.8306	96.4510	.00	100.00
	26-29h	5	50.0000	50.00000	22.36068	-12.0832	112.0832	.00	100.00
	Total	18	68.5183	39.56021	9.32443	48.8455	88.1912	.00	100.00
P_4cell	22-25h	13	67.9485	39.35943	10.91634	44.1638	91.7331	.00	100.00
	26-29h	5	40.0000	54.77226	24.49490	-28.0087	108.0087	.00	100.00
	Total	18	60.1850	44.33208	10.44917	38.1392	82.2308	.00	100.00
P_8cell	22-25h	13	64.1023	43.48612	12.06088	37.8239	90.3807	.00	100.00
	26-29h	5	20.0000	44.72136	20.00000	-35.5289	75.5289	.00	100.00
	Total	18	51.8517	47.10200	11.10205	28.4284	75.2749	.00	100.00
P_Morula	22-25h	13	41.0254	49.35485	13.68857	11.2005	70.8502	.00	100.00
	26-29h	5	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	18	29.6294	45.57387	10.74186	6.9661	52.2928	.00	100.00

Appendix Table 2.30: Effect of oocyte quality (Grade C) and IVM duration on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	22-25h	17	47.3529	41.05002	9.95609	26.2470	68.4589	.00	100.00
	26-29h	10	40.0000	51.63978	16.32993	3.0591	76.9409	.00	100.00
	Total	27	44.6296	44.42023	8.54868	27.0576	62.2017	.00	100.00
P_4cell	22-25h	17	35.5882	38.36494	9.30486	15.8628	55.3137	.00	100.00
	26-29h	10	10.0000	31.62278	10.00000	-12.6216	32.6216	.00	100.00
	Total	27	26.1111	37.55634	7.22772	11.2543	40.9679	.00	100.00
P_8cell	22-25h	17	30.2941	32.76746	7.94728	13.4466	47.1416	.00	100.00
	26-29h	10	10.0000	31.62278	10.00000	-12.6216	32.6216	.00	100.00
	Total	27	22.7778	33.26609	6.40206	9.6182	35.9374	.00	100.00
P_Morula	22-25h	17	2.9412	12.12678	2.94118	-3.2938	9.1762	.00	50.00
	26-29h	10	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	27	1.8519	9.62250	1.85185	-1.9547	5.6584	.00	50.00

Appendix Table 2.31: Effect of different activation regimes after sham injection on oocyte cleavage per ovary

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_SCalcium	GradeA	6	63.8883	42.70966	17.43614	19.0673	108.7094	.00	100.00
	GradeB	6	13.8883	22.15208	9.04355	-9.3589	37.1355	.00	50.00
	GradeC	6	22.2217	40.36849	16.48037	-20.1425	64.5858	.00	100.00
	Total	18	33.3328	40.82483	9.62250	13.0311	53.6345	.00	100.00
P_SDMAP	GradeA	7	32.6529	40.00579	15.12077	-4.3463	69.6520	.00	100.00
	GradeB	7	21.9386	36.67910	13.86340	-11.9839	55.8611	.00	100.00
	GradeC	7	45.4086	41.88956	15.83277	6.6672	84.1500	.00	100.00
	Total	21	33.3333	38.81761	8.47070	15.6638	51.0029	.00	100.00
N_SCalcium	GradeA	6	.8333	.40825	.16667	.4049	1.2618	.00	1.00
	GradeB	6	.3333	.51640	.21082	-.2086	.8753	.00	1.00
	GradeC	6	.3333	.51640	.21082	-.2086	.8753	.00	1.00
	Total	18	.5000	.51450	.12127	.2441	.7559	.00	1.00
N_SDMAP	GradeA	7	1.1429	1.21499	.45922	.0192	2.2665	.00	3.00
	GradeB	7	.5714	.78680	.29738	-.1562	1.2991	.00	2.00
	GradeC	7	1.1429	1.06904	.40406	.1542	2.1316	.00	3.00
	Total	21	.9524	1.02353	.22335	.4865	1.4183	.00	3.00

Appendix Table 2.32: Effect of oocyte quality and different activation regimes after sham injection on the cleavage rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_GradeA	SCalcium	8	41.6663	41.78564	14.77345	6.7326	76.5999	.00	100.00
	SDMAP	6	48.0950	45.90745	18.74164	-.0819	96.2719	.00	100.00
	Total	14	44.4214	41.97201	11.21749	20.1875	68.6553	.00	100.00
P_GradeB	SCalcium	8	12.5000	23.14550	8.18317	-6.8501	31.8501	.00	50.00
	SDMAP	5	50.0000	50.00000	22.36068	-12.0832	112.0832	.00	100.00
	Total	13	26.9231	38.81250	10.76465	3.4689	50.3772	.00	100.00
P_GradeC	SCalcium	6	22.2217	40.36849	16.48037	-20.1425	64.5858	.00	100.00
	SDMAP	7	51.1900	42.60864	16.10455	11.7836	90.5964	.00	100.00
	Total	13	37.8200	42.57562	11.80835	12.0918	63.5482	.00	100.00

Appendix Table 2.33: Effect of different activation regimes after sham injection on the developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	SControl	12	.0000	.00000	.00000	.0000	.0000	.00	.00
	SCalcium	11	17.9227	22.05333	6.64933	3.1071	32.7384	.00	66.67
	SDMAP	8	42.8125	31.22212	11.03869	16.7102	68.9148	.00	87.50
	Total	31	17.4081	26.13409	4.69382	7.8220	26.9941	.00	87.50
P_4cell	SControl	12	.0000	.00000	.00000	.0000	.0000	.00	.00
	SCalcium	11	11.8618	15.50594	4.67522	1.4448	22.2789	.00	42.86
	SDMAP	8	40.3125	28.50124	10.07671	16.4849	64.1401	.00	87.50
	Total	31	14.6123	23.10847	4.15040	6.1360	23.0885	.00	87.50
P_8cell	SControl	12	.0000	.00000	.00000	.0000	.0000	.00	.00
	SCalcium	11	5.7145	13.70315	4.13166	-3.4914	14.9204	.00	42.86
	SDMAP	8	25.8338	27.75744	9.81374	2.6280	49.0395	.00	66.67
	Total	31	8.6945	18.82014	3.38020	1.7912	15.5978	.00	66.67
P_Morula	SControl	12	.0000	.00000	.00000	.0000	.0000	.00	.00
	SCalcium	11	1.2991	4.30860	1.29909	-1.5955	4.1936	.00	14.29
	SDMAP	8	5.2088	9.89834	3.49959	-3.0665	13.4840	.00	25.00
	Total	31	1.8052	5.79102	1.04010	-.3190	3.9293	.00	25.00

Appendix Table 2.34: Effect of oocyte quality (Grade A) and different activation regimes after sham injection on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	SCalcium	8	41.6663	41.78564	14.77345	6.7326	76.5999	.00	100.00
	SDMAP	6	48.0950	45.90745	18.74164	-.0819	96.2719	.00	100.00
	Total	14	44.4214	41.97201	11.21749	20.1875	68.6553	.00	100.00
P_4cell	SCalcium	8	29.1662	36.46040	12.89070	-1.3154	59.6479	.00	100.00
	SDMAP	6	48.0950	45.90745	18.74164	-.0819	96.2719	.00	100.00
	Total	14	37.2786	40.26016	10.75998	14.0330	60.5241	.00	100.00
P_8cell	SCalcium	8	10.4163	19.79503	6.99860	-6.1328	26.9653	.00	50.00
	SDMAP	6	45.7150	47.46585	19.37785	-4.0974	95.5274	.00	100.00
	Total	14	25.5443	37.49866	10.02194	3.8932	47.1954	.00	100.00
P_Morula	SCalcium	8	6.2500	17.67767	6.25000	-8.5289	21.0289	.00	50.00
	SDMAP	6	20.0000	40.00000	16.32993	-21.9774	61.9774	.00	100.00
	Total	14	12.1429	28.87069	7.71602	-4.5266	28.8123	.00	100.00

Appendix Table 2.35: Effect of oocyte quality (Grade B) and different activation regimes after sham injection on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	SCalcium	8	12.5000	23.14550	8.18317	-6.8501	31.8501	.00	50.00
	SDMAP	5	50.0000	50.00000	22.36068	-12.0832	112.0832	.00	100.00
	Total	13	26.9231	38.81250	10.76465	3.4689	50.3772	.00	100.00
P_4cell	SCalcium	8	12.5000	23.14550	8.18317	-6.8501	31.8501	.00	50.00
	SDMAP	5	50.0000	50.00000	22.36068	-12.0832	112.0832	.00	100.00
	Total	13	26.9231	38.81250	10.76465	3.4689	50.3772	.00	100.00
P_8cell	SCalcium	8	6.2500	17.67767	6.25000	-8.5289	21.0289	.00	50.00
	SDMAP	5	30.0000	44.72136	20.00000	-25.5289	85.5289	.00	100.00
	Total	13	15.3846	31.52126	8.74242	-3.6635	34.4327	.00	100.00
P_Morula	SCalcium	8	.0000	.00000	.00000	.0000	.0000	.00	.00
	SDMAP	5	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	13	.0000	.00000	.00000	.0000	.0000	.00	.00

Appendix Table 2.36: Effect of oocyte quality (Grade C) and different activation regimes after sham injection on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	SCalcium	6	22.2217	40.36849	16.48037	-20.1425	64.5858	.00	100.00
	SDMAP	7	51.1900	42.60864	16.10455	11.7836	90.5964	.00	100.00
	Total	13	37.8200	42.57562	11.80835	12.0918	63.5482	.00	100.00
P_4cell	SCalcium	6	5.5550	13.60692	5.55500	-8.7246	19.8346	.00	33.33
	SDMAP	7	44.0471	36.86650	13.93423	9.9513	78.1430	.00	100.00
	Total	13	26.2815	33.99440	9.42835	5.7389	46.8241	.00	100.00
P_8cell	SCalcium	6	5.5550	13.60692	5.55500	-8.7246	19.8346	.00	33.33
	SDMAP	7	22.6186	36.86618	13.93411	-11.4770	56.7141	.00	100.00
	Total	13	14.7431	28.89798	8.01486	-2.7198	32.2060	.00	100.00
P_Morula	SCalcium	6	.0000	.00000	.00000	.0000	.0000	.00	.00
	SDMAP	7	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	13	.0000	.00000	.00000	.0000	.0000	.00	.00

Appendix Table 2.37: Effect of different activation regimes after ICSI on oocyte cleavage per ovary

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_IControl	GradeA	5	20.0000	27.38613	12.24745	-14.0044	54.0044	.00	50.00
	GradeB	5	43.3340	43.46180	19.43671	-10.6309	97.2989	.00	100.00
	GradeC	5	36.6660	41.49973	18.55924	-14.8627	88.1947	.00	100.00
	Total	15	33.3333	36.73176	9.48410	12.9920	53.6747	.00	100.00
P_ICalcium	GradeA	11	51.5155	39.05461	11.77541	25.2782	77.7527	.00	100.00
	GradeB	11	22.7273	34.37758	10.36523	-.3679	45.8224	.00	100.00
	GradeC	11	25.7573	40.38946	12.17788	-1.3767	52.8913	.00	100.00
	Total	33	33.3333	39.08689	6.80415	19.4737	47.1929	.00	100.00
P_IDMAP	GradeA	14	41.1907	36.52946	9.76291	20.0992	62.2822	.00	100.00
	GradeB	14	31.7850	39.43218	10.53869	9.0175	54.5525	.00	100.00
	GradeC	14	27.0236	30.96877	8.27675	9.1427	44.9044	.00	100.00
	Total	42	33.3331	35.43596	5.46789	22.2905	44.3757	.00	100.00
N_IControl	GradeA	5	.6000	.89443	.40000	-.5106	1.7106	.00	2.00
	GradeB	5	.8000	.83666	.37417	-.2389	1.8389	.00	2.00
	GradeC	5	1.0000	1.00000	.44721	-.2417	2.2417	.00	2.00
	Total	15	.8000	.86189	.22254	.3227	1.2773	.00	2.00
N_ICalcium	GradeA	11	.9091	.70065	.21125	.4384	1.3798	.00	2.00
	GradeB	11	.3636	.50452	.15212	.0247	.7026	.00	1.00
	GradeC	11	.3636	.50452	.15212	.0247	.7026	.00	1.00
	Total	33	.5455	.61699	.10740	.3267	.7642	.00	2.00
N_IDMAP	GradeA	14	1.3571	1.15073	.30755	.6927	2.0216	.00	3.00
	GradeB	14	.7143	.72627	.19410	.2949	1.1336	.00	2.00
	GradeC	14	1.0000	1.24035	.33150	.2838	1.7162	.00	4.00
	Total	42	1.0238	1.07040	.16517	.6902	1.3574	.00	4.00

Appendix Table 2.38: Effect of oocyte quality and different activation regimes after ICSI on the cleavage rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
IControl	GradeA	2	41.6650	11.78747	8.33500	-64.2412	147.5712	33.33	50.00
	GradeB	5	30.0000	27.38613	12.24745	-4.0044	64.0044	.00	50.00
	GradeC	7	35.7143	47.55949	17.97580	-8.2709	79.6995	.00	100.00
	Total	14	34.5236	36.08176	9.64326	13.6906	55.3566	.00	100.00
ICalcium	GradeA	11	65.1518	45.00282	13.56886	34.9185	95.3851	.00	100.00
	GradeB	5	56.6660	43.46180	19.43671	2.7011	110.6309	.00	100.00
	GradeC	9	31.4811	42.85345	14.28448	-1.4590	64.4212	.00	100.00
	Total	25	51.3332	44.85589	8.97118	32.8176	69.8488	.00	100.00
IDMAP	GradeA	10	77.5000	35.14775	11.11469	52.3568	102.6432	.00	100.00
	GradeB	8	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00
	GradeC	11	61.0609	42.37583	12.77679	32.5924	89.5294	.00	100.00
	Total	29	77.4714	35.90565	6.66751	63.8136	91.1292	.00	100.00

Appendix Table 2.39: Effect of different activation regimes after ICSI on the developmental rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	IControl	7	38.0943	34.79357	13.15073	5.9156	70.2730	.00	100.00
	ICalcium	14	48.0957	33.86715	9.05138	28.5414	67.6500	.00	100.00
	IDMAP	16	73.2138	33.23365	8.30841	55.5048	90.9227	.00	100.00
	Total	37	57.0654	35.96274	5.91224	45.0748	69.0560	.00	100.00
P_4cell	IControl	7	29.0471	35.83465	13.54423	-4.0944	62.1887	.00	100.00
	ICalcium	14	45.7143	33.63196	8.98852	26.2958	65.1328	.00	100.00
	IDMAP	16	61.7556	34.88644	8.72161	43.1660	80.3453	.00	100.00
	Total	37	49.4978	35.82255	5.88919	37.5540	61.4417	.00	100.00
P_8cell	IControl	7	19.5243	20.85467	7.88233	.2369	38.8116	.00	50.00
	ICalcium	14	38.3336	32.31836	8.63744	19.6735	56.9936	.00	100.00
	IDMAP	16	56.8450	37.41050	9.35263	36.9104	76.7796	.00	100.00
	Total	37	42.7800	35.12227	5.77407	31.0697	54.4903	.00	100.00
P_Morula	IControl	7	.0000	.00000	.00000	.0000	.0000	.00	.00
	ICalcium	14	8.5714	18.33750	4.90090	-2.0163	19.1592	.00	50.00
	IDMAP	16	29.8731	29.35637	7.33909	14.2302	45.5160	.00	100.00
	Total	37	16.1614	25.24403	4.15009	7.7446	24.5781	.00	100.00

Appendix Table 2.40: Effect of oocyte quality (Grade A) and different activation regimes after ICSI on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	IControl	2	41.6650	11.78747	8.33500	-64.2412	147.5712	33.33	50.00
	ICalcium	11	65.1518	45.00282	13.56886	34.9185	95.3851	.00	100.00
	IDMAP	10	77.5000	35.14775	11.11469	52.3568	102.6432	.00	100.00
	Total	23	68.4783	39.24474	8.18309	51.5076	85.4490	.00	100.00
P_4cell	IControl	2	29.1650	5.89020	4.16500	-23.7563	82.0863	25.00	33.33
	ICalcium	11	65.1518	45.00282	13.56886	34.9185	95.3851	.00	100.00
	IDMAP	10	77.5000	35.14775	11.11469	52.3568	102.6432	.00	100.00
	Total	23	67.3913	40.11633	8.36483	50.0437	84.7389	.00	100.00
P_8cell	IControl	2	29.1650	5.89020	4.16500	-23.7563	82.0863	25.00	33.33
	ICalcium	11	37.8791	46.00197	13.87011	6.9746	68.7836	.00	100.00
	IDMAP	10	77.5000	35.14775	11.11469	52.3568	102.6432	.00	100.00
	Total	23	54.3478	43.65504	9.10270	35.4700	73.2257	.00	100.00
P_Morula	IControl	2	.0000	.00000	.00000	.0000	.0000	.00	.00
	ICalcium	11	9.0909	30.15113	9.09091	-11.1649	29.3467	.00	100.00
	IDMAP	10	42.5000	38.17835	12.07305	15.1889	69.8111	.00	100.00
	Total	23	22.8261	36.42993	7.59617	7.0726	38.5796	.00	100.00

Appendix Table 2.41: Effect of oocyte quality (Grade B) and different activation regimes after ICSI on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	IControl	5	30.0000	27.38613	12.24745	-4.0044	64.0044	.00	50.00
	ICalcium	5	56.6660	43.46180	19.43671	2.7011	110.6309	.00	100.00
	IDMAP	8	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00
	Total	18	68.5183	39.56021	9.32443	48.8455	88.1912	.00	100.00
P_4cell	IControl	5	20.0000	27.38613	12.24745	-14.0044	54.0044	.00	50.00
	ICalcium	5	56.6660	43.46180	19.43671	2.7011	110.6309	.00	100.00
	IDMAP	8	87.5000	35.35534	12.50000	57.9422	117.0578	.00	100.00
	Total	18	60.1850	44.33208	10.44917	38.1392	82.2308	.00	100.00
P_8cell	IControl	5	10.0000	22.36068	10.00000	-17.7645	37.7645	.00	50.00
	ICalcium	5	56.6660	43.46180	19.43671	2.7011	110.6309	.00	100.00
	IDMAP	8	75.0000	46.29100	16.36634	36.2998	113.7002	.00	100.00
	Total	18	51.8517	47.10200	11.10205	28.4284	75.2749	.00	100.00
P_Morula	IControl	5	.0000	.00000	.00000	.0000	.0000	.00	.00
	ICalcium	5	26.6660	43.46122	19.43645	-27.2982	80.6302	.00	100.00
	IDMAP	8	50.0000	53.45225	18.89822	5.3128	94.6872	.00	100.00
	Total	18	29.6294	45.57387	10.74186	6.9661	52.2928	.00	100.00

Appendix Table 2.42: Effect of oocyte quality (Grade C) and different activation regimes after ICSI on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	IControl	7	35.7143	47.55949	17.97580	-8.2709	79.6995	.00	100.00
	ICalcium	9	31.4811	42.85345	14.28448	-1.4590	64.4212	.00	100.00
	IDMAP	11	61.0609	42.37583	12.77679	32.5924	89.5294	.00	100.00
	Total	27	44.6296	44.42023	8.54868	27.0576	62.2017	.00	100.00
P_4cell	IControl	7	21.4286	39.33979	14.86904	-14.9547	57.8118	.00	100.00
	ICalcium	9	20.3700	35.13626	11.71209	-6.6381	47.3781	.00	100.00
	IDMAP	11	33.7882	40.51152	12.21468	6.5722	61.0042	.00	100.00
	Total	27	26.1111	37.55634	7.22772	11.2543	40.9679	.00	100.00
P_8cell	IControl	7	14.2857	24.39750	9.22139	-8.2782	36.8496	.00	50.00
	ICalcium	9	20.3700	35.13626	11.71209	-6.6381	47.3781	.00	100.00
	IDMAP	11	30.1518	37.64231	11.34958	4.8634	55.4403	.00	100.00
	Total	27	22.7778	33.26609	6.40206	9.6182	35.9374	.00	100.00
P_Morula	IControl	7	.0000	.00000	.00000	.0000	.0000	.00	.00
	ICalcium	9	.0000	.00000	.00000	.0000	.0000	.00	.00
	IDMAP	11	4.5455	15.07557	4.54545	-5.5824	14.6734	.00	50.00
	Total	27	1.8519	9.62250	1.85185	-1.9547	5.6584	.00	50.00

Appendix Table 2.43: Effect of control activation regime after sham injection and ICSI on the developmental rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	SControl	12	.0000	.00000	.00000	.0000	.0000	.00	.00
	IControl	7	38.0943	34.79357	13.15073	5.9156	70.2730	.00	100.00
	Total	19	14.0347	27.56737	6.32439	.7477	27.3218	.00	100.00
P_4cell	SControl	12	.0000	.00000	.00000	.0000	.0000	.00	.00
	IControl	7	29.0471	35.83465	13.54423	-4.0944	62.1887	.00	100.00
	Total	19	10.7016	25.20465	5.78234	-1.4467	22.8498	.00	100.00
P_8cell	SControl	12	.0000	.00000	.00000	.0000	.0000	.00	.00
	IControl	7	19.5243	20.85467	7.88233	.2369	38.8116	.00	50.00
	Total	19	7.1932	15.44668	3.54371	-.2519	14.6382	.00	50.00
P_Morula	SControl	12	.0000	.00000	.00000	.0000	.0000	.00	.00
	IControl	7	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	19	.0000	.00000	.00000	.0000	.0000	.00	.00

Appendix Table 2.44: Effect of calcium ionophore activation regime after sham injection and ICSI on the developmental rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	SCalcium	11	17.9227	22.05333	6.64933	3.1071	32.7384	.00	66.67
	ICalcium	14	48.0957	33.86715	9.05138	28.5414	67.6500	.00	100.00
	Total	25	34.8196	32.52076	6.50415	21.3957	48.2435	.00	100.00
P_4cell	SCalcium	11	11.8618	15.50594	4.67522	1.4448	22.2789	.00	42.86
	ICalcium	14	45.7143	33.63196	8.98852	26.2958	65.1328	.00	100.00
	Total	25	30.8192	31.73330	6.34666	17.7203	43.9181	.00	100.00
P_8cell	SCalcium	11	5.7145	13.70315	4.13166	-3.4914	14.9204	.00	42.86
	ICalcium	14	38.3336	32.31836	8.63744	19.6735	56.9936	.00	100.00
	Total	25	23.9812	30.28352	6.05670	11.4808	36.4816	.00	100.00
P_Morula	SCalcium	11	1.2991	4.30860	1.29909	-1.5955	4.1936	.00	14.29
	ICalcium	14	8.5714	18.33750	4.90090	-2.0163	19.1592	.00	50.00
	Total	25	5.3716	14.26367	2.85273	-.5162	11.2594	.00	50.00

Appendix Table 2.45: Effect of calcium ionophore and 6-DMAP activation regime after sham injection and ICSI on the developmental rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	SDMAP	8	42.8125	31.22212	11.03869	16.7102	68.9148	.00	87.50
	IDMAP	16	73.2138	33.23365	8.30841	55.5048	90.9227	.00	100.00
	Total	24	63.0800	35.09003	7.16272	48.2628	77.8972	.00	100.00
P_4cell	SDMAP	8	40.3125	28.50124	10.07671	16.4849	64.1401	.00	87.50
	IDMAP	16	61.7556	34.88644	8.72161	43.1660	80.3453	.00	100.00
	Total	24	54.6079	33.87606	6.91492	40.3033	68.9125	.00	100.00
P_8cell	SDMAP	8	25.8338	27.75744	9.81374	2.6280	49.0395	.00	66.67
	IDMAP	16	56.8450	37.41050	9.35263	36.9104	76.7796	.00	100.00
	Total	24	46.5079	37.01678	7.55602	30.8771	62.1387	.00	100.00
P_Morula	SDMAP	8	5.2088	9.89834	3.49959	-3.0665	13.4840	.00	25.00
	IDMAP	16	29.8731	29.35637	7.33909	14.2302	45.5160	.00	100.00
	Total	24	21.6517	27.07255	5.52616	10.2199	33.0834	.00	100.00

APPENDIX 3: SUPPLEMENTARY RESULTS

Appendix Table 3.1: Percentage (%, mean±SEM) of oocytes matured from goat genotypes

Genotypes	No. of ovaries	No. of oocytes matured per ovary	No. of oocytes matured per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
Jamnapari	6	2.00±0.26[a] (n=12)	55.56±20.49[az] (n=6)	5.56±5.56[ay] (n=1)	38.88±20.03[ayz] (n=5)	0.00±0.00[ay] (n=0)	0.00±0.00[ay] (n=0)
Boer crossbred	63	3.60±0.28[a] (n=227)	31.37±3.89[az] (n=81)	29.32±3.94[az] (n=61)	35.87±4.21[az] (n=78)	1.59±0.98[ay] (n=4)	1.85±1.61[ay] (n=3)
Average	34.50 (n=69)	3.46±0.26 (n=239)	33.48±4.00[z] (n=87)	27.25±3.71[z] (n=62)	36.13±4.16[z] (n=83)	1.45±0.89[y] (n=4)	1.69±1.47[y] (n=3)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.2: Number (n, mean±SEM) of oocytes matured from goat genotypes

Genotypes	No. of ovaries	No. of oocytes matured per ovary	Percent of oocytes matured per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
Jamnapari	6	2.00±0.26[a] (n=12)	1.00±0.37[az] (n=6)	0.17±0.17[axy] (n=1)	0.83±0.40[ayz] (n=5)	0.00±0.00[ax] (n=0)	0.00±0.00[ax] (n=0)
Boer crossbred	63	3.60±0.28[a] (n=227)	1.29±0.18[az] (n=81)	0.97±0.13[az] (n=61)	1.24±0.15[az] (n=78)	0.06±0.04[ay] (n=4)	0.05±0.04[ay] (n=3)
Average	34.50 (n=69)	3.46±0.26 (n=239)	1.26±0.17[z] (n=87)	0.90±0.12[y] (n=62)	1.20±0.14[yz] (n=83)	0.06±0.04[x] (n=4)	0.04±0.03[x] (n=3)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[xyz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.3: Maturation rate (%, mean±SEM) of different grades of oocyte at two different goat genotypes

Genotypes	Percent MII oocytes*	Percent maturation**				
		Grade A	Grade B	Grade C	Grade D	Grade E
Jamnapari	20.35±7.86^{a} (12/43)***	73.33±19.44^{az} (6/9)	12.50±12.50^{ay} (1/5)	28.57±14.87^{ay} (5/14)	0.00±0.00^{ay} (0/7)	0.00±0.00^{ay} (0/8)
Boer crossbred	57.39±3.38^{b} (227/377)	75.65±5.22^{az} (81/103)	79.21±4.77^{bz} (61/78)	50.60±4.68^{ay} (78/144)	11.11±6.24^{ax} (4/30)	7.14±5.16^{ax} (3/22)
Average	52.10±3.40 (239/420)	75.42±5.01^{z} (87/112)	73.14±5.32^{z} (62/83)	48.23±4.52^{y} (83/158)	9.20±5.20^{x} (4/37)	5.56±4.04^{x} (3/30)

* Mean percentage of MII oocytes was based on total oocytes from all grades used for IVM, in the respective goat genotypes groups
** Mean percentage of maturation rate was based on oocytes used for IVM, in the respective grades
*** Mean total of matured oocytes per total of oocytes used for IVM
^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.4: Developmental rate (%, mean±SEM) of different grades of oocyte at Boer goat genotype

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	2.15±0.22^{a} (n=43)	73.75±7.77^{b} (n=31)	72.50±8.07^{bz} (n=30)	62.50±9.14^{bz} (n=28)	26.25±8.49^{by} (n=12)
Grade B	1.65±0.21^{a} (n=28)	72.55±8.92^{b} (n=18)	63.73±10.43^{bz} (n=16)	54.90±11.32^{bz} (n=14)	31.37±11.24^{bz} (n=8)
Grade C	1.80±0.23^{a} (n=45)	44.20±8.78^{a} (n=22)	28.20±7.66^{az} (n=18)	24.60±6.79^{az} (n=15)	2.00±2.00^{ay} (n=2)
Average	1.87±0.13 (n=116)	61.51±5.23 (n=71)	52.23±5.50^{z} (n=64)	45.13±5.45^{z} (n=57)	17.88±4.45^{y} (n=22)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades
^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{yz} Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.5: Percentage (%, mean±SEM) of oocytes matured from different body weight groups

Body weight*	No. of ovaries	No. of oocytes matured per ovary	Percent of oocytes matured per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
Light	18	4.44±0.58^{b} (n=80)	30.38±5.91^{ayz} (n=29)	25.11±6.64^{aby} (n=19)	41.73±6.51^{abz} (n=30)	2.78±2.78^{ax} (n=2)	0.00±0.00^{ax} (n=0)
Medium	16	3.75±0.62^{b} (n=60)	40.06±10.11^{az} (n=22)	37.22±8.83^{bz} (n=19)	22.72±6.30^{az} (n=19)	0.00±0.00^{ay} (n=0)	0.00±0.00^{ay} (n=0)
Heavy	6	1.67±0.21^{a} (n=10)	16.67±16.67^{ayz} (n=1)	8.33±8.33^{ay} (n=1)	58.33±20.07^{bz} (n=6)	0.00±0.00^{ay} (n=0)	16.67±16.67^{byz} (n=2)
Average	13.33 (n=40)	3.75±0.39 (n=150)	32.20±5.43^{z} (n=52)	27.43±4.94^{z} (n=39)	36.62±5.12^{z} (n=55)	1.25±1.25^{y} (n=2)	2.50±2.50^{y} (n=2)

*Body weight: light (<20 kg), medium (20-29 kg) and heavy (>29 kg)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)

^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.6: Number (n, mean±SEM) of oocytes matured from different body weight groups

Body weight*	No. of ovaries	No. of oocytes matured per ovary	No. of oocytes matured per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
Light	18	4.44±0.58^{b} (n=80)	1.61±0.37^{bz} (n=29)	1.06±0.25^{abz} (n=19)	1.67±0.28^{az} (n=30)	0.11±0.11^{ay} (n=2)	0.00±0.00^{ay} (n=0)
Medium	16	3.75±0.62^{b} (n=60)	1.38±0.44^{abz} (n=22)	1.19±0.28^{bz} (n=19)	1.19±0.39^{az} (n=19)	0.00±0.00^{ay} (n=0)	0.00±0.00^{ay} (n=0)
Heavy	6	1.67±0.21^{a} (n=10)	0.17±0.17^{ay} (n=1)	0.17±0.17^{ay} (n=1)	1.00±0.37^{az} (n=6)	0.00±0.00^{ay} (n=0)	0.33±0.33^{byz} (n=2)
Average	13.33 (n=40)	3.75±0.39 (n=150)	1.30±0.25^{z} (n=52)	0.98±0.17^{z} (n=39)	1.38±0.21^{z} (n=55)	0.05±0.05^{y} (n=2)	0.05±0.05^{y} (n=2)

*Body weight: light (<20 kg), medium (20-29 kg) and heavy (>29 kg)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)

^{yz} Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.7: Maturation rate (%, mean±SEM) of different grades of oocyte at three body weight groups

Body weight*	Percent MII oocytes**	Percent maturation***				
		Grade A	Grade B	Grade C	Grade D	Grade E
Light	52.80±5.48[a] (80/137) ****	66.77±9.88[az] (29/39)	64.58±10.26[az] (19/27)	50.50±7.64[az] (30/57)	8.33±8.33[ay] (2/12)	0.00±0.00[ay] (0/2)
Medium	50.52±8.19[a] (60/108)	77.32±11.29[az] (22/28)	72.62±11.27[az] (19/24)	45.71±10.68[az] (19/35)	0.00±0.00[ay] (0/10)	0.00±0.00[ay] (0/11)
Heavy	33.57±8.41[a] (10/27)	50.00±50.00[az] (1/2)	50.00 (1/2)	63.33±18.56[az] (6/10)	0.00±0.00[az] (0/7)	22.22±22.22[az] (2/6)
Average	48.83±4.46 (150/272)	69.77±7.33[z] (52/69)	68.21±7.34[z] (39/53)	50.43±5.86[z] (55/102)	3.18±3.18[y] (2/29)	6.67±6.67[y] (2/19)

*Body weight: light (<20 kg), medium (20-29 kg) and heavy (>29 kg)
**Mean percentage of MII oocytes was based on total oocytes from all grades used for IVM, in the respective body weight groups
*** Mean percentage of maturation rate was based on oocytes used for IVM, in the respective grades
**** Mean total of matured oocytes per total of oocytes used for IVM
[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.8: Percentage (%, mean±SEM) of oocytes cleaved from different body weight groups

Body weight	No. of oocytes injected per ovary	No. of oocytes cleaved per ovary	Percent of oocytes cleaved per ovary		
			Grade A	Grade B	Grade C
Light	4.44±1.03[a] (n=40)	2.50±0.63[a] (n=20)	58.33±11.36[az] (n=11)	12.50±8.18[ay] (n=2)	29.17±12.89[ayz] (n=7)
Medium	3.40±0.70[a] (n=34)	2.88±0.72[a] (n=23)	29.17±12.89[az] (n=6)	47.92±13.89[bz] (n=9)	22.92±9.42[az] (n=8)
Average	3.90±0.61[a] (n=74)	2.69±0.46[a] (n=43)	43.75±9.11[z] (n=17)	30.21±9.03[z] (n=11)	26.04±7.75[z] (n=15)

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.9: Number (n, mean±SEM) of oocytes cleaved from different body weight groups

Body weight	No. of oocytes injected per ovary	No. of oocytes cleaved per ovary	No. of oocytes cleaved per ovary		
			Grade A	Grade B	Grade C
Light	4.44±1.03[a] (n=40)	2.50±0.63[a] (n=20)	1.38±0.32[az] (n=11)	0.25±0.16[ay] (n=2)	0.88±0.40[ayz] (n=7)
Medium	3.40±0.70[a] (n=34)	2.88±0.72[a] (n=23)	0.75±0.31[az] (n=6)	1.13±0.30[bz] (n=9)	1.00±0.50[az] (n=8)
Average	3.90±0.61[a] (n=74)	2.69±0.46[a] (n=43)	1.06±0.23[z] (n=17)	0.69±0.20[z] (n=11)	0.94±0.31[z] (n=15)

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.10: Cleavage rate (%, mean±SEM) of different grades of oocyte at two body weight groups

Body weight	Percent cleavage[*]		
	Grade A	Grade B	Grade C
Light	79.76±10.58[az] (11/16)[**]	27.78±14.70[ay] (2/7)	30.56±13.03[ay] (7/17)
Medium	58.33±20.07[az] (6/10)	71.43±14.87[az] (9/13)	69.33±18.45[az] (8/11)
Average	69.87±10.82[z] (17/26)	58.33±12.73[z] (11/20)	44.41±11.45[z] (15/28)

[*] Mean percentage of cleavage was based on oocytes used for ICSI, in the respective grades
[**] Mean total of cleaved embryos per total of oocytes used for ICSI
Mean values within a column with same superscript were not significantly different (P>0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.11: Developmental rate (%, mean±SEM) of different grades of oocyte at two body weight groups

Oocyte grade	Body weight	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
				4-cell	8-cell	Morula
Grade A	Light	2.29±0.52[a] (n=16)	79.76±10.58[a] (n=11)	76.19±12.66[a] (n=10)	61.90±15.84[a] (n=9)	17.86±14.14[a] (n=3)
	Medium	1.67±0.21[a] (n=10)	58.33±20.07[a] (n=6)	58.33±20.07[a] (n=6)	41.67±20.07[a] (n=5)	8.33±8.33[a] (n=1)
	Average	2.00±0.30 (n=26)	69.87±10.82 (n=17)	67.95±11.28 (n=16)	52.56±12.39 (n=14)	13.46±8.31 (n=4)
Grade B	Light	2.33±0.33[a] (n=7)	27.78±14.70[a] (n=2)	27.78±14.70[a] (n=2)	11.11±11.11[a] (n=1)	11.11±11.11[a] (n=1)
	Medium	1.86±0.40[a] (n=13)	71.43±14.87[a] (n=9)	64.29±17.98[a] (n=8)	64.29±17.98[a] (n=8)	28.57±18.44[a] (n=4)
	Average	2.00±0.30 (n=20)	58.33±12.73 (n=11)	53.33±14.01 (n=10)	48.33±15.00 (n=9)	23.33±13.19 (n=5)
Grade C	Light	1.89±0.42[a] (n=17)	30.56±13.03[a] (n=7)	30.56±13.03[a] (n=7)	30.56±13.03[a] (n=7)	5.56±5.56[a] (n=2)
	Medium	2.20±0.80[a] (n=11)	69.33±18.45[a] (n=8)	29.33±18.09[a] (n=6)	21.33±13.73[a] (n=4)	0.00±0.00[a] (n=0)
	Average	2.00±0.38 (n=28)	44.41±11.45 (n=15)	30.12±10.16 (n=13)	27.26±9.45 (n=11)	3.57±3.57 (n=2)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

Appendix Table 3.12: Developmental rate (%, mean±SEM) of different grades of oocyte at light body weight group

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	2.29±0.52[a] (n=16)	79.76±10.58[b] (n=11)	76.19±12.66[az] (n=10)	61.90±15.84[az] (n=9)	17.86±14.14[ay] (n=3)
Grade B	2.33±0.33[a] (n=7)	27.78±14.70[a] (n=2)	27.78±14.70[az] (n=2)	11.11±11.11[az] (n=1)	11.11±11.11[az] (n=1)
Grade C	1.89±0.42[a] (n=17)	30.56±13.03[a] (n=7)	30.56±13.03[az] (n=7)	30.56±13.03[az] (n=7)	5.56±5.56[az] (n=2)
Average	2.11±0.28 (n=40)	48.25±9.25 (n=20)	46.93±9.33[z] (n=19)	39.04±9.39[z] (n=17)	10.97±5.91[y] (n=6)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)

[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.13: Developmental rate (%, mean±SEM) of different grades of oocyte at medium body weight group

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	1.67±0.21[a] (n=10)	58.33±20.07[a] (n=6)	58.33±20.07[az] (n=6)	41.67±20.07[az] (n=5)	8.33±8.33[az] (n=1)
Grade B	1.86±0.40[a] (n=13)	71.43±14.87[a] (n=9)	64.29±17.98[az] (n=8)	64.29±17.98[az] (n=8)	28.57±18.44[az] (n=4)
Grade C	2.20±0.80[a] (n=11)	69.33±18.45[a] (n=8)	29.33±18.09[az] (n=6)	21.33±13.73[az] (n=4)	0.00±0.00[az] (n=0)
Average	1.89±0.27 (n=34)	66.48±9.70 (n=23)	52.59±10.86[z] (n=20)	44.82±10.68[z] (n=17)	13.89±7.89[y] (n=5)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.14: Developmental competence (%, mean±SEM) of *in vitro* produced goat embryos at two body weight groups regardless of the oocyte grades

Body weight	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Light	4.44±1.03[a] (n=40)	51.48±9.65[a] (n=20)	50.37±9.88[a] (n=19)	46.30±10.89[a] (n=17)	11.94±6.48[a] (n=6)
Medium	3.40±0.70[a] (n=34)	59.81±11.80[a] (n=23)	51.14±13.21[a] (n=20)	44.95±13.52[a] (n=17)	10.71±5.76[a] (n=5)
Average	3.90±0.61 (n=74)	55.86±7.56 (n=43)	50.78±8.15 (n=39)	45.59±8.55 (n=34)	11.30±4.19 (n=11)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

Appendix Table 3.15: Percentage (%, mean±SEM) of oocytes matured from different OR cycles

OR cycles	No. of ovaries	No. of oocytes matured per ovary	Percent of oocytes matured per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
OR1	40	3.75±0.39[a] (n=150)	32.20±5.43[az] (n=52)	27.43±4.94[az] (n=39)	36.62±5.12[az] (n=55)	1.25±1.25[ay] (n=2)	2.50±2.50[ay] (n=2)
OR2	25	3.00±0.36[a] (n=75)	38.88±6.56[az] (n=33)	27.65±6.25[az] (n=20)	32.13±7.87[az] (n=20)	0.67±0.67[ay] (n=1)	0.67±0.67[ay] (n=1)
OR3	4	3.50±0.29[a] (n=14)	12.50±7.22[ay] (n=2)	22.92±15.73[ay] (n=3)	56.25±9.24[az] (n=8)	8.33±8.33[by] (n=1)	0.00±0.00[ay] (n=0)
Average	23 (n=69)	3.46±0.26 (n=239)	33.48±4.00[z] (n=87)	27.25±3.71[z] (n=62)	36.13±4.16[z] (n=83)	1.45±0.89[y] (n=4)	1.69±1.47[y] (n=3)

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)

[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.16: Number (n, mean±SEM) of oocytes matured from different OR cycles

OR cycles	No. of ovaries	No. of oocytes matured per ovary	No. of oocytes matured per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
OR1	40	3.75±0.39^{a} (n=150)	1.30±0.25^{az} (n=52)	0.98±0.17^{az} (n=39)	1.38±0.21^{abz} (n=55)	0.05±0.05^{ay} (n=2)	0.05±0.05^{ay} (n=2)
OR2	25	3.00±0.36^{a} (n=75)	1.32±0.23^{az} (n=33)	0.80±0.18^{ay} (n=20)	0.80±0.17^{ay} (n=20)	0.04±0.04^{ax} (n=1)	0.04±0.04^{ax} (n=1)
OR3	4	3.50±0.29^{a} (n=14)	0.50±0.29^{ay} (n=2)	0.75±0.48^{ay} (n=3)	2.00±0.41^{bz} (n=8)	0.25±0.25^{ay} (n=1)	0.00±0.00^{ay} (n=0)
Average	23 (n=69)	3.46±0.26 (n=239)	1.26±0.17^{z} (n=87)	0.90±0.12^{y} (n=62)	1.20±0.14^{yz} (n=83)	0.06±0.04^{x} (n=4)	0.04±0.03^{x} (n=3)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.17: Maturation rate (%, mean±SEM) of different grades of oocyte at three OR cycle groups

OR cycles	Percent MII oocytes*	Percent maturation**				
		Grade A	Grade B	Grade C	Grade D	Grade E
OR1	48.83±4.46^{a} (150/272)***	69.77±7.33^{az} (52/69)	68.21±7.34^{az} (39/53)	48.89±5.95^{az} (55/102)	3.18±3.18^{ay} (2/29)	6.67±6.67^{ay} (2/19)
OR2	57.28±5.38^{a} (75/128)	82.84±5.56^{az} (33/41)	78.45±7.94^{az} (20/27)	40.61±7.92^{ay} (20/44)	16.67±16.67^{abxy} (1/6)	4.76±4.76^{ax} (1/10)
OR3	73.34±9.03^{a} (14/20)	100.00±0.00^{az} (2/2)	100.00±0.00^{az} (3/3)	68.75±11.97^{az} (8/12)	50.00±50.00^{bz} (1/2)	0.00 (0/1)
Average	52.82±3.36 (239/420)	75.42±5.01^{z} (87/112)	73.14±5.32^{z} (62/83)	47.31±4.55^{y} (83/158)	9.20±5.20^{x} (4/37)	5.56±4.04^{x} (3/30)

* Mean percentage of MII oocytes was based on total oocytes from all grades used for IVM, in the respective OR cycles groups
** Mean percentage of maturation rate was based on oocytes used for IVM, in the respective grades
*** Mean total of matured oocytes per total of oocytes used for IVM
^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.18: Percentage (%, mean±SEM) of oocytes cleaved from different OR cycle groups

OR cycles	No. of oocytes injected per ovary	No. of oocytes cleaved per ovary	Percent of oocytes cleaved per ovary		
			Grade A	Grade B	Grade C
OR1	3.64±0.54[a] (n=80)	2.69±0.46[a] (n=43)	43.75±9.11[az] (n=17)	30.21±9.03[az] (n=11)	26.04±7.75[az] (n=15)
OR2	2.92±0.42[a] (n=35)	2.33±0.36[a] (n=28)	45.28±10.80[az] (n=15)	27.36±10.89[az] (n=6)	27.36±10.89[az] (n=7)
OR3	3.00±0.58[a] (n=9)	1.00±0.00[a] (n=2)	0.00±0.00[az] (n=0)	50.00±50.00[az] (n=1)	50.00±50.00[az] (n=1)
Average	3.35±0.35 (n=124)	2.43±0.29 (n=73)	41.45±6.70[z] (n=32)	30.39±6.87[z] (n=18)	28.17±6.44[z] (n=23)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Appendix Table 3.19: Number (n, mean±SEM) of oocytes cleaved from different OR cycle groups

OR cycles	No. of oocytes injected per ovary	No. of oocytes cleaved per ovary	No. of oocytes cleaved per ovary		
			Grade A	Grade B	Grade C
OR1	3.64±0.54[a] (n=80)	2.69±0.46[a] (n=43)	1.06±0.23[az] (n=17)	0.69±0.20[az] (n=11)	0.94±0.31[az] (n=15)
OR2	2.92±0.42[a] (n=35)	2.33±0.36[a] (n=28)	1.25±0.31[az] (n=15)	0.50±0.15[ay] (n=6)	0.58±0.19[ay] (n=7)
OR3	3.00±0.58[a] (n=9)	1.00±0.00[a] (n=2)	0.00±0.00[az] (n=0)	0.50±0.50[az] (n=1)	0.50±0.50[az] (n=1)
Average	3.35±0.35 (n=124)	2.43±0.29 (n=73)	1.07±0.18[z] (n=32)	0.60±0.12[z] (n=18)	0.77±0.18[z] (n=23)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.20: Cleavage rate (%, mean±SEM) of different grades of oocyte at three different OR cycle groups

OR cycles	Percent cleavage[*]		
	Grade A	Grade B	Grade C
OR1	69.87±10.82[az] (17/26)[**]	53.03±12.68[az] (11/21)	36.57±10.28[abz] (15/33)
OR2	74.07±12.14[az] (15/20)	100.00±0.00[bz] (6/6)	78.57±14.87[bz] (7/9)
OR3	0.00 (0/1)	50.00 (1/2)	11.11±11.11[a] (1/6)
Average	68.48±8.18[z] (32/47)	68.52±9.32[z] (18/29)	44.63±8.55[z] (23/48)

[*] Mean percentage of cleavage was based on oocytes used for ICSI, in the respective grades
[**] Mean total of cleaved embryos per total of oocytes used for ICSI
[ab] Mean values within a column with different superscripts were significantly different (P<0.05)
[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Appendix Table 3.21: Developmental rate (%, mean±SEM) of different grades of oocytes at three different OR cycle groups

Oocyte grade	OR cycles	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
				4-cell	8-cell	Morula
Grade A	OR1	2.00±0.30[a] (n=26)	69.87±10.82[a] (n=17)	67.95±11.28[a] (n=16)	52.56±12.39[a] (n=14)	13.46±8.31[a] (n=4)
	OR2	2.22±0.28[a] (n=20)	74.07±12.14[a] (n=15)	74.07±12.14[a] (n=15)	62.96±14.10[a] (n=14)	38.89±14.16[a] (n=8)
	OR3	1.00 (n=1)	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)
	Average	2.04±0.20 (n=47)	68.48±8.18 (n=32)	67.39±8.37 (n=31)	54.35±9.10 (n=28)	22.83±7.60 (n=12)
Grade B	OR1	1.91±0.29[a] (n=21)	53.03±12.68[a] (n=11)	48.49±13.57[a] (n=10)	43.94±14.26[a] (n=9)	21.21±12.12[a] (n=5)
	OR2	1.00±0.00[a] (n=6)	100.00±0.00[b] (n=6)	83.33±16.67[a] (n=5)	66.67±21.08[a] (n=4)	50.00±22.36[a] (n=3)
	OR3	2.00 (n=2)	50.00 (n=1)	50.00 (n=1)	50.00 (n=1)	0.00 (n=0)
	Average	1.61±0.20 (n=29)	68.52±9.32 (n=17)	60.19±10.45 (n=16)	51.85±11.10 (n=14)	29.63±10.74 (n=8)

(continued)

(continued)

Oocyte grade	OR cycles	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
				4-cell	8-cell	Morula
Grade C	OR1	1.94±0.32[a] (n=33)	36.57±10.28[ab] (n=15)	24.80±8.79[a] (n=13)	22.45±8.16[a] (n=11)	2.94±2.94[a] (n=2)
	OR2	1.29±0.18[a] (n=9)	78.57±14.87[b] (n=7)	35.71±17.98[a] (n=4)	28.57±14.87[a] (n=3)	0.00±0.00[a] (n=0)
	OR3	2.00±0.58[a] (n=6)	11.11±11.11[a] (n=1)	11.11±11.11[a] (n=1)	11.11±11.11[a] (n=1)	0.00±0.00[a] (n=0)
	Average	1.78±0.22 (n=48)	44.63±8.55 (n=23)	26.11±7.23 (n=18)	22.78±6.40 (n=15)	1.85±1.85 (n=2)

*Mean percentage of development was based on oocytes used for sham injection, in the respective grades
[ab] Mean values within a column with different superscripts were significantly different (P<0.05)

Appendix Table 3.22: Developmental rate (%, mean±SEM) of different grades of oocyte at OR1 group

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	2.00±0.30[a] (n=26)	69.87±10.82[a] (n=17)	67.95±11.28[bz] (n=16)	52.56±12.39[az] (n=14)	13.46±8.31[ay] (n=4)
Grade B	1.91±0.29[a] (n=21)	53.03±12.68[a] (n=11)	48.49±13.57[abz] (n=10)	43.94±14.26[az] (n=9)	21.21±12.12[az] (n=5)
Grade C	1.94±0.32[a] (n=33)	36.57±10.28[a] (n=15)	24.80±8.79[az] (n=13)	22.45±8.16[ayz] (n=11)	2.94±2.94[ay] (n=2)
Average	1.95±0.17 (n=80)	51.55±6.67 (n=43)	44.84±6.76[z] (n=39)	37.76±6.61[z] (n=34)	11.18±4.39[y] (n=11)

*Mean percentage of development was based on oocytes used for ICSI, in the respective grades
[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.23: Developmental rate (%, mean±SEM) of different grades of oocyte at OR2 group

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	2.22±0.28^{b} (n=20)	74.07±12.14^{a} (n=15)	74.07±12.14^{az} (n=15)	62.96±14.10^{az} (n=14)	38.89±14.16^{abz} (n=8)
Grade B	1.00±0.00^{a} (n=6)	100.00±0.00^{a} (n=6)	83.33±16.67^{az} (n=5)	66.67±21.08^{az} (n=4)	50.00±22.36^{bz} (n=3)
Grade C	1.29±0.18^{a} (n=9)	78.57±14.87^{a} (n=7)	35.71±17.98^{az} (n=4)	28.57±14.87^{az} (n=3)	0.00±0.00^{az} (n=0)
Average	1.59±0.17 (n=35)	82.58±6.98 (n=28)	64.39±9.45^{z} (n=24)	53.03±9.66^{yz} (n=21)	29.55±9.17^{y} (n=11)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)

^{yz} Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.24: Developmental rate (%, mean±SEM) of different grades of oocyte at OR3 group

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	1.00^{a} (n=1)	0.00^{a} (n=0)	0.00^{a} (n=0)	0.00^{a} (n=0)	0.00 (n=0)
Grade B	2.00^{a} (n=2)	50.00^{a} (n=1)	50.00^{a} (n=1)	50.00^{a} (n=1)	0.00 (n=0)
Grade C	2.00±0.58^{a} (n=6)	11.11±11.11^{a} (n=1)	11.11±11.11^{az} (n=1)	11.11±11.11^{az} (n=1)	0.00±0.00^{z} (n=0)
Average	1.80±0.37 (n=9)	16.67±10.54 (n=2)	16.67±10.54^{z} (n=2)	16.67±10.54^{z} (n=2)	0.00±0.00^{z} (n=0)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

^{a} Mean values within a column with same superscript were not significantly different (P>0.05)

^{z} Mean values within a row with same superscripts were not significantly different (P>0.05)

Appendix Table 3.25: Developmental competence (%, mean±SEM) of *in vitro* produced goat embryos at three different OR cycle groups regardless of the oocyte grades

OR cycle	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
OR1	3.64±0.54[a] (n=80)	48.25±7.74[ab] (n=43)	43.85±7.98[ab] (n=39)	39.37±8.11[a] (n=34)	9.76±3.71[ab] (n=11)
OR2	2.92±0.42[a] (n=35)	82.64±5.84[b] (n=28)	63.36±8.24[b] (n=24)	54.86±8.61[a] (n=21)	31.94±9.50[b] (n=11)
OR3	3.00±0.58[a] (n=9)	19.44±10.02[a] (n=2)	19.44±10.02[a] (n=2)	19.44±10.02[a] (n=2)	0.00±0.00[a] (n=0)
Average	3.35±0.35 (n=124)	57.07±5.91 (n=73)	49.50±5.89 (n=65)	42.78±5.77 (n=57)	16.16±4.15 (n=22)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

Appendix Table 3.26: Percentage (%, mean±SEM) of oocytes matured from different age groups

Age*	No. of ovaries	No. of oocytes matured per ovary	Percent of oocytes matured per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
Group 1	11	4.18±0.60[a] (n=46)	24.87±7.13[az] (n=13)	34.11±10.10[az] (n=12)	36.47±6.53[az] (n=19)	4.55±4.55[ay] (n=2)	0.00±0.00[ay] (n=0)
Group 2	14	3.93±0.74[a] (n=55)	34.42±8.86[az] (n=21)	32.99±8.67[az] (n=18)	32.59±7.39[az] (n=16)	0.00±0.00[ay] (n=0)	0.00±0.00[ay] (n=0)
Group 3	7	5.00±1.02[a] (n=35)	42.74±15.69[az] (n=14)	18.13±6.64[ayz] (n=7)	39.13±14.34[az] (n=14)	0.00±0.00[ay] (n=0)	0.00±0.00[ay] (n=0)
Average	10.67 (n=32)	4.25±0.44 (n=136)	32.96±5.65[z] (n=48)	30.13±5.32[z] (n=37)	35.35±4.87[z] (n=49)	1.56±1.56[y] (n=2)	0.00±0.00[y] (n=0)

*Age: group 1 (<18 months), group 2 (18-24 months) and group 3 (>24 months)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.27: Number (n, mean±SEM) of oocytes matured from different age groups

Age*	No. of ovaries	No. of oocytes matured per ovary	No. of oocytes matured per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
Group 1	11	4.18±0.60[a] (n=46)	1.18±0.38[az] (n=13)	1.09±0.29[az] (n=12)	1.73±0.38[az] (n=19)	0.18±0.18[ay] (n=2)	0.00±0.00[y] (n=0)
Group 2	14	3.93±0.74[a] (n=55)	1.50±0.44[az] (n=21)	1.29±0.34[az] (n=18)	1.14±0.29[az] (n=16)	0.00±0.00[ay] (n=0)	0.00±0.00[y] (n=0)
Group 3	7	5.00±1.02[a] (n=35)	2.00±0.90[az] (n=14)	1.00±0.38[ayz] (n=7)	2.00±0.72[az] (n=14)	0.00±0.00[ay] (n=0)	0.00±0.00[y] (n=0)
Average	10.67 (n=32)	4.25±0.44 (n=136)	1.50±0.30[z] (n=48)	1.16±0.19[z] (n=37)	1.53±0.24[z] (n=49)	0.06±0.06[y] (n=2)	0.00±0.00[y] (n=0)

*Age: group 1 (<18 months), group 2 (18-24 months) and group 3 (>24 months)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.28: Maturation rate (%, mean±SEM) of different grades of oocyte at three age groups

Age	Percent MII oocytes[*]	Percent maturation[**]				
		Grade A	Grade B	Grade C	Grade D	Grade E
Group 1	50.53±8.07^{a} (46/82)[***]	45.46±12.60^{ayz} (13/22)	68.52±14.29^{az} (12/16)	56.39±11.48^{az} (19/33)	13.33±13.33^{ayz} (2/9)	0.00±0.00^{ay} (0/2)
Group 2	59.97±3.01^{a} (55/87)	93.15±3.50^{bz} (21/24)	75.00±6.80^{az} (18/24)	48.21±8.62^{ay} (16/33)	0.00±0.00^{ax} (0/6)	N/A (0/0)
Group 3	56.46±13.97^{a} (35/18)	79.63±16.33^{abz} (14/18)	80.00±20.00^{az} (7/8)	49.52±14.37^{ayz} (14/24)	0.00±0.00^{ay} (0/4)	0.00±0.00^{ay} (0/5)
Average	55.57±4.75 (136/228)	68.99±7.77^{z} (48/64)	73.55±7.23^{z} (37/48)	51.56±6.20^{z} (49/90)	5.13±5.13^{y} (2/19)	0.00±0.00^{y} (0/7)

[*] Mean percentage of MII oocytes was based on total oocytes from all grades used for IVM, in the respective age groups

[**] Mean percentage of maturation rate was based on oocytes used for IVM, in the respective grades

[***] Mean total of matured oocytes per total of oocytes used for IVM

NA: not applicable

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)

[xyz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.29: Percentage (%, mean±SEM) of oocytes cleaved from different age groups

Age	No. of oocytes injected per ovary	No. of oocytes cleaved per ovary	Percent of oocytes cleaved per ovary		
			Grade A	Grade B	Grade C
Group 1	4.50±1.19[a] (n=18)	3.00±1.08[a] (n=12)	41.67±14.43[az] (n=6)	37.50±23.94[az] (n=2)	20.83±12.50[az] (n=4)
Group 2	4.44±0.99[a] (n=40)	2.50±0.63[a] (n=20)	47.92±13.53[az] (n=8)	25.00±9.96[az] (n=6)	27.08±12.57[az] (n=6)
Group 3	3.67±1.67[a] (n=11)	4.00±2.00[a] (n=8)	50.00±50.00[az] (n=2)	16.67±16.67[az] (n=2)	33.34±33.34[az] (n=4)
Average	4.31±0.66 (n=69)	2.86±0.51 (n=40)	46.43±9.91[z] (n=16)	27.38±8.67[z] (n=10)	26.19±8.51[z] (n=14)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Appendix Table 3.30: Number (n, mean±SEM) of oocytes cleaved from different age groups

Age	No. of oocytes injected per ovary	No. of oocytes cleaved per ovary	No. of oocytes cleaved per ovary		
			Grade A	Grade B	Grade C
Group 1	4.50±1.19[a] (n=18)	3.00±1.08[a] (n=12)	1.50±0.65[az] (n=6)	0.50±0.29[az] (n=2)	1.00±0.71[az] (n=4)
Group 2	4.44±0.99[a] (n=40)	2.50±0.63[a] (n=20)	1.00±0.27[az] (n=8)	0.75±0.31[az] (n=6)	0.75±0.31[az] (n=6)
Group 3	3.67±1.67[a] (n=11)	4.00±2.00[a] (n=8)	1.00±1.00[az] (n=2)	1.00±1.00[az] (n=2)	2.00±2.00[az] (n=4)
Average	4.31±0.66 (n=69)	2.86±0.51 (n=40)	1.14±0.25[z] (n=16)	0.71±0.22[z] (n=10)	1.00±0.35[z] (n=14)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Appendix Table 3.31: Cleavage rate (%, mean±SEM) of different grades of oocyte at three different age groups

Age	Percent cleavage*		
	Grade A	Grade B	Grade C
Group 1	75.00±14.43^{az} (6/8) **	75.00±25.00^{az} (2/3)	41.67±22.05^{az} (4/7)
Group 2	80.56±12.49^{az} (8/12)	38.89±15.32^{az} (6/14)	35.19±14.82^{az} (6/14)
Group 3	100.00 (2/2)	100.00 (2/2)	40.00±40.00^{a} (4/7)
Average	80.83±8.43^{z} (16/22)	53.70±13.26^{yz} (10/19)	37.26±11.00^{y} (14/28)

* Mean percentage of cleavage was based on oocytes used for ICSI, in the respective grades

** Mean total of cleaved embryos per total of oocytes used for ICSI

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Appendix Table 3.32: Developmental rate (%, mean±SEM) of different grades of oocytes at three different age groups

Oocyte grade	Age	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
				4-cell	8-cell	Morula
Grade A	Group 1	2.67±0.67[a] (n=8)	75.00±14.43[a] (n=6)	75.00±14.43[a] (n=6)	75.00±14.43[a] (n=6)	41.67±30.05[a] (n=3)
	Group 2	2.00±0.52[a] (n=12)	80.56±12.49[a] (n=8)	76.39±14.97[a] (n=7)	59.72±18.56[a] (n=6)	0.00±0.00[a] (n=0)
	Group 3	2.00 (n=2)	100.00 (n=2)	100.00 (n=2)	100.00 (n=2)	50.00 (n=1)
	Average	2.20±0.36 (n=22)	80.83±8.43 (n=16)	78.33±9.72 (n=15)	68.33±12.10 (n=14)	17.50±10.57 (n=4)
Grade B	Group 1	1.50±0.50 [a] (n=3)	75.00±25.00[a] (n=2)	50.00±50.00[a] (n=1)	50.00±50.00[a] (n=1)	0.00±0.00[a] (n=0)
	Group 2	2.33±0.42[a] (n=14)	38.89±15.32[a] (n=6)	38.89±15.32[a] (n=6)	30.56±16.34[a] (n=5)	22.22±16.48[a] (n=3)
	Group 3	2.00 (n=2)	100.00 (n=2)	100.00 (n=2)	100.00 (n=2)	100.00 (n=2)
	Average	2.11±0.31 (n=19)	53.70±13.26 (n=10)	48.15±14.56 (n=9)	42.59±15.49 (n=8)	25.93±14.46 (n=5)

(continued)

(continued)

Oocyte grade	Age	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
				4-cell	8-cell	Morula
Grade C	Group 1	2.33±0.88[a] (n=7)	41.67±22.05[a] (n=4)	41.67±22.05[a] (n=4)	41.67±22.05[a] (n=4)	16.67±16.67[a] (n=2)
	Group 2	1.56±0.38[a] (n=14)	35.19±14.82[a] (n=6)	24.07±12.76[a] (n=5)	24.07±12.76[a] (n=5)	0.00±0.00[a] (n=0)
	Group 3	3.50±1.50[a] (n=7)	40.00±40.00[a] (n=4)	40.00±40.00[a] (n=4)	20.00±20.00[a] (n=2)	0.00±0.00[a] (n=0)
	Average	2.00±0.38 (n=28)	37.26±11.00 (n=14)	30.12±10.16 (n=13)	27.26±9.45 (n=11)	3.57±3.57 (n=2)

*Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

Appendix Table 3.33: Developmental rate (%, mean±SEM) of different grades of oocyte at Group 1 age group

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	2.67±0.67[a] (n=8)	75.00±14.43[a] (n=6)	75.00±14.43[az] (n=6)	75.00±14.43[az] (n=6)	41.67±30.05[az] (n=3)
Grade B	1.50±0.50[a] (n=3)	75.00±25.00[a] (n=2)	50.00±50.00[az] (n=1)	50.00±50.00[az] (n=1)	0.00±0.00[az] (n=0)
Grade C	2.33±0.89[a] (n=7)	41.67±22.05[a] (n=4)	41.67±22.05[az] (n=4)	41.67±22.05[az] (n=4)	16.67±16.67[az] (n=2)
Average	2.25±0.41 (n=18)	62.50±11.57 (n=12)	56.25±13.98[z] (n=11)	56.25±13.98[z] (n=11)	21.88±12.89[z] (n=5)

*Mean percentage of development was based on oocytes used for ICSI, in the respective grades
[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Appendix Table 3.34: Developmental rate (%, mean±SEM) of different grades of oocyte at Group 2 age group

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	2.00±0.52[a] (n=12)	80.56±12.49[a] (n=8)	76.39±14.97[bz] (n=7)	59.72±18.56[az] (n=6)	0.00±0.00[ay] (n=0)
Grade B	2.33±0.42[a] (n=14)	38.89±15.32[a] (n=6)	38.89±15.32[abz] (n=6)	30.56±16.34[az] (n=5)	22.22±16.48[az] (n=3)
Grade C	1.56±0.38[a] (n=14)	35.19±14.82[a] (n=6)	24.07±12.76[az] (n=5)	24.07±12.76[az] (n=5)	0.00±0.00[az] (n=0)
Average	1.91±0.25 (n=40)	49.21±9.24 (n=20)	43.25±9.19[z] (n=18)	36.11±9.12[z] (n=16)	6.35±4.94[y] (n=3)

*Mean percentage of development was based on oocytes used for ICSI, in the respective grades
[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Appendix Table 3.35: Developmental rate (%, mean±SEM) of different grades of oocyte at Group 3 age group

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	2.00 (n=2)	100.00 (n=2)	100.00 (n=2)	100.00 (n=2)	50.00 (n=1)
Grade B	2.00 (n=2)	100.00 (n=2)	100.00 (n=2)	100.00 (n=2)	100.00 (n=2)
Grade C	3.50±1.50 (n=7)	40.00±40.00 (n=4)	40.00±40.00^{z} (n=4)	20.00±20.00^{z} (n=2)	0.00±0.00^{z} (n=0)
Average	2.75±0.75 (n=11)	70.00±23.81 (n=8)	70.00±23.81^{z} (n=8)	60.00±24.50^{z} (n=6)	37.50±23.94^{z} (n=3)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

^{z} Mean values within a row with same superscripts were not significantly different (P>0.05)

Appendix Table 3.36: Developmental competence (%, mean±SEM) of *in vitro* produced goat embryos at three different age groups regardless of the oocyte grades

Age	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Group 1	4.50±1.19^{a} (n=18)	62.50±9.92^{a} (n=12)	54.17±18.16^{a} (n=11)	54.17±18.16^{a} (n=11)	21.88±12.89^{a} (n=5)
Group 2	4.44±0.99^{a} (n=40)	51.04±9.75^{a} (n=20)	47.67±9.93^{a} (n=18)	43.60±10.81^{a} (n=16)	5.40±3.64^{a} (n=3)
Group 3	3.67±1.67^{a} (n=11)	61.90±31.23^{a} (n=8)	61.90±31.23^{a} (n=8)	52.38±28.97^{a} (n=6)	26.19±14.48^{a} (n=3)
Average	4.31±0.66 (n=69)	55.92±7.74 (n=40)	51.96±8.51 (n=37)	47.89±8.61 (n=33)	13.42±4.81 (n=11)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

^{a} Mean values within a column with same superscript were not significantly different (P>0.05)

Appendix Table 3.37: Percentage (%, mean±SEM) of oocytes matured from different stimulation response groups

Stimulation response	No. of ovaries	No. of oocytes matured per ovary	Percent of oocytes matured per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
Excellent	9	4.44±0.85^{b} (n=40)	40.67±11.69^{az} (n=17)	24.47±8.74^{ayz} (n=8)	29.30±6.98^{az} (n=13)	5.56±5.56^{axy} (n=2)	0.00±0.00^{ax} (n=0)
Good	47	3.64±0.31^{ab} (n=171)	32.14±4.19^{az} (n=60)	30.70±4.49^{az} (n=48)	36.44±4.89^{az} (n=61)	0.36±0.36^{ay} (n=1)	0.36±0.36^{ay} (n=1)
Satisfactory	13	2.15±0.39^{a} (n=28)	33.33±13.07^{ayz} (n=10)	16.67±9.25^{axyz} (n=6)	39.74±12.74^{az} (n=9)	2.57±2.56^{ax} (n=1)	7.69±7.69^{axy} (n=2)
Average	23 (n=69)	3.46±0.26 (n=239)	33.48±4.00^{z} (n=87)	27.25±3.71^{z} (n=62)	36.13±4.16^{z} (n=83)	1.45±0.89^{y} (n=4)	1.69±1.47^{y} (n=3)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.38: Number (n, mean±SEM) of oocytes matured from different stimulation response groups

Stimulation response	No. of ovaries	No. of oocytes matured per ovary	No. of oocytes matured per ovary				
			Grade A	Grade B	Grade C	Grade D	Grade E
Excellent	9	4.44±0.85^{b} (n=40)	1.89±0.70^{bz} (n=17)	0.89±0.31^{axyz} (n=8)	1.44±0.44^{ayz} (n=13)	0.22±0.22^{axy} (n=2)	0.00±0.00^{ax} (n=0)
Good	47	3.64±0.31^{ab} (n=171)	1.28±0.19^{abz} (n=60)	1.02±0.14^{az} (n=48)	1.30±0.18^{az} (n=61)	0.02±0.02^{ay} (n=1)	0.02±0.02^{ay} (n=1)
Satisfactory	13	2.15±0.39^{a} (n=28)	0.77±0.30^{az} (n=10)	0.46±0.27^{ayz} (n=6)	0.69±0.21^{ayz} (n=9)	0.08±0.08^{ay} (n=1)	0.15±0.15^{ayz} (n=2)
Average	23 (n=69)	3.46±0.26 (n=239)	1.26±0.17^{z} (n=87)	0.90±0.12^{y} (n=62)	1.20±0.14^{yz} (n=83)	0.06±0.04^{x} (n=4)	0.04±0.03^{x} (n=3)

^{ab} Mean values within a column with different superscripts were significantly different (P<0.05)
^{xyz} Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.39: Maturation rate (%, mean±SEM) of different grades of oocyte at three stimulation response groups

Stimulation response	Percent MII oocytes*	Percent maturation**				
		Grade A	Grade B	Grade C	Grade D	Grade E
Excellent	51.53±8.78[a] (40/73)***	68.06±12.28[az] (17/23)	61.11±15.32[az] (8/12)	45.67±11.10[ayz] (13/28)	13.33±13.33[ay] (2/7)	0.00 (0/3)
Good	53.39±4.07[a] (171/292)	77.36±5.97[az] (60/76)	75.05±5.82[az] (48/64)	49.52±5.54[ay] (61/111)	5.26±5.26[ax] (1/25)	3.70±3.70[ax] (1/16)
Satisfactory	51.67±8.50[a] (28/55)	73.61±15.87[az] (10/13)	75.00±25.00[az] (6/7)	45.00±11.93[ayz] (9/19)	20.00±20.00[ay] (1/5)	8.33±8.33[ay] (2/11)
Average	52.82±3.36 (239/420)	75.42±5.01[z] (87/112)	73.14±5.32[z] (62/83)	48.23±4.52[y] (83/158)	9.20±5.20[x] (4/37)	5.56±4.04[x] (3/30)

* Mean percentage of MII oocytes was based on total oocytes from all grades used for IVM, in the respective stimulation responses groups
** Mean percentage of maturation rate was based on oocytes used for IVM, in the respective grades
*** Mean total of matured oocytes per total of oocytes used for IVM
[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.40: Percentage (%, mean±SEM) of oocytes cleaved from different stimulation response groups

Stimulation response	No. of oocytes injected per ovary	No. of oocytes cleaved per ovary	Percent of oocytes cleaved per ovary		
			Grade A	Grade B	Grade C
Excellent	4.33±1.45[a] (n=13)	4.50±1.50[b] (n=9)	50.00±16.67[az] (n=4)	16.67±16.67[az] (n=2)	33.33±0.00[az] (n=3)
Good	3.56±0.44[a] (n=96)	2.42±0.32[ab] (n=58)	39.31±7.11[az] (n=24)	32.43±7.67[az] (n=15)	28.26±7.19[az] (n=19)
Satisfactory	2.14±0.26[a] (n=15)	1.50±0.29[a] (n=6)	50.00±28.87[az] (n=4)	25.00±25.00[az] (n=1)	25.00±25.00[az] (n=1)
Average	3.35±0.35 (n=124)	2.43±0.29 (n=73)	41.45±6.70[z] (n=32)	30.39±6.87[z] (n=18)	28.17±6.44[z] (n=23)

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)
[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Appendix Table 3.41: Number (n, mean±SEM) of oocytes cleaved from different stimulation response groups

Stimulation response	No. of oocytes injected per ovary	No. of oocytes cleaved per ovary	No. of oocytes cleaved per ovary		
			Grade A	Grade B	Grade C
Excellent	4.33±1.45[a] (n=13)	4.50±1.50[b] (n=9)	2.00±0.00[az] (n=4)	1.00±1.00[az] (n=2)	1.50±0.50[az] (n=3)
Good	3.56±0.44[a] (n=96)	2.42±0.32[ab] (n=58)	1.00±0.20[az] (n=24)	0.63±0.13[az] (n=15)	0.79±0.22[az] (n=19)
Satisfactory	2.14±0.26[a] (n=15)	1.50±0.29[a] (n=6)	1.00±0.58[az] (n=4)	0.25±0.25[az] (n=1)	0.25±0.25[az] (n=1)
Average	3.35±0.35 (n=124)	2.43±0.29 (n=73)	1.07±0.18[z] (n=32)	0.60±0.12[z] (n=18)	0.77±0.18[z] (n=23)

[ab] Mean values within a column with different superscripts were significantly different (P<0.05)
[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Appendix Table 3.42: Cleavage rate (%, mean±SEM) of different grades of oocyte at three stimulation response groups

Stimulation response	Percent cleavage[*]		
	Grade A	Grade B	Grade C
Excellent	100.00±0.00[az] (4/4)[**]	50.00±50.00[az] (2/3)	38.89±20.03[az] (3/6)
Good	67.13±9.25[az] (24/36)	72.22±9.85[az] (15/24)	49.42±10.17[az] (19/36)
Satisfactory	55.56±29.40[az] (4/7)	50.00 (1/2)	25.00±25.00[az] (1/6)
Average	68.48±8.18[z] (32/47)	68.52±9.32[z] (18/29)	44.63±8.55[z] (23/48)

[*] Mean percentage of cleavage was based on oocytes used for ICSI, in the respective grades
[**] Mean total of cleaved embryos per total of oocytes used for ICSI
[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Appendix Table 3.43: Developmental rate (%, mean±SEM) of different grades of oocytes at three stimulation response groups

Oocyte grade	Stimulation response	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
				4-cell	8-cell	Morula
Grade A	Excellent	2.00±0.00[a] (n=4)	100.00±0.00[a] (n=4)	100.00±0.00[a] (n=4)	100.00±0.00[a] (n=4)	50.00±50.00[a] (n=2)
	Good	2.00±0.26[a] (n=36)	67.13±9.25[a] (n=24)	65.74±9.50[a] (n=23)	49.07±10.24[a] (n=20)	20.83±8.26[a] (n=9)
	Satisfactory	2.33±0.33[a] (n=7)	55.56±29.40[a] (n=4)	55.56±29.40[a] (n=4)	55.56±29.40[a] (n=4)	16.67±16.67[a] (n=1)
	Average	2.04±0.20 (n=47)	68.48±8.18 (n=32)	67.39±8.37 (n=31)	54.35±9.10 (n=28)	22.83±7.60 (n=12)
Grade B	Excellent	1.50±0.50[a] (n=3)	50.00±50.00[a] (n=2)	50.00±50.00[a] (n=2)	50.00±50.00[a] (n=2)	50.00±50.00[a] (n=2)
	Good	1.60±0.24[a] (n=24)	72.22±9.85[a] (n=15)	62.22±11.56[a] (n=13)	52.22±12.48[a] (n=11)	28.89±11.67[a] (n=6)
	Satisfactory	2.00 (n=2)	50.00 (n=1)	50.00 (n=1)	50.00 (n=1)	0.00 (n=0)
	Average	1.61±0.20 (n=29)	68.52±9.32 (n=18)	60.19±10.45 (n=16)	51.85±11.10 (n=14)	29.63±10.74 (n=8)

(continued)

(continued)

Oocyte grade	Stimulation response	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
				4-cell	8-cell	Morula
Grade C	Excellent	2.00±0.58[a] (n=6)	38.89±20.03[a] (n=3)	38.89±20.03[a] (n=3)	38.89±20.03[a] (n=3)	0.00±0.00[a] (n=0)
	Good	1.80±0.28[a] (n=36)	49.42±10.17[a] (n=19)	29.42±9.01[a] (n=15)	24.92±7.85[a] (n=12)	2.50±2.50[a] (n=2)
	Satisfactory	1.50±0.29[a] (n=6)	25.00±25.00[a] (n=1)	0.00±0.00[a] (n=0)	0.00±0.00[a] (n=0)	0.00±0.00[a] (n=0)
	Average	1.78±0.22 (n=48)	44.63±8.55 (n=23)	26.11±7.23 (n=18)	22.78±6.40 (n=15)	1.85±1.85 (n=2)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

Appendix Table 3.44: Developmental rate (%, mean±SEM) of different grades of oocyte at excellent stimulation response

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	2.00±0.00[a] (n=4)	100.00±0.00[a] (n=4)	100.00±0.00[az] (n=4)	100.00±0.00[az] (n=4)	50.00±50.00[az] (n=2)
Grade B	1.50±0.50[a] (n=3)	50.00±50.00[a] (n=2)	50.00±50.00[az] (n=2)	50.00±50.00[az] (n=2)	50.00±50.00[az] (n=2)
Grade C	2.00±0.58[a] (n=6)	38.89±20.03[a] (n=3)	38.89±20.03[az] (n=3)	38.89±20.03[az] (n=3)	0.00±0.00[az] (n=0)
Average	1.86±0.26 (n=13)	59.52±17.00 (n=9)	59.52±17.00[z] (n=9)	59.52±17.00[z] (n=9)	28.57±18.44[z] (n=4)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Appendix Table 3.45: Developmental rate (%, mean±SEM) of different grades of oocyte at good stimulation response

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	2.00±0.26[a] (n=36)	67.13±9.25[a] (n=24)	65.74±9.50[bz] (n=23)	49.07±10.24[az] (n=20)	20.83±8.26[aby] (n=9)
Grade B	1.60±0.24[a] (n=24)	72.22±9.85[a] (n=15)	62.22±11.56[bz] (n=13)	52.22±12.48[az] (n=11)	28.89±11.67[bz] (n=6)
Grade C	1.80±0.28[a] (n=36)	49.42±10.17[a] (n=19)	29.42±9.01[az] (n=15)	24.92±7.85[az] (n=12)	2.50±2.50[ay] (n=2)
Average	1.81±0.15 (n=96)	61.89±5.75 (n=58)	51.04±6.07[z] (n=51)	40.85±5.92[z] (n=43)	16.20±4.60[y] (n=17)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades
[ab] Mean values within a column with different superscripts were significantly different (P<0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.46: Developmental rate (%, mean±SEM) of different grades of oocyte at satisfactory stimulation response

Oocyte grade	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Grade A	2.33±0.33[a] (n=7)	55.56±29.40[a] (n=4)	55.56±29.40[az] (n=4)	55.56±29.40[az] (n=4)	16.67±16.67[az] (n=1)
Grade B	2.00 (n=2)	50.00 (n=1)	50.00 (n=1)	50.00 (n=1)	0.00 (n=0)
Grade C	1.50±0.29[a] (n=6)	25.00±25.00[a] (n=1)	0.00±0.00[a] (n=0)	0.00±0.00[a] (n=0)	0.00±0.00[a] (n=0)
Average	1.88±0.23 (n=15)	39.58±16.04 (n=6)	27.08±14.06[z] (n=5)	27.08±14.06[z] (n=5)	6.25±6.25[z] (n=1)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades
[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[z] Mean values within a row with same superscripts were not significantly different (P>0.05)

Appendix Table 3.47: Developmental competence (%, mean±SEM) of *in vitro* produced goat embryos at three stimulation response groups regardless of the oocyte grades

Stimulation response	No. of oocytes injected	Percent cleavage (2-cell)	Percent development*		
			4-cell	8-cell	Morula
Excellent	4.33±1.45[a] (n=13)	53.57±26.96[a] (n=9)	53.57±26.96[a] (n=9)	53.57±26.96[a] (n=9)	26.19±14.48[a] (n=4)
Good	3.56±0.44[a] (n=96)	61.14±6.27[a] (n=58)	54.47±6.34[a] (n=51)	45.27±6.35[a] (n=43)	17.39±5.15[a] (n=17)
Satisfactory	2.14±0.26[a] (n=15)	42.86±17.39[a] (n=6)	28.57±15.31[a] (n=5)	28.57±15.31[a] (n=5)	7.14±7.14[a] (n=1)
Average	3.35±0.35 (n=124)	57.07±5.91 (n=73)	49.50±5.89 (n=65)	42.78±5.77 (n=57)	16.16±4.15 (n=22)

* Mean percentage of development was based on oocytes used for ICSI, in the respective grades

[a] Mean values within a column with same superscript were not significantly different (P>0.05)

Appendix Table 3.48: Percentage (%, mean ±SEM) of immature oocyte following sham injection using different activation regimes at 22 to 25 hours of IVM duration

Treatment	No. of immature oocytes injected per ovary	Percent of immature oocytes injected per ovary				
		Grade A	Grade B	Grade C	Grade D	Grade E
Control	4.00 (n=4)	25.00 (n=1)	25.00 (n=1)	50.00 (n=2)	0.00 (n=0)	0.00 (n=0)
Ca^{2+} ionophore	1.33±0.33[a] (n=4)	0.00±0.00[ay] (n=0)	16.67±16.67[ay] (n=1)	83.33±16.67[az] (n=3)	0.00±0.00[ay] (n=0)	0.00±0.00[ay] (n=0)
Ca^{2+} ionophore + 6-DMAP	2.17±0.31[a] (n=13)	11.11±11.11[ayz] (n=2)	0.00±0.00[ay] (n=0)	47.22±18.47[az] (n=6)	25.00±17.08[ayz] (n=4)	16.67±16.67[ayz] (n=1)
Average	2.10±0.32 (n=21)	9.17±6.86[y] (n=3)	7.50±5.34[y] (n=2)	58.33±12.73[z] (n=11)	15.00±10.68[y] (n=4)	10.00±10.00[y] (n=1)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.49: Number (n, mean ±SEM) of immature oocyte following sham injection using different activation regimes at 22 to 25 hours of IVM duration

Treatment	No. of immature oocytes injected per ovary	No. of immature oocytes injected per ovary				
		Grade A	Grade B	Grade C	Grade D	Grade E
Control	4.00 (n=4)	1.00 (n=1)	1.00 (n=1)	2.00 (n=2)	0.00 (n=0)	0.00 (n=0)
Ca^{2+} ionophore	1.33±0.33[a] (n=4)	0.00±0.00[ay] (n=0)	0.33±0.33[ay] (n=1)	1.00±0.00[az] (n=3)	0.00±0.00[ay] (n=0)	0.00±0.00[ay] (n=0)
Ca^{2+} ionophore + 6-DMAP	2.17±0.31[a] (n=13)	0.33±0.33[az] (n=2)	0.00±0.00[az] (n=0)	1.00±0.37[az] (n=6)	0.67±0.49[az] (n=4)	0.17±0.17[az] (n=1)
Average	2.10±0.32 (n=21)	0.30±0.21[y] (n=3)	0.20±0.13[y] (n=2)	1.10±0.23[z] (n=11)	0.40±0.31[y] (n=4)	0.10±0.10[y] (n=1)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.50: Percentage (%, mean ±SEM) of immature oocyte following ICSI using different activation regimes at 22 to 25 hours of IVM duration

Treatment	No. of immature oocytes injected per ovary	Percent of immature oocytes injected per ovary				
		Grade A	Grade B	Grade C	Grade D	Grade E
Control	3.33±1.20[a] (n=10)	15.00±7.64[ay] (n=2)	6.67±6.67[ay] (n=1)	70.00±15.28[az] (n=6)	8.33±8.33[ay] (n=1)	0.00±0.00[ay] (n=0)
Ca^{2+} ionophore	1.80±0.29[a] (n=18)	10.00±10.00[ay] (n=1)	7.50±5.34[ay] (n=2)	52.50±10.83[az] (n=10)	25.00±11.18[ay] (n=4)	5.00±5.00[ay] (n=1)
Ca^{2+} ionophore + 6-DMAP	1.83±0.40[a] (n=11)	22.22±16.48[az] (n=2)	5.56±5.56[az] (n=1)	47.22±16.34[az] (n=6)	8.33±8.33[az] (n=1)	16.67±16.67[az] (n=1)
Average	2.05±0.28 (n=39)	14.65±7.27[y] (n=5)	6.75±3.32[y] (n=4)	53.60±7.84[z] (n=22)	17.11±6.64[y] (n=6)	7.90±5.75[y] (n=2)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.51: Number (n, mean ±SEM) of immature oocyte following ICSI using different activation regimes at 22 to 25 hours of IVM duration

Treatment	No. of immature oocytes injected per ovary	No. of immature oocytes injected per ovary				
		Grade A	Grade B	Grade C	Grade D	Grade E
Control	3.33±1.20[a] (n=10)	0.67±0.33[ay] (n=2)	0.33±0.33[ay] (n=1)	2.00±0.58[az] (n=6)	0.33±0.33[ay] (n=1)	0.00±0.00[ay] (n=0)
Ca^{2+} ionophore	1.80±0.29[a] (n=18)	0.10±0.10[ay] (n=1)	0.20±0.13[ay] (n=2)	1.00±0.26[az] (n=10)	0.40±0.16[ay] (n=4)	0.10±0.10[ay] (n=1)
Ca^{2+} ionophore + 6-DMAP	1.83±0.40[a] (n=11)	0.33±0.21[ay] (n=2)	0.17±0.17[ay] (n=1)	1.00±0.37[az] (n=6)	0.17±0.17[ay] (n=1)	0.17±0.17[ay] (n=1)
Average	2.05±0.28 (n=39)	0.26±0.10[z] (n=5)	0.21±0.10[z] (n=4)	1.16±0.21[y] (n=22)	0.32±0.11[z] (n=6)	0.11±0.07[z] (n=2)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.52: Percentage (%, mean ±SEM) of immature oocyte following sham injection using different activation regimes at 26 to 29 hours of IVM duration

Treatment	No. of immature oocytes injected per ovary	Percent of immature oocytes injected per ovary				
		Grade A	Grade B	Grade C	Grade D	Grade E
Control	2.50±0.40[a] (n=25)	19.50 ±10.39[az] (n=4)	15.33 ±6.80[az] (n=4)	36.50 ±10.25[az] (n=10)	12.83 ±5.76[az] (n=4)	15.83 ±10.13[az] (n=3)
Ca^{2+} ionophore	2.75±0.68[a] (n=22)	25.42 ±12.57[az] (n=4)	16.67 ±9.45[az] (n=4)	32.50 ±12.31[az] (n=8)	18.75 ±12.38[az] (n=3)	6.67 ±4.54[az] (n=3)
Ca^{2+} ionophore + 6-DMAP	1.75±0.48[a] (n=7)	8.33 ±8.33[az] (n=1)	0.00 ±0.00[az] (n=0)	33.33 ±23.57[az] (n=2)	20.83 ±12.50[az] (n=2)	37.50 ±23.94[az] (n=2)
Average	2.46±0.31 (n=54)	19.62 ±6.61[yz] (n=9)	13.03 ±4.65[y] (n=8)	34.47 ±7.31[z] (n=20)	16.44 ±5.44[yz] (n=9)	16.44 ±6.55[yz] (n=8)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.53: Number (n, mean ±SEM) of immature oocyte following sham injection using different activation regimes at 26 to 29 hours of IVM duration

Treatment	No. of immature oocytes injected per ovary	No. of immature oocytes injected per ovary				
		Grade A	Grade B	Grade C	Grade D	Grade E
Control	2.50±0.40[a] (n=25)	0.40±0.16[ay] (n=4)	0.40±0.16[ay] (n=4)	1.00±0.26[az] (n=10)	0.40±0.16[ay] (n=4)	0.30±0.15[ay] (n=3)
Ca^{2+} ionophore	2.75±0.68[a] (n=22)	0.50±0.19[az] (n=4)	0.50±0.27[az] (n=4)	1.00±0.38[az] (n=8)	0.38±0.18[az] (n=3)	0.38±0.26[az] (n=3)
Ca^{2+} ionophore + 6-DMAP	1.75±0.48[a] (n=7)	0.25±0.25[az] (n=1)	0.00 ±0.00[az] (n=0)	0.50±0.29[az] (n=2)	0.50±0.29[az] (n=2)	0.50±0.29[az] (n=2)
Average	2.46±0.31 (n=54)	0.41±0.11[z] (n=9)	0.36±0.12[z] (n=8)	0.91±0.19[y] (n=20)	0.41±0.11[z] (n=9)	0.36±0.12[z] (n=8)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.54: Percentage (%, mean ±SEM) of immature oocyte following ICSI using different activation regimes at 26 to 29 hours of IVM duration

Treatment	No. of immature oocytes injected per ovary	Percent of immature oocytes injected per ovary				
		Grade A	Grade B	Grade C	Grade D	Grade E
Control	2.00±0.00[a] (n=6)	16.67±16.67[az] (n=1)	16.67±16.67[az] (n=1)	16.67±16.67[az] (n=1)	33.33±16.67[az] (n=2)	16.67±16.67[az] (n=1)
Ca^{2+} ionophore	3.00±0.66[a] (n=24)	5.00±5.00[az] (n=2)	9.17±4.70[az] (n=3)	20.00±12.54[az] (n=4)	32.50±12.75[az] (n=7)	33.33±12.86[az] (n=8)
Ca^{2+} ionophore + 6-DMAP	2.85±0.47[a] (n=37)	11.15±5.64[ay] (n=5)	7.31±4.26[ay] (n=3)	47.44±10.02[az] (n=17)	14.87±8.71[ay] (n=5)	19.23±10.65[ay] (n=7)
Average	2.79±0.33 (n=67)	9.79±3.90[y] (n=8)	9.10±3.28[y] (n=7)	34.44±7.49[az] (n=22)	23.06±6.67[ayz] (n=14)	23.61±7.53[ayz] (n=16)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

Appendix Table 3.55: Number (n, mean ±SEM) of immature oocyte following ICSI using different activation regimes at 26 to 29 hours of IVM duration

Treatment	No. of immature oocytes injected per ovary	No. of immature oocytes injected per ovary				
		Grade A	Grade B	Grade C	Grade D	Grade E
Control	2.00±0.00[a] (n=6)	0.33±0.33[az] (n=1)	0.33±0.33[az] (n=1)	0.33±0.33[az] (n=1)	0.67±0.33[az] (n=2)	0.33±0.33[az] (n=1)
Ca^{2+} ionophore	3.00±0.66[a] (n=24)	0.25±0.25[az] (n=2)	0.38±0.18[az] (n=3)	0.50±0.27[az] (n=4)	0.88±0.30[az] (n=7)	1.00±0.42[az] (n=8)
Ca^{2+} ionophore + 6-DMAP	2.85±0.47[a] (n=37)	0.39±0.18[ay] (n=5)	0.23±0.12[ay] (n=3)	1.31±0.29[az] (n=17)	0.39±0.24[ay] (n=5)	0.54±0.29[ay] (n=7)
Average	2.79±0.33 (n=67)	0.33±0.13[y] (n=8)	0.29±0.10[y] (n=7)	0.92±0.20[z] (n=22)	0.58±0.17[yz] (n=14)	0.67±0.21[yz] (n=16)

[a] Mean values within a column with same superscript were not significantly different (P>0.05)
[yz] Mean values within a row with different superscripts were significantly different (P<0.05)

APPENDIX 4: STATISTICAL DATA OF SUPPLEMENTARY RESULTS

Appendix Table 4.1: Effect of goat genotype on oocyte maturation per ovary

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_Jamnapari	GradeA	6	55.5550	50.18514	20.48800	2.8889	108.2211	.00	100.00
	GradeB	6	5.5550	13.60692	5.55500	-8.7246	19.8346	.00	33.33
	GradeC	6	38.8883	49.06541	20.03087	-12.6027	90.3793	.00	100.00
	GradeD	6	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeE	6	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	30	19.9997	37.75286	6.89270	5.9025	34.0968	.00	100.00
P_Boer	GradeA	63	31.3737	30.90175	3.89325	23.5911	39.1562	.00	100.00
	GradeB	63	29.3160	31.25255	3.93745	21.4452	37.1869	.00	100.00
	GradeC	63	35.8713	33.37576	4.20495	27.4657	44.2768	.00	100.00
	GradeD	63	1.5873	7.75642	.97722	-.3661	3.5407	.00	50.00
	GradeE	63	1.8519	12.73921	1.60499	-1.3564	5.0602	.00	100.00
	Total	315	20.0000	29.55147	1.66504	16.7240	23.2761	.00	100.00
N_Jamnapari	GradeA	6	1.0000	.89443	.36515	.0614	1.9386	.00	2.00
	GradeB	6	.1667	.40825	.16667	-.2618	.5951	.00	1.00
	GradeC	6	.8333	.98319	.40139	-.1985	1.8651	.00	2.00
	GradeD	6	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeE	6	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	30	.4000	.72397	.13218	.1297	.6703	.00	2.00
N_Boer	GradeA	63	1.2857	1.44165	.18163	.9226	1.6488	.00	7.00
	GradeB	63	.9683	.99949	.12592	.7165	1.2200	.00	4.00
	GradeC	63	1.2381	1.20100	.15131	.9356	1.5406	.00	5.00
	GradeD	63	.0635	.30443	.03835	-.0132	.1402	.00	2.00
	GradeE	63	.0476	.27989	.03526	-.0229	.1181	.00	2.00
	Total	315	.7206	1.11077	.06258	.5975	.8438	.00	7.00

Appendix Table 4.2: Effect of oocyte quality and goat genotype on the maturation rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_GradeA	Jamnapari	5	73.3340	43.46122	19.43645	19.3698	127.2982	.00	100.00
	Boer	45	75.6544	35.00889	5.21882	65.1366	86.1723	.00	100.00
	Total	50	75.4224	35.42948	5.01048	65.3535	85.4913	.00	100.00
P_GradeB	Jamnapari	4	12.5000	25.00000	12.50000	-27.2806	52.2806	.00	50.00
	Boer	40	79.2088	30.16553	4.76959	69.5613	88.8562	.00	100.00
	Total	44	73.1443	35.28799	5.31987	62.4158	83.8728	.00	100.00
P_GradeC	Jamnapari	7	28.5714	39.33979	14.86904	-7.8118	64.9547	.00	100.00
	Boer	58	50.6036	35.66390	4.68290	41.2263	59.9810	.00	100.00
	Total	65	48.2309	36.40415	4.51538	39.2104	57.2514	.00	100.00
P_GradeD	Jamnapari	5	.0000	.00000	.00000	.0000	.0000	.00	.00
	Boer	24	11.1113	30.56131	6.23830	-1.7937	24.0162	.00	100.00
	Total	29	9.1955	28.02596	5.20429	-1.4650	19.8560	.00	100.00
P_GradeE	Jamnapari	4	.0000	.00000	.00000	.0000	.0000	.00	.00
	Boer	14	7.1429	19.29825	5.15767	-3.9996	18.2853	.00	66.67
	Total	18	5.5556	17.15024	4.04235	-2.9731	14.0842	.00	66.67

Appendix Table 4.3: Effect of Boer goat on the developmental rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	GradeA	20	73.7500	34.75880	7.77230	57.4824	90.0176	.00	100.00
	GradeB	17	72.5488	36.77146	8.91839	53.6427	91.4550	.00	100.00
	GradeC	25	44.2000	43.89371	8.77874	26.0816	62.3184	.00	100.00
	Total	62	61.5053	41.16901	5.22847	51.0504	71.9603	.00	100.00
P_4cell	GradeA	20	72.5000	36.08218	8.06822	55.6130	89.3870	.00	100.00
	GradeB	17	63.7253	42.99372	10.42751	41.6200	85.8306	.00	100.00
	GradeC	25	28.2000	38.29696	7.65939	12.3918	44.0082	.00	100.00
	Total	62	52.2311	43.30820	5.50015	41.2329	63.2294	.00	100.00
P_8cell	GradeA	20	62.5000	40.86985	9.13878	43.3723	81.6277	.00	100.00
	GradeB	17	54.9018	46.68339	11.32239	30.8994	78.9041	.00	100.00
	GradeC	25	24.6000	33.94344	6.78869	10.5888	38.6112	.00	100.00
	Total	62	45.1344	42.93579	5.45285	34.2307	56.0380	.00	100.00
P_Morula	GradeA	20	26.2500	37.97473	8.49141	8.4773	44.0227	.00	100.00
	GradeB	17	31.3724	46.35399	11.24249	7.5393	55.2054	.00	100.00
	GradeC	25	2.0000	10.00000	2.00000	-2.1278	6.1278	.00	50.00
	Total	62	17.8763	35.05940	4.45255	8.9729	26.7797	.00	100.00

Appendix Table 4.4: Effect of body weight on oocyte maturation per ovary

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_Light	GradeA	18	30.3833	25.05111	5.90460	17.9257	42.8410	.00	83.33
	GradeB	18	25.1056	28.16123	6.63767	11.1013	39.1098	.00	100.00
	GradeC	18	41.7333	27.61892	6.50984	27.9988	55.4679	.00	100.00
	GradeD	18	2.7778	11.78511	2.77778	-3.0828	8.6384	.00	50.00
	GradeE	18	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	90	20.0000	26.59096	2.80293	14.4306	25.5694	.00	100.00
P_Medium	GradeA	16	40.0669	40.43380	10.10845	18.5212	61.6125	.00	100.00
	GradeB	16	37.2169	35.32554	8.83138	18.3932	56.0405	.00	100.00
	GradeC	16	22.7156	25.18867	6.29717	9.2935	36.1377	.00	71.43
	GradeD	16	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeE	16	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	80	19.9999	31.19202	3.48737	13.0584	26.9413	.00	100.00
P_Heavy	GradeA	6	16.6667	40.82483	16.66667	-26.1764	59.5097	.00	100.00
	GradeB	6	8.3333	20.41241	8.33333	-13.0882	29.7548	.00	50.00
	GradeC	6	58.3333	49.15960	20.06932	6.7435	109.9232	.00	100.00
	GradeD	6	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeE	6	16.6667	40.82483	16.66667	-26.1764	59.5097	.00	100.00
	Total	30	20.0000	38.50660	7.03031	5.6214	34.3786	.00	100.00
N_Light	GradeA	18	1.6111	1.57700	.37170	.8269	2.3953	.00	5.00
	GradeB	18	1.0556	1.05564	.24882	.5306	1.5805	.00	3.00
	GradeC	18	1.6667	1.18818	.28006	1.0758	2.2575	.00	4.00
	GradeD	18	.1111	.47140	.11111	-.1233	.3455	.00	2.00
	GradeE	18	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	90	.8889	1.23110	.12977	.6310	1.1467	.00	5.00
N_Medium	GradeA	16	1.3750	1.74642	.43661	.4444	2.3056	.00	7.00
	GradeB	16	1.1875	1.10868	.27717	.5967	1.7783	.00	4.00
	GradeC	16	1.1875	1.55858	.38964	.3570	2.0180	.00	5.00
	GradeD	16	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeE	16	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	80	.7500	1.28772	.14397	.4634	1.0366	.00	7.00
N_Heavy	GradeA	6	.1667	.40825	.16667	-.2618	.5951	.00	1.00
	GradeB	6	.1667	.40825	.16667	-.2618	.5951	.00	1.00
	GradeC	6	1.0000	.89443	.36515	.0614	1.9386	.00	2.00
	GradeD	6	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeE	6	.3333	.81650	.33333	-.5235	1.1902	.00	2.00
	Total	30	.3333	.66089	.12066	.0866	.5801	.00	2.00

Appendix Table 4.5: Effect of oocyte quality and body weight on the maturation rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_GradeA	Light	17	66.7647	40.72456	9.87716	45.8261	87.7033	.00	100.00
	Medium	12	77.3150	39.10175	11.28770	52.4709	102.1591	.00	100.00
	Heavy	2	50.0000	70.71068	50.00000	-585.3102	685.3102	.00	100.00
	Total	31	69.7671	40.81365	7.33035	54.7965	84.7377	.00	100.00
P_GradeB	Light	12	64.5842	35.55571	10.26405	41.9931	87.1752	.00	100.00
	Medium	14	72.6193	42.16729	11.26968	48.2726	96.9660	.00	100.00
	Heavy	1	50.0000	.	.	.	.	50.00	50.00
	Total	27	68.2104	38.12070	7.33633	53.1303	83.2904	.00	100.00
P_GradeC	Light	20	50.5005	34.16574	7.63969	34.5104	66.4906	.00	100.00
	Medium	14	45.7143	39.94502	10.67575	22.6507	68.7779	.00	100.00
	Heavy	5	63.3340	41.49973	18.55924	11.8053	114.8627	.00	100.00
	Total	39	50.4277	36.61838	5.86363	38.5574	62.2980	.00	100.00
P_GradeD	Light	8	8.3338	23.57140	8.33375	-11.3724	28.0399	.00	66.67
	Medium	8	.0000	.00000	.00000	.0000	.0000	.00	.00
	Heavy	5	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	21	3.1748	14.54859	3.17476	-3.4477	9.7972	.00	66.67
P_GradeE	Light	2	.0000	.00000	.00000	.0000	.0000	.00	.00
	Medium	5	.0000	.00000	.00000	.0000	.0000	.00	.00
	Heavy	3	22.2233	38.49194	22.22333	-73.3960	117.8426	.00	66.67
	Total	10	6.6670	21.08291	6.66700	-8.4148	21.7488	.00	66.67

Appendix Table 4.6: Effect of body weight on oocyte cleavage per ovary

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_Light	GradeA	8	58.3338	32.12093	11.35646	31.4800	85.1875	.00	100.00
	GradeB	8	12.5000	23.14550	8.18317	-6.8501	31.8501	.00	50.00
	GradeC	8	29.1662	36.46040	12.89070	-1.3154	59.6479	.00	100.00
	Total	24	33.3333	35.44077	7.23432	18.3680	48.2987	.00	100.00
P_Medium	GradeA	8	29.1662	36.46040	12.89070	-1.3154	59.6479	.00	100.00
	GradeB	8	47.9163	39.27728	13.88661	15.0796	80.7529	.00	100.00
	GradeC	8	22.9162	26.63357	9.41639	.6500	45.1825	.00	66.67
	Total	24	33.3329	34.75268	7.09386	18.6581	48.0077	.00	100.00
N_Light	GradeA	8	1.3750	.91613	.32390	.6091	2.1409	.00	3.00
	GradeB	8	.2500	.46291	.16366	-.1370	.6370	.00	1.00
	GradeC	8	.8750	1.12599	.39810	-.0664	1.8164	.00	3.00
	Total	24	.8333	.96309	.19659	.4267	1.2400	.00	3.00
N_Medium	GradeA	8	.7500	.88641	.31339	.0089	1.4911	.00	2.00
	GradeB	8	1.1250	.83452	.29505	.4273	1.8227	.00	2.00
	GradeC	8	1.0000	1.41421	.50000	-.1823	2.1823	.00	4.00
	Total	24	.9583	1.04170	.21264	.5185	1.3982	.00	4.00

Appendix Table 4.7: Effect of oocyte quality and body weight on the cleavage rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_GradeA	Light	7	79.7614	27.99610	10.58153	53.8694	105.6535	33.33	100.00
	Medium	6	58.3333	49.15960	20.06932	6.7435	109.9232	.00	100.00
	Total	13	69.8715	39.01866	10.82183	46.2928	93.4503	.00	100.00
P_GradeB	Light	3	27.7767	25.45839	14.69841	-35.4655	91.0188	.00	50.00
	Medium	7	71.4286	39.33979	14.86904	35.0453	107.8118	.00	100.00
	Total	10	58.3330	40.25405	12.72945	29.5370	87.1290	.00	100.00
P_GradeC	Light	9	30.5556	39.08680	13.02893	.5108	60.6003	.00	100.00
	Medium	5	69.3340	41.25794	18.45111	18.1055	120.5625	.00	100.00
	Total	14	44.4050	42.84555	11.45095	19.6667	69.1433	.00	100.00

Appendix Table 4.8: Effect of body weight on the developmental rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	Light	9	51.4811	28.95718	9.65239	29.2227	73.7396	.00	100.00
	Medium	10	59.8090	37.30045	11.79544	33.1259	86.4921	.00	100.00
	Total	19	55.8642	32.96343	7.56233	39.9763	71.7521	.00	100.00
P_4cell	Light	9	50.3700	29.63667	9.87889	27.5892	73.1508	.00	100.00
	Medium	10	51.1420	41.75743	13.20486	21.2705	81.0135	.00	100.00
	Total	19	50.7763	35.52982	8.15110	33.6515	67.9011	.00	100.00
P_8cell	Light	9	46.2967	32.66775	10.88925	21.1860	71.4073	.00	100.00
	Medium	10	44.9520	42.75154	13.51922	14.3694	75.5346	.00	100.00
	Total	19	45.5889	37.26427	8.54901	27.6281	63.5498	.00	100.00
P_Morula	Light	9	11.9444	19.43651	6.47884	-2.9958	26.8847	.00	50.00
	Medium	10	10.7140	18.21047	5.75866	-2.3130	23.7410	.00	50.00
	Total	19	11.2968	18.27868	4.19342	2.4868	20.1069	.00	50.00

Appendix Table 4.9: Effect of oocyte quality (Grade A) and body weight on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	Light	7	79.7614	27.99610	10.58153	53.8694	105.6535	33.33	100.00
	Medium	6	58.3333	49.15960	20.06932	6.7435	109.9232	.00	100.00
	Total	13	69.8715	39.01866	10.82183	46.2928	93.4503	.00	100.00
P_4cell	Light	7	76.1900	33.48252	12.65520	45.2238	107.1562	25.00	100.00
	Medium	6	58.3333	49.15960	20.06932	6.7435	109.9232	.00	100.00
	Total	13	67.9485	40.66118	11.27738	43.3772	92.5198	.00	100.00
P_8cell	Light	7	61.9043	41.90446	15.83840	23.1491	100.6595	.00	100.00
	Medium	6	41.6667	49.15960	20.06932	-9.9232	93.2565	.00	100.00
	Total	13	52.5638	44.66770	12.38859	25.5714	79.5563	.00	100.00
P_Morula	Light	7	17.8571	37.40066	14.13612	-16.7327	52.4470	.00	100.00
	Medium	6	8.3333	20.41241	8.33333	-13.0882	29.7548	.00	50.00
	Total	13	13.4615	29.95723	8.30864	-4.6414	31.5645	.00	100.00

Appendix Table 4.10: Effect of oocyte quality (Grade B) and body weight on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	Light	3	27.7767	25.45839	14.69841	-35.4655	91.0188	.00	50.00
	Medium	7	71.4286	39.33979	14.86904	35.0453	107.8118	.00	100.00
	Total	10	58.3330	40.25405	12.72945	29.5370	87.1290	.00	100.00
P_4cell	Light	3	27.7767	25.45839	14.69841	-35.4655	91.0188	.00	50.00
	Medium	7	64.2857	47.55949	17.97580	20.3005	108.2709	.00	100.00
	Total	10	53.3330	44.30551	14.01063	21.6388	85.0272	.00	100.00
P_8cell	Light	3	11.1100	19.24308	11.11000	-36.6925	58.9125	.00	33.33
	Medium	7	64.2857	47.55949	17.97580	20.3005	108.2709	.00	100.00
	Total	10	48.3330	47.43428	15.00004	14.4006	82.2654	.00	100.00
P_Morula	Light	3	11.1100	19.24308	11.11000	-36.6925	58.9125	.00	33.33
	Medium	7	28.5714	48.79500	18.44278	-16.5564	73.6993	.00	100.00
	Total	10	23.3330	41.72210	13.19369	-6.5132	53.1792	.00	100.00

Appendix Table 4.11: Effect of oocyte quality (Grade C) and body weight on the cleavage and developmental rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	Light	9	30.5556	39.08680	13.02893	.5108	60.6003	.00	100.00
	Medium	5	69.3340	41.25794	18.45111	18.1055	120.5625	.00	100.00
	Total	14	44.4050	42.84555	11.45095	19.6667	69.1433	.00	100.00
P_4cell	Light	9	30.5556	39.08680	13.02893	.5108	60.6003	.00	100.00
	Medium	5	29.3340	40.44277	18.08656	-20.8823	79.5503	.00	80.00
	Total	14	30.1193	37.99746	10.15525	8.1802	52.0584	.00	100.00
P_8cell	Light	9	30.5556	39.08680	13.02893	.5108	60.6003	.00	100.00
	Medium	5	21.3340	30.69687	13.72806	-16.7812	59.4492	.00	66.67
	Total	14	27.2621	35.37138	9.45340	6.8393	47.6850	.00	100.00
P_Morula	Light	9	5.5556	16.66667	5.55556	-7.2556	18.3667	.00	50.00
	Medium	5	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	14	3.5714	13.36306	3.57143	-4.1442	11.2870	.00	50.00

Appendix Table 4.12: Effect of age on oocyte maturation per ovary

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_Group1	GradeA	11	24.8700	23.65024	7.13081	8.9816	40.7584	.00	66.67
	GradeB	11	34.1127	33.49097	10.09791	11.6132	56.6123	.00	100.00
	GradeC	11	36.4718	21.67145	6.53419	21.9127	51.0309	.00	66.67
	GradeD	11	4.5455	15.07557	4.54545	-5.5824	14.6734	.00	50.00
	GradeE	11	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	55	20.0000	25.90673	3.49326	12.9964	27.0036	.00	100.00
P_Group2	GradeA	14	34.4214	33.13428	8.85551	15.2903	53.5526	.00	100.00
	GradeB	14	32.9929	32.44846	8.67222	14.2577	51.7280	.00	100.00
	GradeC	14	32.5857	27.63381	7.38545	16.6304	48.5410	.00	100.00
	GradeD	14	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeE	14	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	70	20.0000	28.63565	3.42261	13.1721	26.8279	.00	100.00
P_Group3	GradeA	7	42.7386	41.51313	15.69049	4.3453	81.1318	.00	100.00
	GradeB	7	18.1286	17.56710	6.63974	1.8817	34.3754	.00	40.00
	GradeC	7	39.1329	37.94477	14.34177	4.0398	74.2259	.00	100.00
	GradeD	7	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeE	7	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	35	20.0000	30.98233	5.23697	9.3572	30.6428	.00	100.00
N_Group1	GradeA	11	1.1818	1.25045	.37703	.3418	2.0219	.00	4.00
	GradeB	11	1.0909	.94388	.28459	.4568	1.7250	.00	2.00
	GradeC	11	1.7273	1.27208	.38355	.8727	2.5819	.00	4.00
	GradeD	11	.1818	.60302	.18182	-.2233	.5869	.00	2.00
	GradeE	11	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	55	.8364	1.11826	.15079	.5341	1.1387	.00	4.00
N_Group2	GradeA	14	1.5000	1.65250	.44165	.5459	2.4541	.00	5.00
	GradeB	14	1.2857	1.26665	.33853	.5544	2.0171	.00	4.00
	GradeC	14	1.1429	1.09945	.29384	.5081	1.7777	.00	4.00
	GradeD	14	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeE	14	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	70	.7857	1.21456	.14517	.4961	1.0753	.00	5.00
N_Group3	GradeA	7	2.0000	2.38048	.89974	-.2016	4.2016	.00	7.00
	GradeB	7	1.0000	1.00000	.37796	.0752	1.9248	.00	2.00
	GradeC	7	2.0000	1.91485	.72375	.2291	3.7709	.00	5.00
	GradeD	7	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeE	7	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	35	1.0000	1.62698	.27501	.4411	1.5589	.00	7.00

Appendix Table 4.13: Effect of oocyte quality and age on the maturation rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_GradeA	Group 1	12	45.5558	43.63160	12.59536	17.8336	73.2780	.00	100.00
	Group 2	9	93.1478	10.48995	3.49665	85.0845	101.2111	75.00	100.00
	Group 3	6	79.6300	40.01027	16.33412	37.6418	121.6182	.00	100.00
	Total	27	68.9919	40.35747	7.76680	53.0270	84.9567	.00	100.00
P_GradeB	Group 1	9	68.5189	42.85345	14.28448	35.5788	101.4590	.00	100.00
	Group 2	9	75.0011	20.41190	6.80397	59.3111	90.6911	50.00	100.00
	Group 3	5	80.0000	44.72136	20.00000	24.4711	135.5289	.00	100.00
	Total	23	73.5513	34.69299	7.23399	58.5489	88.5537	.00	100.00
P_GradeC	Group 1	12	56.3892	39.75840	11.47726	31.1279	81.6504	.00	100.00
	Group 2	13	48.2054	31.07252	8.61797	29.4284	66.9823	.00	100.00
	Group 3	7	49.5243	38.02697	14.37285	14.3552	84.6934	.00	100.00
	Total	32	51.5628	35.06009	6.19781	38.9223	64.2033	.00	100.00
P_GradeD	Group 1	5	13.3340	29.81573	13.33400	-23.6871	50.3551	.00	66.67
	Group 2	6	.0000	.00000	.00000	.0000	.0000	.00	.00
	Group 3	2	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	13	5.1285	18.49093	5.12846	-6.0455	16.3024	.00	66.67
P_GradeE	Group 1	2	.0000	.00000	.00000	.0000	.0000	.00	.00
	Group 2	0	.	.	.	.	.	.	.
	Group 3	2	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	4	.0000	.00000	.00000	.0000	.0000	.00	.00

Appendix Table 4.14: Effect of age on oocyte cleavage per ovary

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_Group1	GradeA	4	41.6675	28.86848	14.43424	-4.2687	87.6037	.00	66.67
	GradeB	4	37.5000	47.87136	23.93568	-38.6740	113.6740	.00	100.00
	GradeC	4	20.8325	24.99944	12.49972	-18.9472	60.6122	.00	50.00
	Total	12	33.3333	33.33364	9.62259	12.1542	54.5125	.00	100.00
P_Group2	GradeA	8	47.9163	38.25320	13.52455	15.9358	79.8967	.00	100.00
	GradeB	8	25.0000	28.17237	9.96044	1.4473	48.5527	.00	66.67
	GradeC	8	27.0825	35.56500	12.57413	-2.6506	56.8156	.00	100.00
	Total	24	33.3329	34.40326	7.02254	18.8057	47.8601	.00	100.00
P_Group3	GradeA	2	50.0000	70.71068	50.00000	-585.3102	685.3102	.00	100.00
	GradeB	2	16.6650	23.56787	16.66500	-195.0839	228.4139	.00	33.33
	GradeC	2	33.3350	47.14281	33.33500	-390.2263	456.8963	.00	66.67
	Total	6	33.3333	42.16423	17.21347	-10.9153	77.5820	.00	100.00
N_Group1	GradeA	4	1.5000	1.29099	.64550	-.5543	3.5543	.00	3.00
	GradeB	4	.5000	.57735	.28868	-.4187	1.4187	.00	1.00
	GradeC	4	1.0000	1.41421	.70711	-1.2503	3.2503	.00	3.00
	Total	12	1.0000	1.12815	.32567	.2832	1.7168	.00	3.00
N_Group2	GradeA	8	1.0000	.75593	.26726	.3680	1.6320	.00	2.00
	GradeB	8	.7500	.88641	.31339	.0089	1.4911	.00	2.00
	GradeC	8	.7500	.88641	.31339	.0089	1.4911	.00	2.00
	Total	24	.8333	.81650	.16667	.4886	1.1781	.00	2.00
N_Group3	GradeA	2	1.0000	1.41421	1.00000	-11.7062	13.7062	.00	2.00
	GradeB	2	1.0000	1.41421	1.00000	-11.7062	13.7062	.00	2.00
	GradeC	2	2.0000	2.82843	2.00000	-23.4124	27.4124	.00	4.00
	Total	6	1.3333	1.63299	.66667	-.3804	3.0471	.00	4.00

Appendix Table 4.15: Effect of oocyte quality and age on the cleavage rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_GradeA	Group 1	3	75.0000	25.00000	14.43376	12.8966	137.1034	50.00	100.00
	Group 2	6	80.5550	30.58183	12.48498	48.4613	112.6487	33.33	100.00
	Group 3	1	100.0000	.	.	.	.	100.00	100.00
	Total	10	80.8330	26.65864	8.43020	61.7626	99.9034	33.33	100.00
P_GradeB	Group 1	2	75.0000	35.35534	25.00000	-242.6551	392.6551	50.00	100.00
	Group 2	6	38.8883	37.51553	15.31565	-.4818	78.2585	.00	100.00
	Group 3	1	100.0000	.	.	.	.	100.00	100.00
	Total	9	53.7033	39.77194	13.25731	23.1319	84.2748	.00	100.00
P_GradeC	Group 1	3	41.6667	38.18813	22.04793	-53.1979	136.5312	.00	75.00
	Group 2	9	35.1856	44.44474	14.81491	1.0223	69.3488	.00	100.00
	Group 3	2	40.0000	56.56854	40.00000	-468.2482	548.2482	.00	80.00
	Total	14	37.2621	41.16696	11.00233	13.4930	61.0312	.00	100.00

Appendix Table 4.16: Effect of age on the developmental rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	Group 1	4	62.5000	19.83917	9.91958	30.9315	94.0685	33.33	75.00
	Group 2	9	51.0044	29.24896	9.74965	28.5217	73.4872	.00	100.00
	Group 3	3	61.9033	54.08390	31.22535	-72.4485	196.2552	.00	100.00
	Total	16	55.9219	30.95478	7.73870	39.4272	72.4165	.00	100.00
P_4cell	Group 1	4	54.1675	36.32454	18.16227	-3.6329	111.9679	.00	75.00
	Group 2	9	47.6711	29.79992	9.93331	24.7649	70.5774	.00	100.00
	Group 3	3	61.9033	54.08390	31.22535	-72.4485	196.2552	.00	100.00
	Total	16	51.9638	34.05337	8.51334	33.8180	70.1095	.00	100.00
P_8cell	Group 1	4	54.1675	36.32454	18.16227	-3.6329	111.9679	.00	75.00
	Group 2	9	43.5978	32.43686	10.81229	18.6646	68.5310	.00	100.00
	Group 3	3	52.3800	50.16964	28.96546	-72.2483	177.0083	.00	100.00
	Total	16	47.8869	34.44175	8.61044	29.5342	66.2396	.00	100.00
P_Morula	Group 1	4	21.8750	25.76941	12.88471	-19.1299	62.8799	.00	50.00
	Group 2	9	5.3967	10.92090	3.64030	-2.9979	13.7912	.00	28.57
	Group 3	3	26.1900	25.08482	14.48273	-36.1242	88.5042	.00	50.00
	Total	16	13.4150	19.25157	4.81289	3.1566	23.6734	.00	50.00

Appendix Table 4.17: Effect of oocyte quality (Grade A) and age on the cleavage and developmental rates

Descriptives

						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
P_2cell	Group 1	3	75.0000	25.00000	14.43376	12.8966	137.1034	50.00	100.00
	Group 2	6	80.5550	30.58183	12.48498	48.4613	112.6487	33.33	100.00
	Group 3	1	100.0000	.	.	.	.	100.00	100.00
	Total	10	80.8330	26.65864	8.43020	61.7626	99.9034	33.33	100.00
P_4cell	Group 1	3	75.0000	25.00000	14.43376	12.8966	137.1034	50.00	100.00
	Group 2	6	76.3883	36.67376	14.97200	37.9016	114.8751	25.00	100.00
	Group 3	1	100.0000	.	.	.	.	100.00	100.00
	Total	10	78.3330	30.73236	9.71842	56.3484	100.3176	25.00	100.00
P_8cell	Group 1	3	75.0000	25.00000	14.43376	12.8966	137.1034	50.00	100.00
	Group 2	6	59.7217	45.46608	18.56145	12.0079	107.4354	.00	100.00
	Group 3	1	100.0000	.	.	.	.	100.00	100.00
	Total	10	68.3330	38.24904	12.09541	40.9713	95.6947	.00	100.00
P_Morula	Group 1	3	41.6667	52.04165	30.04626	-87.6120	170.9453	.00	100.00
	Group 2	6	.0000	.00000	.00000	.0000	.0000	.00	.00
	Group 3	1	50.0000	.	.	.	.	50.00	50.00
	Total	10	17.5000	33.43734	10.57381	-6.4196	41.4196	.00	100.00

Appendix Table 4.18: Effect of oocyte quality (Grade B) and age on the cleavage and developmental rates

Descriptives

						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
P_2cell	Group 1	2	75.0000	35.35534	25.00000	-242.6551	392.6551	50.00	100.00
	Group 2	6	38.8883	37.51553	15.31565	-.4818	78.2585	.00	100.00
	Group 3	1	100.0000	.	.	.	.	100.00	100.00
	Total	9	53.7033	39.77194	13.25731	23.1319	84.2748	.00	100.00
P_4cell	Group 1	2	50.0000	70.71068	50.00000	-585.3102	685.3102	.00	100.00
	Group 2	6	38.8883	37.51553	15.31565	-.4818	78.2585	.00	100.00
	Group 3	1	100.0000	.	.	.	.	100.00	100.00
	Total	9	48.1478	43.65635	14.55212	14.5905	81.7050	.00	100.00
P_8cell	Group 1	2	50.0000	70.71068	50.00000	-585.3102	685.3102	.00	100.00
	Group 2	6	30.5550	40.02310	16.33936	-11.4467	72.5567	.00	100.00
	Group 3	1	100.0000	.	.	.	.	100.00	100.00
	Total	9	42.5922	46.48120	15.49373	6.8636	78.3208	.00	100.00
P_Morula	Group 1	2	.0000	.00000	.00000	.0000	.0000	.00	.00
	Group 2	6	22.2217	40.36849	16.48037	-20.1425	64.5858	.00	100.00
	Group 3	1	100.0000	.	.	.	.	100.00	100.00
	Total	9	25.9256	43.39020	14.46340	-7.4271	59.2782	.00	100.00

Appendix Table 4.19: Effect of oocyte quality (Grade C) and age on the cleavage and developmental rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	Group 1	3	41.6667	38.18813	22.04793	-53.1979	136.5312	.00	75.00
	Group 2	9	35.1856	44.44474	14.81491	1.0223	69.3488	.00	100.00
	Group 3	2	40.0000	56.56854	40.00000	-468.2482	548.2482	.00	80.00
	Total	14	37.2621	41.16696	11.00233	13.4930	61.0312	.00	100.00
P_4cell	Group 1	3	41.6667	38.18813	22.04793	-53.1979	136.5312	.00	75.00
	Group 2	9	24.0744	38.28949	12.76316	-5.3575	53.5064	.00	100.00
	Group 3	2	40.0000	56.56854	40.00000	-468.2482	548.2482	.00	80.00
	Total	14	30.1193	37.99746	10.15525	8.1802	52.0584	.00	100.00
P_8cell	Group 1	3	41.6667	38.18813	22.04793	-53.1979	136.5312	.00	75.00
	Group 2	9	24.0744	38.28949	12.76316	-5.3575	53.5064	.00	100.00
	Group 3	2	20.0000	28.28427	20.00000	-234.1241	274.1241	.00	40.00
	Total	14	27.2621	35.37138	9.45340	6.8393	47.6850	.00	100.00
P_Morula	Group 1	3	16.6667	28.86751	16.66667	-55.0442	88.3775	.00	50.00
	Group 2	9	.0000	.00000	.00000	.0000	.0000	.00	.00
	Group 3	2	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	14	3.5714	13.36306	3.57143	-4.1442	11.2870	.00	50.00

Appendix Table 4.20: Effect of OR cycle on oocyte maturation per ovary

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_OR1	GradeA	40	32.1992	34.35207	5.43154	21.2129	43.1856	.00	100.00
	GradeB	40	27.4343	31.25640	4.94207	17.4380	37.4305	.00	100.00
	GradeC	40	36.6163	32.40798	5.12415	26.2517	46.9808	.00	100.00
	GradeD	40	1.2500	7.90569	1.25000	-1.2784	3.7784	.00	50.00
	GradeE	40	2.5000	15.81139	2.50000	-2.5567	7.5567	.00	100.00
	Total	200	20.0000	30.30767	2.14308	15.7739	24.2260	.00	100.00
P_OR2	GradeA	25	38.8760	32.78677	6.55735	25.3423	52.4097	.00	100.00
	GradeB	25	27.6480	31.24131	6.24826	14.7522	40.5438	.00	100.00
	GradeC	25	32.1428	39.33818	7.86764	15.9048	48.3808	.00	100.00
	GradeD	25	.6668	3.33400	.66680	-.7094	2.0430	.00	16.67
	GradeE	25	.6668	3.33400	.66680	-.7094	2.0430	.00	16.67
	Total	125	20.0001	31.06209	2.77828	14.5011	25.4991	.00	100.00
P_OR3	GradeA	4	12.5000	14.43376	7.21688	-10.4673	35.4673	.00	25.00
	GradeB	4	22.9175	31.45919	15.72959	-27.1411	72.9761	.00	66.67
	GradeC	4	56.2500	18.47997	9.23998	26.8442	85.6558	33.33	75.00
	GradeD	4	8.3325	16.66500	8.33250	-18.1852	34.8502	.00	33.33
	GradeE	4	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	20	20.0000	26.26941	5.87402	7.7055	32.2945	.00	75.00
N_OR1	GradeA	40	1.3000	1.58842	.25115	.7920	1.8080	.00	7.00
	GradeB	40	.9750	1.04973	.16598	.6393	1.3107	.00	4.00
	GradeC	40	1.3750	1.31437	.20782	.9546	1.7954	.00	5.00
	GradeD	40	.0500	.31623	.05000	-.0511	.1511	.00	2.00
	GradeE	40	.0500	.31623	.05000	-.0511	.1511	.00	2.00
	Total	200	.7500	1.19778	.08470	.5830	.9170	.00	7.00
N_OR2	GradeA	25	1.3200	1.14455	.22891	.8476	1.7924	.00	3.00
	GradeB	25	.8000	.91287	.18257	.4232	1.1768	.00	3.00
	GradeC	25	.8000	.86603	.17321	.4425	1.1575	.00	3.00
	GradeD	25	.0400	.20000	.04000	-.0426	.1226	.00	1.00
	GradeE	25	.0400	.20000	.04000	-.0426	.1226	.00	1.00
	Total	125	.6000	.90696	.08112	.4394	.7606	.00	3.00
N_OR3	GradeA	4	.5000	.57735	.28868	-.4187	1.4187	.00	1.00
	GradeB	4	.7500	.95743	.47871	-.7735	2.2735	.00	2.00
	GradeC	4	2.0000	.81650	.40825	.7008	3.2992	1.00	3.00
	GradeD	4	.2500	.50000	.25000	-.5456	1.0456	.00	1.00
	GradeE	4	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	20	.7000	.92338	.20647	.2678	1.1322	.00	3.00

Appendix Table 4.21: Effect of oocyte quality and OR cycle on the maturation rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_GradeA	OR1	31	69.7671	40.81365	7.33035	54.7965	84.7377	.00	100.00
	OR2	17	82.8435	22.91110	5.55676	71.0637	94.6233	33.33	100.00
	OR3	2	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00
	Total	50	75.4224	35.42948	5.01048	65.3535	85.4913	.00	100.00
P_GradeB	OR1	27	68.2104	38.12070	7.33633	53.1303	83.2904	.00	100.00
	OR2	15	78.4447	30.75410	7.94067	61.4136	95.4757	.00	100.00
	OR3	2	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00
	Total	44	73.1443	35.28799	5.31987	62.4158	83.8728	.00	100.00
P_GradeC	OR1	39	48.8892	37.17421	5.95264	36.8387	60.9397	.00	100.00
	OR2	22	40.6059	37.15616	7.92172	24.1318	57.0800	.00	100.00
	OR3	4	68.7500	23.93568	11.96784	30.6630	106.8370	50.00	100.00
	Total	65	47.3078	36.68888	4.55070	38.2168	56.3989	.00	100.00
P_GradeD	OR1	21	3.1748	14.54859	3.17476	-3.4477	9.7972	.00	66.67
	OR2	6	16.6667	40.82483	16.66667	-26.1764	59.5097	.00	100.00
	OR3	2	50.0000	70.71068	50.00000	-585.3102	685.3102	.00	100.00
	Total	29	9.1955	28.02596	5.20429	-1.4650	19.8560	.00	100.00
P_GradeE	OR1	10	6.6670	21.08291	6.66700	-8.4148	21.7488	.00	66.67
	OR2	7	4.7614	12.59756	4.76143	-6.8894	16.4122	.00	33.33
	OR3	1	.0000	.	.	.	.	.00	.00
	Total	18	5.5556	17.15024	4.04235	-2.9731	14.0842	.00	66.67

Appendix Table 4.22: Effect of OR cycle on oocyte cleavage per ovary

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_OR1	GradeA	16	43.7500	36.45159	9.11290	24.3263	63.1737	.00	100.00
	GradeB	16	30.2081	36.11664	9.02916	10.9629	49.4533	.00	100.00
	GradeC	16	26.0412	31.01312	7.75328	9.5155	42.5670	.00	100.00
	Total	48	33.3331	34.72302	5.01184	23.2506	43.4156	.00	100.00
P_OR2	GradeA	12	45.2783	37.40229	10.79711	21.5140	69.0426	.00	100.00
	GradeB	12	27.3608	37.72208	10.88943	3.3934	51.3283	.00	100.00
	GradeC	12	27.3608	37.72208	10.88943	3.3934	51.3283	.00	100.00
	Total	36	33.3333	37.51630	6.25272	20.6396	46.0270	.00	100.00
P_OR3	GradeA	2	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeB	2	50.0000	70.71068	50.00000	-585.3102	685.3102	.00	100.00
	GradeC	2	50.0000	70.71068	50.00000	-585.3102	685.3102	.00	100.00
	Total	6	33.3333	51.63978	21.08185	-20.8593	87.5260	.00	100.00
N_OR1	GradeA	16	1.0625	.92871	.23218	.5676	1.5574	.00	3.00
	GradeB	16	.6875	.79320	.19830	.2648	1.1102	.00	2.00
	GradeC	16	.9375	1.23659	.30915	.2786	1.5964	.00	4.00
	Total	48	.8958	.99444	.14354	.6071	1.1846	.00	4.00
N_OR2	GradeA	12	1.2500	1.05529	.30464	.5795	1.9205	.00	3.00
	GradeB	12	.5000	.52223	.15076	.1682	.8318	.00	1.00
	GradeC	12	.5833	.66856	.19300	.1586	1.0081	.00	2.00
	Total	36	.7778	.83190	.13865	.4963	1.0593	.00	3.00
N_OR3	GradeA	2	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeB	2	.5000	.70711	.50000	-5.8531	6.8531	.00	1.00
	GradeC	2	.5000	.70711	.50000	-5.8531	6.8531	.00	1.00
	Total	6	.3333	.51640	.21082	-.2086	.8753	.00	1.00

Appendix Table 4.23: Effect of oocyte quality and OR cycle on the cleavage rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_GradeA	OR1	13	69.8715	39.01866	10.82183	46.2928	93.4503	.00	100.00
	OR2	9	74.0744	36.43051	12.14350	46.0715	102.0774	.00	100.00
	OR3	1	.0000	.	.	.	.	.00	.00
	Total	23	68.4783	39.24474	8.18309	51.5076	85.4490	.00	100.00
P_GradeB	OR1	11	53.0300	42.04390	12.67671	24.7845	81.2755	.00	100.00
	OR2	6	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00
	OR3	1	50.0000	.	.	.	.	50.00	50.00
	Total	18	68.5183	39.56021	9.32443	48.8455	88.1912	.00	100.00
P_GradeC	OR1	17	36.5688	42.37935	10.27850	14.7794	58.3583	.00	100.00
	OR2	7	78.5714	39.33979	14.86904	42.1882	114.9547	.00	100.00
	OR3	3	11.1100	19.24308	11.11000	-36.6925	58.9125	.00	33.33
	Total	27	44.6296	44.42023	8.54868	27.0576	62.2017	.00	100.00

Appendix Table 4.24: Effect of OR cycle on the developmental rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	OR1	22	48.2464	36.28217	7.73538	32.1598	64.3330	.00	100.00
	OR2	12	82.6392	20.24371	5.84385	69.7769	95.5014	50.00	100.00
	OR3	3	19.4433	17.34588	10.01465	-23.6462	62.5329	.00	33.33
	Total	37	57.0654	35.96274	5.91224	45.0748	69.0560	.00	100.00
P_4cell	OR1	22	43.8523	37.41822	7.97759	27.2620	60.4426	.00	100.00
	OR2	12	67.3617	28.52818	8.23538	49.2357	85.4876	.00	100.00
	OR3	3	19.4433	17.34588	10.01465	-23.6462	62.5329	.00	33.33
	Total	37	49.4978	35.82255	5.88919	37.5540	61.4417	.00	100.00
P_8cell	OR1	22	39.3723	38.03513	8.10912	22.5084	56.2361	.00	100.00
	OR2	12	54.8617	29.82666	8.61022	35.9107	73.8126	.00	100.00
	OR3	3	19.4433	17.34588	10.01465	-23.6462	62.5329	.00	33.33
	Total	37	42.7800	35.12227	5.77407	31.0697	54.4903	.00	100.00
P_Morula	OR1	22	9.7564	17.38176	3.70580	2.0497	17.4630	.00	50.00
	OR2	12	31.9442	32.92072	9.50339	11.0273	52.8610	.00	100.00
	OR3	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	37	16.1614	25.24403	4.15009	7.7446	24.5781	.00	100.00

Appendix Table 4.25: Effect of oocyte quality (Grade A) and OR cycle on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	OR1	13	69.8715	39.01866	10.82183	46.2928	93.4503	.00	100.00
	OR2	9	74.0744	36.43051	12.14350	46.0715	102.0774	.00	100.00
	OR3	1	.0000	.	.	.	.	.00	.00
	Total	23	68.4783	39.24474	8.18309	51.5076	85.4490	.00	100.00
P_4cell	OR1	13	67.9485	40.66118	11.27738	43.3772	92.5198	.00	100.00
	OR2	9	74.0744	36.43051	12.14350	46.0715	102.0774	.00	100.00
	OR3	1	.0000	.	.	.	.	.00	.00
	Total	23	67.3913	40.11633	8.36483	50.0437	84.7389	.00	100.00
P_8cell	OR1	13	52.5638	44.66770	12.38859	25.5714	79.5563	.00	100.00
	OR2	9	62.9633	42.31022	14.10341	30.4408	95.4858	.00	100.00
	OR3	1	.0000	.	.	.	.	.00	.00
	Total	23	54.3478	43.65504	9.10270	35.4700	73.2257	.00	100.00
P_Morula	OR1	13	13.4615	29.95723	8.30864	-4.6414	31.5645	.00	100.00
	OR2	9	38.8889	42.49216	14.16405	6.2265	71.5513	.00	100.00
	OR3	1	.0000	.	.	.	.	.00	.00
	Total	23	22.8261	36.42993	7.59617	7.0726	38.5796	.00	100.00

Appendix Table 4.26: Effect of oocyte quality (Grade B) and OR cycle on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	OR1	11	53.0300	42.04390	12.67671	24.7845	81.2755	.00	100.00
	OR2	6	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00
	OR3	1	50.0000	.	.	.	.	50.00	50.00
	Total	18	68.5183	39.56021	9.32443	48.8455	88.1912	.00	100.00
P_4cell	OR1	11	48.4845	45.00292	13.56889	18.2512	78.7179	.00	100.00
	OR2	6	83.3333	40.82483	16.66667	40.4903	126.1764	.00	100.00
	OR3	1	50.0000	.	.	.	.	50.00	50.00
	Total	18	60.1850	44.33208	10.44917	38.1392	82.2308	.00	100.00
P_8cell	OR1	11	43.9391	47.30096	14.26178	12.1619	75.7163	.00	100.00
	OR2	6	66.6667	51.63978	21.08185	12.4740	120.8593	.00	100.00
	OR3	1	50.0000	.	.	.	.	50.00	50.00
	Total	18	51.8517	47.10200	11.10205	28.4284	75.2749	.00	100.00
P_Morula	OR1	11	21.2118	40.20141	12.12118	-5.7959	48.2195	.00	100.00
	OR2	6	50.0000	54.77226	22.36068	-7.4800	107.4800	.00	100.00
	OR3	1	.0000	.	.	.	.	.00	.00
	Total	18	29.6294	45.57387	10.74186	6.9661	52.2928	.00	100.00

Appendix Table 4.27: Effect of oocyte quality (Grade C) and OR cycle on the cleavage and developmental rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	OR1	17	36.5688	42.37935	10.27850	14.7794	58.3583	.00	100.00
	OR2	7	78.5714	39.33979	14.86904	42.1882	114.9547	.00	100.00
	OR3	3	11.1100	19.24308	11.11000	-36.6925	58.9125	.00	33.33
	Total	27	44.6296	44.42023	8.54868	27.0576	62.2017	.00	100.00
P_4cell	OR1	17	24.8041	36.23770	8.78893	6.1724	43.4358	.00	100.00
	OR2	7	35.7143	47.55949	17.97580	-8.2709	79.6995	.00	100.00
	OR3	3	11.1100	19.24308	11.11000	-36.6925	58.9125	.00	33.33
	Total	27	26.1111	37.55634	7.22772	11.2543	40.9679	.00	100.00
P_8cell	OR1	17	22.4512	33.63494	8.15767	5.1577	39.7447	.00	100.00
	OR2	7	28.5714	39.33979	14.86904	-7.8118	64.9547	.00	100.00
	OR3	3	11.1100	19.24308	11.11000	-36.6925	58.9125	.00	33.33
	Total	27	22.7778	33.26609	6.40206	9.6182	35.9374	.00	100.00
P_Morula	OR1	17	2.9412	12.12678	2.94118	-3.2938	9.1762	.00	50.00
	OR2	7	.0000	.00000	.00000	.0000	.0000	.00	.00
	OR3	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	27	1.8519	9.62250	1.85185	-1.9547	5.6584	.00	50.00

Appendix Table 4.28: Effect of ovarian stimulation response on oocyte maturation per ovary

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_Excellent	GradeA	9	40.6744	35.05852	11.68617	13.7261	67.6228	.00	100.00
	GradeB	9	24.4711	26.21215	8.73738	4.3227	44.6196	.00	66.67
	GradeC	9	29.2989	20.92634	6.97545	13.2135	45.3843	.00	50.00
	GradeD	9	5.5556	16.66667	5.55556	-7.2556	18.3667	.00	50.00
	GradeE	9	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	45	20.0000	26.68748	3.97833	11.9822	28.0178	.00	100.00
P_Good	GradeA	47	32.1377	28.72309	4.18969	23.7042	40.5711	.00	100.00
	GradeB	47	30.7091	30.80699	4.49366	21.6639	39.7544	.00	100.00
	GradeC	47	36.4438	33.54245	4.89267	26.5954	46.2923	.00	100.00
	GradeD	47	.3547	2.43157	.35468	-.3593	1.0686	.00	16.67
	GradeE	47	.3547	2.43157	.35468	-.3593	1.0686	.00	16.67
	Total	235	20.0000	28.88289	1.88411	16.2880	23.7120	.00	100.00
P_Satisfactory	GradeA	13	33.3331	47.14045	13.07441	4.8464	61.8198	.00	100.00
	GradeB	13	16.6669	33.33375	9.24512	-3.4765	36.8103	.00	100.00
	GradeC	13	39.7438	45.91588	12.73477	11.9972	67.4905	.00	100.00
	GradeD	13	2.5638	9.24408	2.56385	-3.0223	8.1500	.00	33.33
	GradeE	13	7.6923	27.73501	7.69231	-9.0678	24.4524	.00	100.00
	Total	65	20.0000	37.29115	4.62540	10.7597	29.2403	.00	100.00
N_Excellent	GradeA	9	1.8889	2.08833	.69611	.2837	3.4941	.00	7.00
	GradeB	9	.8889	.92796	.30932	.1756	1.6022	.00	2.00
	GradeC	9	1.4444	1.33333	.44444	.4196	2.4693	.00	4.00
	GradeD	9	.2222	.66667	.22222	-.2902	.7347	.00	2.00
	GradeE	9	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	45	.8889	1.36885	.20406	.4776	1.3001	.00	7.00
N_Good	GradeA	47	1.2766	1.29719	.18921	.8957	1.6575	.00	5.00
	GradeB	47	1.0213	.98884	.14424	.7309	1.3116	.00	4.00
	GradeC	47	1.2979	1.23209	.17972	.9361	1.6596	.00	5.00
	GradeD	47	.0213	.14586	.02128	-.0216	.0641	.00	1.00
	GradeE	47	.0213	.14586	.02128	-.0216	.0641	.00	1.00
	Total	235	.7277	1.08323	.07066	.5884	.8669	.00	5.00
N_Satisfactory	GradeA	13	.7692	1.09193	.30285	.1094	1.4291	.00	3.00
	GradeB	13	.4615	.96742	.26831	-.1231	1.0461	.00	3.00
	GradeC	13	.6923	.75107	.20831	.2384	1.1462	.00	2.00
	GradeD	13	.0769	.27735	.07692	-.0907	.2445	.00	1.00
	GradeE	13	.1538	.55470	.15385	-.1814	.4890	.00	2.00
	Total	65	.4308	.80950	.10041	.2302	.6314	.00	3.00

Appendix Table 4.29: Effect of oocyte quality and ovarian stimulation response on the maturation rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_GradeA	Excellent	8	68.0563	34.72626	12.27759	39.0244	97.0881	.00	100.00
	Good	36	77.3611	35.82452	5.97075	65.2398	89.4824	.00	100.00
	Satisfactory	6	73.6117	38.87885	15.87222	32.8108	114.4125	.00	100.00
	Total	50	75.4224	35.42948	5.01048	65.3535	85.4913	.00	100.00
P_GradeB	Excellent	6	61.1117	37.51553	15.31565	21.7415	100.4818	.00	100.00
	Good	34	75.0494	33.93509	5.81982	63.2089	86.8899	.00	100.00
	Satisfactory	4	75.0000	50.00000	25.00000	-4.5612	154.5612	.00	100.00
	Total	44	73.1443	35.28799	5.31987	62.4158	83.8728	.00	100.00
P_GradeC	Excellent	10	45.6670	35.10324	11.10062	20.5557	70.7783	.00	100.00
	Good	45	49.5187	37.13493	5.53575	38.3621	60.6752	.00	100.00
	Satisfactory	10	45.0000	37.72087	11.92839	18.0161	71.9839	.00	100.00
	Total	65	48.2309	36.40415	4.51538	39.2104	57.2514	.00	100.00
P_GradeD	Excellent	5	13.3340	29.81573	13.33400	-23.6871	50.3551	.00	66.67
	Good	19	5.2632	22.94157	5.26316	-5.7943	16.3206	.00	100.00
	Satisfactory	5	20.0000	44.72136	20.00000	-35.5289	75.5289	.00	100.00
	Total	29	9.1955	28.02596	5.20429	-1.4650	19.8560	.00	100.00
P_GradeE	Excellent	1	.0000	.	.	.	.	.00	.00
	Good	9	3.7033	11.11000	3.70333	-4.8366	12.2432	.00	33.33
	Satisfactory	8	8.3338	23.57140	8.33375	-11.3724	28.0399	.00	66.67
	Total	18	5.5556	17.15024	4.04235	-2.9731	14.0842	.00	66.67

Appendix Table 4.30: Effect of ovarian stimulation response on oocyte cleavage per ovary

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_Excellent	GradeA	2	50.0000	23.57494	16.67000	-161.8124	261.8124	33.33	66.67
	GradeB	2	16.6650	23.56787	16.66500	-195.0839	228.4139	.00	33.33
	GradeC	2	33.3300	.00000	.00000	33.3300	33.3300	33.33	33.33
	Total	6	33.3317	21.08291	8.60706	11.2065	55.4568	.00	66.67
P_Good	GradeA	24	39.3058	34.83565	7.11080	24.5960	54.0156	.00	100.00
	GradeB	24	32.4304	37.56160	7.66723	16.5695	48.2913	.00	100.00
	GradeC	24	28.2637	35.21334	7.18789	13.3945	43.1330	.00	100.00
	Total	72	33.3333	35.67717	4.20460	24.9496	41.7171	.00	100.00
P_Satisfactory	GradeA	4	50.0000	57.73503	28.86751	-41.8693	141.8693	.00	100.00
	GradeB	4	25.0000	50.00000	25.00000	-54.5612	104.5612	.00	100.00
	GradeC	4	25.0000	50.00000	25.00000	-54.5612	104.5612	.00	100.00
	Total	12	33.3333	49.23660	14.21338	2.0499	64.6168	.00	100.00
N_Excellent	GradeA	2	2.0000	.00000	.00000	2.0000	2.0000	2.00	2.00
	GradeB	2	1.0000	1.41421	1.00000	-11.7062	13.7062	.00	2.00
	GradeC	2	1.5000	.70711	.50000	-4.8531	7.8531	1.00	2.00
	Total	6	1.5000	.83666	.34157	.6220	2.3780	.00	2.00
N_Good	GradeA	24	1.0000	.97802	.19964	.5870	1.4130	.00	3.00
	GradeB	24	.6250	.64690	.13205	.3518	.8982	.00	2.00
	GradeC	24	.7917	1.06237	.21685	.3431	1.2403	.00	4.00
	Total	72	.8056	.91373	.10768	.5908	1.0203	.00	4.00
N_Satisfactory	GradeA	4	1.0000	1.15470	.57735	-.8374	2.8374	.00	2.00
	GradeB	4	.2500	.50000	.25000	-.5456	1.0456	.00	1.00
	GradeC	4	.2500	.50000	.25000	-.5456	1.0456	.00	1.00
	Total	12	.5000	.79772	.23028	-.0068	1.0068	.00	2.00

Appendix Table 4.31: Effect of oocyte quality and ovarian stimulation response on the cleavage rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_GradeA	Excellent	2	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00
	Good	18	67.1294	39.24069	9.24912	47.6155	86.6434	.00	100.00
	Satisfacatory	3	55.5567	50.91787	29.39745	-70.9303	182.0437	.00	100.00
	Total	23	68.4783	39.24474	8.18309	51.5076	85.4490	.00	100.00
P_GradeB	Excellent	2	50.0000	70.71068	50.00000	-585.3102	685.3102	.00	100.00
	Good	15	72.2220	38.14505	9.84901	51.0980	93.3460	.00	100.00
	Satisfacatory	1	50.0000	.	.	.	.	50.00	50.00
	Total	18	68.5183	39.56021	9.32443	48.8455	88.1912	.00	100.00
P_GradeC	Excellent	3	38.8900	34.69577	20.03161	-47.2991	125.0791	.00	66.67
	Good	20	49.4165	45.47763	10.16911	28.1323	70.7007	.00	100.00
	Satisfacatory	4	25.0000	50.00000	25.00000	-54.5612	104.5612	.00	100.00
	Total	27	44.6296	44.42023	8.54868	27.0576	62.2017	.00	100.00

Appendix Table 4.32: Effect of ovarian stimulation response on the developmental rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	Excellent	3	53.5700	46.70101	26.96284	-62.4417	169.5817	.00	85.71
	Good	27	61.1374	32.58209	6.27043	48.2484	74.0265	.00	100.00
	Satisfactory	7	42.8571	46.00477	17.38817	.3098	85.4045	.00	100.00
	Total	37	57.0654	35.96274	5.91224	45.0748	69.0560	.00	100.00
P_4cell	Excellent	3	53.5700	46.70101	26.96284	-62.4417	169.5817	.00	85.71
	Good	27	54.4707	32.93985	6.33928	41.4402	67.5013	.00	100.00
	Satisfactory	7	28.5714	40.49998	15.30756	-8.8848	66.0277	.00	100.00
	Total	37	49.4978	35.82255	5.88919	37.5540	61.4417	.00	100.00
P_8cell	Excellent	3	53.5700	46.70101	26.96284	-62.4417	169.5817	.00	85.71
	Good	27	45.2648	32.97787	6.34659	32.2192	58.3104	.00	100.00
	Satisfactory	7	28.5714	40.49998	15.30756	-8.8848	66.0277	.00	100.00
	Total	37	42.7800	35.12227	5.77407	31.0697	54.4903	.00	100.00
P_Morula	Excellent	3	26.1900	25.08482	14.48273	-36.1242	88.5042	.00	50.00
	Good	27	17.3852	26.76720	5.15135	6.7964	27.9739	.00	100.00
	Satisfactory	7	7.1429	18.89822	7.14286	-10.3351	24.6208	.00	50.00
	Total	37	16.1614	25.24403	4.15009	7.7446	24.5781	.00	100.00

Appendix Table 4.33: Effect of oocyte quality (Grade A) and ovarian stimulation response on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	Excellent	2	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00
	Good	18	67.1294	39.24069	9.24912	47.6155	86.6434	.00	100.00
	Satisfactory	3	55.5567	50.91787	29.39745	-70.9303	182.0437	.00	100.00
	Total	23	68.4783	39.24474	8.18309	51.5076	85.4490	.00	100.00
P_4cell	Excellent	2	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00
	Good	18	65.7406	40.31048	9.50127	45.6946	85.7865	.00	100.00
	Satisfactory	3	55.5567	50.91787	29.39745	-70.9303	182.0437	.00	100.00
	Total	23	67.3913	40.11633	8.36483	50.0437	84.7389	.00	100.00
P_8cell	Excellent	2	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00
	Good	18	49.0739	43.43232	10.23710	27.4755	70.6723	.00	100.00
	Satisfactory	3	55.5567	50.91787	29.39745	-70.9303	182.0437	.00	100.00
	Total	23	54.3478	43.65504	9.10270	35.4700	73.2257	.00	100.00
P_Morula	Excellent	2	50.0000	70.71068	50.00000	-585.3102	685.3102	.00	100.00
	Good	18	20.8333	35.03635	8.25815	3.4102	38.2565	.00	100.00
	Satisfactory	3	16.6667	28.86751	16.66667	-55.0442	88.3775	.00	50.00
	Total	23	22.8261	36.42993	7.59617	7.0726	38.5796	.00	100.00

Appendix Table 4.34: Effect of oocyte quality (Grade B) and ovarian stimulation response on the cleavage and developmental rates

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	Excellent	2	50.0000	70.71068	50.00000	-585.3102	685.3102	.00	100.00
	Good	15	72.2220	38.14505	9.84901	51.0980	93.3460	.00	100.00
	Satisfactory	1	50.0000	.	.	.	.	50.00	50.00
	Total	18	68.5183	39.56021	9.32443	48.8455	88.1912	.00	100.00
P_4cell	Excellent	2	50.0000	70.71068	50.00000	-585.3102	685.3102	.00	100.00
	Good	15	62.2220	44.75108	11.55468	37.4397	87.0043	.00	100.00
	Satisfactory	1	50.0000	.	.	.	.	50.00	50.00
	Total	18	60.1850	44.33208	10.44917	38.1392	82.2308	.00	100.00
P_8cell	Excellent	2	50.0000	70.71068	50.00000	-585.3102	685.3102	.00	100.00
	Good	15	52.2220	48.33206	12.47928	25.4566	78.9874	.00	100.00
	Satisfactory	1	50.0000	.	.	.	.	50.00	50.00
	Total	18	51.8517	47.10200	11.10205	28.4284	75.2749	.00	100.00
P_Morula	Excellent	2	50.0000	70.71068	50.00000	-585.3102	685.3102	.00	100.00
	Good	15	28.8887	45.19210	11.66855	3.8621	53.9152	.00	100.00
	Satisfactory	1	.0000	.	.	.	.	.00	.00
	Total	18	29.6294	45.57387	10.74186	6.9661	52.2928	.00	100.00

Appendix Table 4.35: Effect of oocyte quality (Grade C) and ovarian stimulation response on the cleavage and developmental rate

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_2cell	Excellent	3	38.8900	34.69577	20.03161	-47.2991	125.0791	.00	66.67
	Good	20	49.4165	45.47763	10.16911	28.1323	70.7007	.00	100.00
	Satisfactory	4	25.0000	50.00000	25.00000	-54.5612	104.5612	.00	100.00
	Total	27	44.6296	44.42023	8.54868	27.0576	62.2017	.00	100.00
P_4cell	Excellent	3	38.8900	34.69577	20.03161	-47.2991	125.0791	.00	66.67
	Good	20	29.4165	40.28143	9.00720	10.5642	48.2688	.00	100.00
	Satisfactory	4	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	27	26.1111	37.55634	7.22772	11.2543	40.9679	.00	100.00
P_8cell	Excellent	3	38.8900	34.69577	20.03161	-47.2991	125.0791	.00	66.67
	Good	20	24.9165	35.10829	7.85045	8.4853	41.3477	.00	100.00
	Satisfactory	4	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	27	22.7778	33.26609	6.40206	9.6182	35.9374	.00	100.00
P_Morula	Excellent	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Good	20	2.5000	11.18034	2.50000	-2.7326	7.7326	.00	50.00
	Satisfactory	4	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	27	1.8519	9.62250	1.85185	-1.9547	5.6584	.00	50.00

Appendix Table 4.36: Effect of oocyte quality and different activation regimes at 22 to 25 hours of IVM duration on the immature oocyte following sham injection

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_SControl	GradeA	1	25.0000	.	.	.	.	25.00	25.00
	GradeB	1	25.0000	.	.	.	.	25.00	25.00
	GradeC	1	50.0000	.	.	.	.	50.00	50.00
	GradeD	1	.0000	.	.	.	.	.00	.00
	GradeE	1	.0000	.	.	.	.	.00	.00
	Total	5	20.0000	20.91650	9.35414	-5.9713	45.9713	.00	50.00
P_SCalcium	GradeA	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeB	3	16.6667	28.86751	16.66667	-55.0442	88.3775	.00	50.00
	GradeC	3	83.3333	28.86751	16.66667	11.6225	155.0442	50.00	100.00
	GradeD	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeE	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	15	20.0000	36.83942	9.51190	-.4010	40.4010	.00	100.00
P_SDMAP	GradeA	6	11.1117	27.21791	11.11167	-17.4518	39.6751	.00	66.67
	GradeB	6	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeC	6	47.2217	45.23621	18.46761	-.2508	94.6942	.00	100.00
	GradeD	6	25.0000	41.83300	17.07825	-18.9010	68.9010	.00	100.00
	GradeE	6	16.6667	40.82483	16.66667	-26.1764	59.5097	.00	100.00
	Total	30	20.0000	36.46244	6.65710	6.3847	33.6153	.00	100.00
N_SControl	GradeA	1	1.0000	.	.	.	.	1.00	1.00
	GradeB	1	1.0000	.	.	.	.	1.00	1.00
	GradeC	1	2.0000	.	.	.	.	2.00	2.00
	GradeD	1	.0000	.	.	.	.	.00	.00
	GradeE	1	.0000	.	.	.	.	.00	.00
	Total	5	.8000	.83666	.37417	-.2389	1.8389	.00	2.00
N_SCalcium	GradeA	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeB	3	.3333	.57735	.33333	-1.1009	1.7676	.00	1.00
	GradeC	3	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	GradeD	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeE	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	15	.2667	.45774	.11819	.0132	.5202	.00	1.00
N_SDMAP	GradeA	6	.3333	.81650	.33333	-.5235	1.1902	.00	2.00
	GradeB	6	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeC	6	1.0000	.89443	.36515	.0614	1.9386	.00	2.00
	GradeD	6	.6667	1.21106	.49441	-.6043	1.9376	.00	3.00
	GradeE	6	.1667	.40825	.16667	-.2618	.5951	.00	1.00
	Total	30	.4333	.81720	.14920	.1282	.7385	.00	3.00

Appendix Table 4.37: Effect of oocyte quality and different activation regimes at 22 to 25 hours of IVM duration on the immature oocyte following ICSI

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_IControl	GradeA	3	15.0000	13.22876	7.63763	-17.8621	47.8621	.00	25.00
	GradeB	3	6.6667	11.54701	6.66667	-22.0177	35.3510	.00	20.00
	GradeC	3	70.0000	26.45751	15.27525	4.2759	135.7241	50.00	100.00
	GradeD	3	8.3333	14.43376	8.33333	-27.5221	44.1888	.00	25.00
	GradeE	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	15	20.0000	29.45942	7.60639	3.6859	36.3141	.00	100.00
P_ICalcium	GradeA	10	10.0000	31.62278	10.00000	-12.6216	32.6216	.00	100.00
	GradeB	10	7.5000	16.87371	5.33594	-4.5707	19.5707	.00	50.00
	GradeC	10	52.5000	34.25801	10.83333	27.9933	77.0067	.00	100.00
	GradeD	10	25.0000	35.35534	11.18034	-.2917	50.2917	.00	100.00
	GradeE	10	5.0000	15.81139	5.00000	-6.3108	16.3108	.00	50.00
	Total	50	20.0000	32.34066	4.57366	10.8089	29.1911	.00	100.00
P_IDMAP	GradeA	6	22.2217	40.36849	16.48037	-20.1425	64.5858	.00	100.00
	GradeB	6	5.5550	13.60692	5.55500	-8.7246	19.8346	.00	33.33
	GradeC	6	47.2233	40.02379	16.33964	5.2209	89.2257	.00	100.00
	GradeD	6	8.3333	20.41241	8.33333	-13.0882	29.7548	.00	50.00
	GradeE	6	16.6667	40.82483	16.66667	-26.1764	59.5097	.00	100.00
	Total	30	20.0000	34.29665	6.26168	7.1934	32.8066	.00	100.00
N_IControl	GradeA	3	.6667	.57735	.33333	-.7676	2.1009	.00	1.00
	GradeB	3	.3333	.57735	.33333	-1.1009	1.7676	.00	1.00
	GradeC	3	2.0000	1.00000	.57735	-.4841	4.4841	1.00	3.00
	GradeD	3	.3333	.57735	.33333	-1.1009	1.7676	.00	1.00
	GradeE	3	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	15	.6667	.89974	.23231	.1684	1.1649	.00	3.00
N_ICalcium	GradeA	10	.1000	.31623	.10000	-.1262	.3262	.00	1.00
	GradeB	10	.2000	.42164	.13333	-.1016	.5016	.00	1.00
	GradeC	10	1.0000	.81650	.25820	.4159	1.5841	.00	3.00
	GradeD	10	.4000	.51640	.16330	.0306	.7694	.00	1.00
	GradeE	10	.1000	.31623	.10000	-.1262	.3262	.00	1.00
	Total	50	.3600	.59796	.08456	.1901	.5299	.00	3.00
N_IDMAP	GradeA	6	.3333	.51640	.21082	-.2086	.8753	.00	1.00
	GradeB	6	.1667	.40825	.16667	-.2618	.5951	.00	1.00
	GradeC	6	1.0000	.89443	.36515	.0614	1.9386	.00	2.00
	GradeD	6	.1667	.40825	.16667	-.2618	.5951	.00	1.00
	GradeE	6	.1667	.40825	.16667	-.2618	.5951	.00	1.00
	Total	30	.3667	.61495	.11227	.1370	.5963	.00	2.00

Appendix Table 4.38: Effect of oocyte quality and different activation regimes at 26 to 29 hours of IVM duration on the immature oocyte following sham injection

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_SControl	GradeA	10	19.5000	32.86758	10.39364	-4.0120	43.0120	.00	100.00
	GradeB	10	15.3330	21.49904	6.79859	-.0465	30.7125	.00	50.00
	GradeC	10	36.5000	32.41789	10.25144	13.3096	59.6904	.00	100.00
	GradeD	10	12.8330	18.22401	5.76294	-.2037	25.8697	.00	50.00
	GradeE	10	15.8330	32.02265	10.12645	-7.0746	38.7406	.00	100.00
	Total	50	19.9998	28.28025	3.99943	11.9626	28.0370	.00	100.00
P_SCalcium	GradeA	8	25.4162	35.54274	12.56625	-4.2982	55.1307	.00	100.00
	GradeB	8	16.6675	26.72702	9.44943	-5.6768	39.0118	.00	66.67
	GradeC	8	32.4988	34.81239	12.30804	3.3949	61.6026	.00	100.00
	GradeD	8	18.7500	35.00261	12.37529	-10.5129	48.0129	.00	100.00
	GradeE	8	6.6663	12.84733	4.54222	-4.0744	17.4069	.00	33.33
	Total	40	19.9998	29.98564	4.74115	10.4099	29.5896	.00	100.00
P_SDMAP	GradeA	4	8.3325	16.66500	8.33250	-18.1852	34.8502	.00	33.33
	GradeB	4	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeC	4	33.3325	47.14045	23.57023	-41.6785	108.3435	.00	100.00
	GradeD	4	20.8325	24.99944	12.49972	-18.9472	60.6122	.00	50.00
	GradeE	4	37.5000	47.87136	23.93568	-38.6740	113.6740	.00	100.00
	Total	20	19.9995	32.71332	7.31492	4.6892	35.3098	.00	100.00
N_SControl	GradeA	10	.4000	.51640	.16330	.0306	.7694	.00	1.00
	GradeB	10	.4000	.51640	.16330	.0306	.7694	.00	1.00
	GradeC	10	1.0000	.81650	.25820	.4159	1.5841	.00	2.00
	GradeD	10	.4000	.51640	.16330	.0306	.7694	.00	1.00
	GradeE	10	.3000	.48305	.15275	-.0456	.6456	.00	1.00
	Total	50	.5000	.61445	.08690	.3254	.6746	.00	2.00
N_SCalcium	GradeA	8	.5000	.53452	.18898	.0531	.9469	.00	1.00
	GradeB	8	.5000	.75593	.26726	-.1320	1.1320	.00	2.00
	GradeC	8	1.0000	1.06904	.37796	.1063	1.8937	.00	3.00
	GradeD	8	.3750	.51755	.18298	-.0577	.8077	.00	1.00
	GradeE	8	.3750	.74402	.26305	-.2470	.9970	.00	2.00
	Total	40	.5500	.74936	.11848	.3103	.7897	.00	3.00
N_SDMAP	GradeA	4	.2500	.50000	.25000	-.5456	1.0456	.00	1.00
	GradeB	4	.0000	.00000	.00000	.0000	.0000	.00	.00
	GradeC	4	.5000	.57735	.28868	-.4187	1.4187	.00	1.00
	GradeD	4	.5000	.57735	.28868	-.4187	1.4187	.00	1.00
	GradeE	4	.5000	.57735	.28868	-.4187	1.4187	.00	1.00
	Total	20	.3500	.48936	.10942	.1210	.5790	.00	1.00

Appendix Table 4.39: Effect of oocyte quality and different activation regimes at 26 to 29 hours of IVM duration on the immature oocyte following ICSI

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
P_IContol	GradeA	3	16.6667	28.86751	16.66667	-55.0442	88.3775	.00	50.00
	GradeB	3	16.6667	28.86751	16.66667	-55.0442	88.3775	.00	50.00
	GradeC	3	16.6667	28.86751	16.66667	-55.0442	88.3775	.00	50.00
	GradeD	3	33.3333	28.86751	16.66667	-38.3775	105.0442	.00	50.00
	GradeE	3	16.6667	28.86751	16.66667	-55.0442	88.3775	.00	50.00
	Total	15	20.0000	25.35463	6.54654	5.9591	34.0409	.00	50.00
P_ICalcium	GradeA	8	5.0000	14.14214	5.00000	-6.8231	16.8231	.00	40.00
	GradeB	8	9.1663	13.30267	4.70321	-1.9551	20.2876	.00	33.33
	GradeC	8	20.0000	35.45621	12.53566	-9.6421	49.6421	.00	100.00
	GradeD	8	32.5000	36.06696	12.75159	2.3473	62.6527	.00	100.00
	GradeE	8	33.3338	39.19872	13.85884	.5628	66.1047	.00	100.00
	Total	40	20.0000	30.68097	4.85109	10.1877	29.8123	.00	100.00
P_IDMAP	GradeA	13	11.1538	20.33771	5.64067	-1.1361	23.4438	.00	66.67
	GradeB	13	7.3077	15.35895	4.25981	-1.9736	16.5890	.00	50.00
	GradeC	13	47.4354	36.13663	10.02250	25.5982	69.2725	.00	100.00
	GradeD	13	14.8715	31.40564	8.71036	-4.1067	33.8498	.00	100.00
	GradeE	13	19.2308	38.39738	10.64952	-3.9725	42.4341	.00	100.00
	Total	65	19.9998	32.17114	3.99034	12.0282	27.9715	.00	100.00
N_IControl	GradeA	3	.3333	.57735	.33333	-1.1009	1.7676	.00	1.00
	GradeB	3	.3333	.57735	.33333	-1.1009	1.7676	.00	1.00
	GradeC	3	.3333	.57735	.33333	-1.1009	1.7676	.00	1.00
	GradeD	3	.6667	.57735	.33333	-.7676	2.1009	.00	1.00
	GradeE	3	.3333	.57735	.33333	-1.1009	1.7676	.00	1.00
	Total	15	.4000	.50709	.13093	.1192	.6808	.00	1.00
N_ICalcium	GradeA	8	.2500	.70711	.25000	-.3412	.8412	.00	2.00
	GradeB	8	.3750	.51755	.18298	-.0577	.8077	.00	1.00
	GradeC	8	.5000	.75593	.26726	-.1320	1.1320	.00	2.00
	GradeD	8	.8750	.83452	.29505	.1773	1.5727	.00	2.00
	GradeE	8	1.0000	1.19523	.42258	.0008	1.9992	.00	3.00
	Total	40	.6000	.84124	.13301	.3310	.8690	.00	3.00
N_IDMAP	GradeA	13	.3846	.65044	.18040	-.0084	.7777	.00	2.00
	GradeB	13	.2308	.43853	.12163	-.0342	.4958	.00	1.00
	GradeC	13	1.3077	1.03155	.28610	.6843	1.9311	.00	3.00
	GradeD	13	.3846	.86972	.24122	-.1410	.9102	.00	3.00
	GradeE	13	.5385	1.05003	.29123	-.0961	1.1730	.00	3.00
	Total	65	.5692	.90085	.11174	.3460	.7925	.00	3.00