# EFFECT OF SPERM AND OOCYTE FACTORS ON INTRACYTOPLASMIC SPERM INJECTION (ICSI) PERFORMANCE IN MICE AND GOATS

## NOR FARIZAH BINTI ABDUL HAMID

### FACULTY OF SCIENCE
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## NOR FARIZAH BINTI ABDUL HAMID

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I.C/Passport No: **850415-04-5106**

Registration/Matric No: **SGR080023**

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# ABSTRACT

Selected sperm and oocyte factors related were evaluated in ICSI experiments involving mouse and goat species in the present study. The mouse oocyte collection was conducted through oviduct retrieval, while the goat oocytes were retrieved via LOPU technique. The effectiveness of sperm capacitation chemical treatment (Heparin and Theophylline) for 1 hour and sperm movement (Rapid and Slow) were identified in both species. For oocyte factors, the effects of post-hCG injection durations (13-15 hours and 16-18 hours) in mice were studied. LOPU cycles (OR1, OR2 and OR3) and IVM durations (18-21 hours and 22-25 hours) were investigated in goats.

In Experiments 1, the effects of different sperm capacitation chemicals and sperm movement of mouse sperm were studied. Mouse ICSI-embryonic development at early stages (2- and 4-cell stage) were improved by using Heparin compared to Theophylline ($64.67\pm1.69\%$ versus $60.50\pm1.21\%$ and $50.55\pm2.01\%$ versus $45.09\pm1.60\%$, respectively). Both sperm movement factors had same potential to produce mouse ICSI-derived embryos.

In Experiments 2, the effect of post-hCG injection duration in mouse oocytes on embryonic development rates were studied. Two different durations were significantly different ($P<0.05$) on all cleavage rates (2-, 4-, 8-cell and morula stage) whereby at 13-15 hours were higher than at 16-18 hours duration ($67.95\pm1.06\%$ versus $57.02\pm1.33\%$, $53.51\pm1.13\%$ versus $41.90\pm1.98\%$, $39.40\pm1.01\%$ versus $22.21\pm1.62\%$, and $14.83\pm1.09\%$ versus $9.77\pm1.02\%$, respectively).

Experiment 3 evaluated the effect of two different sperm capacitation chemicals (Heparin and Theophylline) and sperm movement (Rapid and Slow) of goat sperm were studied. Both sperm capacitation chemicals gave same potential of goat ICSI-embryo

development (P>0.05). Rapid sperm movement gave better goat ICSI-embryonic development (4-, 8- cell and morula stage), whereby the respective values were 56.62±4.69% versus 41.80±4.49%, 39.51±4.70% versus 24.24±4.05% and 9.21±2.85% versus 2.24±0.94%.

Experiment 4 investigated the effect of LOPU cycles on the yield of different oocyte grades (Grades A, B, C, D and E) from 16 donors goat. Lower number of Grades D and E (31 and 19, respectively) oocytes were obtained compared to Grades A, B and C. Higher number of Grade C oocytes were obtained compared to Grades A and B from LOPU technique (153 versus 106 and 91, respectively). OR1 gave better quantity and quality oocytes, followed by OR2 and OR3 (number of oocytes per ovary were 5.47±0.67, 3.94±0.44 and 3.09±0.50, respectively). In another sub-experiment, maturation rate of goat oocytes at 18 to 21 hours was higher than 22 to 25 hours of IVM duration (97.34±7.86% versus 89.01±2.41%, respectively). However, both IVM durations had the same potential in ICSI-derived embryo development.

In conclusion, for mouse study, embryonic development can be achieved from Heparin-sperm capacitation treatment, using any sperm movement for 13 to 15 hours of post-hCG suntikan oocytes. For goat study, LOPU is good procedure to provide consistent high quality oocytes (Grades A, B and C) and it usage can be repeated on the same donor up to 3 times. Rapid sperm movement is a better choice for ICSI-embryonic development. Both sperm capacitation chemical treatments have the same potential to produce ICSI-embryonic development.

# ABSTRAK

Faktor sperma dan oosit tertentu berkaitan telah dinilai dalam eksperimen ICSI melibatkan spesies mencit dan kambing dalam kajian kini. Pengumpulan oosit mencit dilakukan melalui perolehan oviduk, manakala oosit kambing diperolehi melalui teknik LOPU. Keberkesanan perlakuan kimia kapasitasi sperma (Heparin dan Theophylline) selama 1 jam dan pergerakan sperma (laju dan perlahan) dikenalpasti pada kedua-dua spesies. Bagi faktor oosit, kesan tempoh suntikan pasca-hCG (13-15 jam dan 16-18 jam) pada mencit dikaji. Kitaran LOPU (OR1, OR2 and OR3) dan tempoh IVM (18-21 jam dan 22-25 jam) disiasat dalam kambing.

Dalam Eksperimen 1, kesan perbezaan kimia kapasitasi sperma dan pergerakan sperma dikaji. Perkembangan embrio-ICSI mencit pada peringkat awal (2- dan 4-peringkat sel) boleh diperbaiki dengan penggunaan Heparin berbanding Theophylline ($64.67\pm1.69\%$ berbanding $60.50\pm1.21\%$ dan $50.55\pm2.01\%$ berbanding $45.09\pm1.60\%$, masing-masing). Kedua-dua faktor pergerakan sperma menunjukkan potensi yang sama untuk menghasilkan embrio melalui ICSI.

Dalam Eksperimen 2, kesan tempoh suntikan pasca-hCG terhadap oosit mencit terhadap kadar perkembangan embrio dikaji. Terdapat perbezaan ketara pada dua tempoh yang berbeza ($P<0.05$), ke atas semua kadar pembahagian sel (2-, 4-, 8-sel dan morula) di mana pada tempoh 13-15 jam adalah berbeza berbanding pada tempoh 16-18 jam ($67.95\pm1.06\%$ berbanding $57.02\pm1.33\%$, $53.51\pm1.13\%$ berbanding $41.90\pm1.98\%$, $39.40\pm1.01\%$ berbanding $22.21\pm1.62\%$, dan $14.83\pm1.09\%$ berbanding $9.77\pm1.02\%$, masing-masing).

Eksperimen 3 menilai kesan perbezaaan dua kimia kapasitasi sperma dan pergerakan sperma (laju dan perlahan)pada sperma kambing dikaji. Kedua-dua kimia

kapasitasi sperma memberi potensi sama pada perkembangan embrio-ICSI (P>0.05). Pergerakan sperma yang laju memberi perkembangan embrio-ICSI yang lebih baik (peringkat 4-, 8- dan morula )berbanding pergerakan sperma yang perlahan, di mana nilai masing-masing ialah 56.62±4.69% berbanding 41.80±4.49%, 39.51±4.70% berbanding 24.24±4.05% and 9.21±2.85% berbanding 2.24±0.94%.

Eksperimen 4, menyiasat kesan kitaran LOPU terhadap hasil gred oosit (Gred A, B, C, D dan E) yang berbeza daripada 16 kambing penderma. Oosit Gred D dan E adalah yang terendah (31 dan 19, masing-masing) diperolehi berbanding dengan Gred A, B dan C. Gred C menunjukkan hasilan oosit yang tertinggi berbanding oosit Gred A, dan B daripada teknik LOPU (153 berbanding 106 dan 91, masing-masing). OR1 memberikan kuantiti dan kualiti oosit yang baik, diikuti oleh OR2 dan OR3 (bilangan oosit per ovari adalah 5.47±0.67, 3.94±0.44 dan 3.09±0.50, masing-masing). Pada sub-eksperimen yang lain, kadar kematangan oosit kambing pada tempoh 18 ke 21 jam adalah tinggi berbanding pada tempoh IVM 22 ke 25 jam (97.34±7.86% berbanding 89.01±2.41%, masing-masing). Walau bagaimanapun, kedua-dua tempoh IVM mempunyai kadar potensi yang sama dalam perkembangan embrio perolehan ICSI.

Kesimpulannya, bagi kajian mencit, perkembangan embrio boleh dicapai daripada perlakuan kapasitasi Heparin-kapasitasi sperma, dengan menggunakan mana-mana pergerakan sperma dengan mengguna 13 ke 15 jam tempoh suntikan pasca-hCG. Bagi kajian kambing, LOPU adalah prosedur yang baik untuk membekalkan oosit berkualiti tinggi secara konsisten (Gred A, B dan C) dan penggunaanya boleh diulang pada penderma yang sama sehingga 3 kali. Pergerakan sperm yang laju adalah pilihan yang lebih baik untuk perkembangan embrio-ICSI. Kedua-dua perlakuan kimia kapasitasi sperma memberi potensi yang sama untuk menghasilkan perkembangan embrio-ICSI.

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| | |
|---|---|
| % | percentage |
| µ/µm | Micron/Micrometre |
| µl | Microlitre |
| µm | Micrometre |
| µM | Micromolar |
| °C | Degree Celsius |
| 6-DMAP | 6-dimethilaminopurine |
| ABEL | Animal Biotechnology-Embryo Laboratory |
| AI | Artificial Insemination |
| ANOVA | One-way analysis of variance |
| AR | Acrosome reaction |
| ART | Assisted reproduction techniques |
| ATP | Adenosine triphosphate |
| bFSH | Bovine FSH |
| BME | Basal media eagle |
| BO | Bracket and Oliphant |
| BSA | Bovine serum albumin |
| BSA-FAF | Bovine serum albumin-Fatty acid free |
| $Ca^{2+}$ | Calcium |
| CaI | Calcium ionophore |
| cAMP | Cyclic adenosine monophosphate |
| CB | Cytochalasin B |
| CC | Cumulus cells |
| CFO | Cumulus free occytes |
| cFSH | caprine FSH (follicle stimulating hormone) |
| CHX | Cycloheximide |

| | |
|---|---|
| CIDR | Controlled internal drug release |
| CL | Corpura lutea |
| $CO_2$ | Carbon dioxide |
| COC | Cumulus oocytes complexes |
| COCs | Cumulus-oocyte complex(es) (i.e. CCs and oocyte cytoplasm) |
| COM | Cumulus oocyte mass |
| CR1aa | Charles Roskenkran's 1 amino acid |
| CR2 | Charles Roskenkran's 2 |
| CZB | Chatot-Ziomek-Bavister |
| D-Glucose | Deoxy-Glucose |
| DM | Defined medium |
| DMRT | Duncan's multiple range test |
| DMSO | Dimethysulfoxyde anhydrous |
| DNA | Deoxyribonucleic acid |
| DPBS | Diphosphate-buffered saline |
| DTT | Dithiothreitol |
| e.g | For example |
| $E_2$ | Estradiols |
| eCG | Equine chorionic gonadotrophin |
| EDI | Electrode ionization |
| EDTA | Ethylene diamine tetraacetic acid |
| EMiL | Embryo Micromanipulation Laboratory |
| et al. | et alii/alia (and others) |
| ET | Embryo transfer |
| etc. | et cetera (and so forth) |
| $F_1$ | Phenotype |
| FBS | Foetal bovine serum |
| FCS | Foetal calf serum |

| | |
|---|---|
| FF | Follicular fluid |
| FGA | Fluorogestone acetate |
| FPN | female pronucleas |
| FSH | Follicle stimulating hormone |
| G | Gauge |
| g | Gram |
| *g* | Gravity |
| G1-G2 | Gardner's sequential media |
| G6PD | Glucose -6-phosphate dehydrogenase |
| GCs | Granulosa cell(s) |
| GnRH | Gonadotrophin –releasing hormone |
| GOEC | Goat oviduct ephithelial cells |
| GSH | Oocyte glutathione hormone |
| GV | Germinal vesicle |
| GVBD | Germinal vesicle breakdown |
| hCG | Human chorionic ganadotrophin |
| hr | Hour |
| HWM | Hepes Whitten's medium |
| i.m. | Intramuscular |
| i.p. | Intraperitoneal |
| ICSI | Intracytoplasmic sperm injection |
| ID | Inner diameter |
| IGF-I | Insulin-like growth factor-I |
| IPPP | Institute of Research, Management and Monitoring |
| ISB | Institute of Biological Science |
| IU | International unit |
| IVC | *In vitro* culture |
| IVF | *In vitro* fertilisation |

| | |
|---|---|
| IVM | *In vitro* maturation |
| IVP | *In vitro* production |
| K$^+$-ATPase | Kalium-Adenosine Triphosphatease |
| KSOM | Potassium simplex optimization medium |
| LED | Light emitting diode |
| L-Glutamine | (Left)- glutamine |
| LH | Luteinising hormone |
| LN$_2$ | Liquid nitrogen |
| LOPU | Laparoscopic oocyte pick-up |
| m | Meter |
| M | Molar |
| MAP | Medroxyprogestrone acetate |
| MAPK | Mitogen-activated protein kinase |
| mDM | Modified defined medium |
| mean±SEM | Mean plus or minus standard error of means |
| MEM | Minimum essential medium |
| mg | Milligram |
| MI | Metaphase I |
| MIC | Microdrop individual culture |
| MII | Metaphase II |
| ml | Milliliter |
| mm | Millimeter |
| mM | Millimolar |
| mOsm/kg | Miliosmol per kilogram |
| MPF | Maturation-promoting factor |
| MPN | Male pronucleus |
| mSOF | Modified synthetic oviductal fluid |
| mTALP | Modified Tyrode-Albumin-Lactate-Pyruvate |

| | |
|---|---|
| MΩ-cm | Milliohm-centimeter |
| n | Number |
| Na Lactate | Natrium lactate |
| Na pyruvate | Natrium pyruvate |
| $Na^+$ | Natrium |
| NaTuRe | Nuclear Transfer and Reprogramming Laboratory |
| NT | Nuclear transfer |
| OD | Outer diameter |
| oFSH | Ovine FSH (follicle stimulating hormone) |
| OGS | Oestrus goat serum |
| OPU | Oocyte pick-up |
| OR | Oocyte recovery / oocyte retrieval |
| OS | Oestrus serum |
| p.H | Negative logarithm of the effective hydrogen-ion concentration in gram |
| PB | Polar body |
| PB-I | First polar body |
| PB-II | Second polar body |
| PBS | Phosphate buffer saline |
| PCC | premature chromosome condensation |
| PMSG | Pregnant mare's serum gonadotrophin |
| PN | Pronucleus |
| PVA | Polyvinylalcohol |
| PVP | Polyvinylpyrrolidone |
| $r^2$ | Regression coefficients |
| RNA | Ribonucleic acid |
| RO | Reverse osmosis |
| rpm | Rotation per minute |
| s.c. | Subcutaneous |

| | |
|---|---|
| SOAF | Sperm-borne-oocyte-activating-factor |
| SOF | Synthetic Oviductal Fluid |
| SOR | Surgical oocytes retrieval |
| SPSS | Statistical Package for Social Science |
| sp-TALP | Sperm-tyrode-Albumin-Lactate-Pyruvate |
| $SrCL_2$ | Strontium Cloride |
| SS | Steer serum |
| TCM-199 | Tissue culture medium-199 |
| TCM-Py | Tissue culture media-Pyruvate |
| TUGA | Transvaginal ultrasound-guided aspiration |
| TYH | Toyoda, Yokohama and Hosi's |
| UV | Ultraviolet |
| Vs. | Versus |
| w/v | Weight: volume ration |
| WID | Well-in-drop |
| WM | Whitte's medium |
| ZD | Zona drilling |
| ZP | Zona pellucida |
| β | Beta |

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# Chapter 1

## 1.0   INTRODUCTION

**Chapter 1**

**1.0     INTRODUCTION**

**1.1     INTRODUCTION**

Assisted reproduction techniques (ART) have been applied to solve reproductive problems laboratory animals, livestock animals, wildlife animals and human. The common ART include artificial insemination (AI), oestrus synchronisation, superovulation, laparoscopic oocyte pick-up (LOPU), *in vitro* maturation (IVM), *in vitro* fertilisation (IVF), *in vitro* culture (IVC), intracytoplasmic sperm injection (ICSI), embryo transfer (ET), gametes and embryo cryopreservation, nuclear transfer (NT), gene transfer and stem cells technique.

Laboratory animals such as the mice are commonly used to study reproductive technologies. They are smaller in size and easier in handling and management of this animals as well as they are good models to study reproduction in larger mammalian species such as livestock animals.

The first successful fertilisation of mammalian oocyte by ICSI procedure was reported in hamster about 20 years ago (Uehara and Yanagimachi, 1976). Among the many laboratory animals, mice are commonly used as model animals. Mice are ideal experimental subjects because the oocytes and embryos are easier and more hardy to culture *in vitro* and they are available in abundance (Kimura and Yanagimachi, 1995).

Goat is an important small ruminant economically for in the production of goat meat, goat milk and their products. However, the productivity of goats depends on breed, season and environmental conditions.

Advances in goat reproductive technologies are comparatively slower than those of other domestic animals (Keskintepe *et al., 1996*) even though there are successful reports on *in vitro* maturation, fertilisation and culture condition (Keskintepe *et al., 1994*; Martino *et al., 1995*; Ongeri *et al., 2001*; Baldassarre *et al., 2003*; Wang *et al., 2003*; Rahman, 2008a).

In the human, ICSI is a popular ART that has been used routinely in fertility centres worldwide in an effort to alleviate infertility problems, especially in oligospermia cases (Palermo *et al., 1992*). In farm animals, ICSI technique has been used successfully to produce offspring in cattle (Goto *et al., 1990*; Hamano *et al., 1999*; Horiuchi *et al., 2002*; Wei and Fukui, 2002; Oikawa *et al., 2005*), sheep (Catt *et al., 1996*; Gomez *et al., 1998*) and goats (Wang *et al., 2003*). However, the reproductive efficiency is still unsatisfactory (Ock *et al., 2003*).

## 1.2    BACKGROUND

### 1.2.1    Intracytoplasmic Sperm Injection (ICSI)

The ICSI technique is generally classified as an extended, special version of *in vitro* fertilisation in mammalian species. This technique can bypass the process of sperm penetration of cumulus cells, corolla oophorus, zona pellucida and oolema during fertilisation by directly depositing the sperm into the ooplasma. This technique requires the micromanipulator unit and skilled operator. There is good historical background regarding of ICSI, where the fertilisation was occurred and the live births of offspring was obtained from mammalian species such as hamsters (Uehara and Yanagimachi, 1976); rabbits (Hosoi *et al., 1988*); cattles (Goto *et al., 1990*); humans (Palermo *et al., 1992*); mice (Kimura and Yanagimachi, 1995); sheep (Catt *et al., 1996*); horses

(Cochran *et al.,* 1998); cats (Pope *et al.,* 1998); monkeys (Hewitson *et al.,* 1999) and pigs (Martin, 2000).

ICSI procedure includes sperm immobilisation, sperm insemination into the oolemma and oocyte activation. The orientation of polar body during sperm insemination at position 6 and 12 o'clock (Keskintepe *et al.,* 1997; Wang *et al.,* 2003; Jimenez-Macedo *et al.,* 2005, 2006 and 2007; Rahman, 2008a), the flexibility of the oolemma and the maturity of oocytes also contribute and play major role to determine the success of the ICSI procedure. Many attempts have been made to improve the ICSI performance. Among them is to use the assisted machine for ICSI called piezo-driven ICSI. This device helps to avoid the high elasticity of oolemma especially in mice (Kimura and Yanagimachi, 1995). In goat, this technique is still not popular, hence, many researchers are still using the conventional ICSI protocol without piezo-driven. Usually sperm or oocytes are chemically treated to obtain better fertilisation and embryo development rates (Keskintepe *et al.,* 1997; Zhou *et al.,* 2004; Jimenez-Macedo *et al.,* 2006 and 2007; Rahman, 2008). Wang *et al,* (2003) used the piezo-driven without any activation treatment.

## 1.2.2 Application of ICSI

ICSI technique has been described as depositing a single sperm directly into the ooplasm of oocytes for the process of fertilisation and further developmental competence of embryos, foetuses and offspring. One advantage of using ICSI technique is to avoid the occurrence of polyspermy during the process of fertilisation (Palomo *et al.,* 1999; Bhatia *et al.,* 2002). In addition through this good criteria, ICSI become major application for animal production includes use of genetically important but biologically inferior male gametes for creating wild and domestic animal (Iritani, 1991).

Recently, ICSI was adapted in human therapy to overcome the male infertility and unexplained fertilisation failure starting of the successful research done by Palermo *et al.,* (1992).

The introduction of ICSI in assisted reproductive has contributed the understanding of early events of fertilisation especially during the two gametes interacting such as capacitation, the acrosome reaction and pronucleus formation. In addition, ICSI also can be a valuable research device for studying the mechanism of fertilisation in normal versus abnormal and *in vivo* versus in vitro condition, both in animal and human. This technique has been used to produce farm animals' offspring, such as cattle (Goto *et al.,* 1990; Hamano *et al.,* 1999; Horiuchi *et al.,* 2002; Wei and Fukui, 2002 and Oikawa *et al.,* 2005), sheep (Catt *et al.,* 1996 and Gomez *et al.,* 1998) and goat (Wang *et al.,* 2003).

ICSI intriguing perspective of being able to predetermine the sex of the offspring by using sperm as carriers for altered chromosomal material, this may become the useful way of generating transgenic animals (Perry *et al.,* 2001).

## 1.3   STATEMENT OF PROBLEMS

ICSI has become one of the famous ART which has been used widely in various species. Physiology of oocyte and sperm aspects become one of the limitation for the successful of ICSI procedure include:

a) How efficient and frequent of laparoscopic oocyte pick-up (LOPU) procedure can be carried out for collection of the oocytes from each goat?

b) How does the quality of oocytes after different oocytes retrieval (OR) cycles affect the ICSI performance?

c) What is the optimal maturation duration for OR goat oocytes before ICSI can be performed?

d) Are there any differences in ICSI-derived embryo development for different types of sperm movement in mice and goat?

e) Which sperm capacitation chemical gives the optimal ICSI performance in mice and goat?

f) Does hormonal treatments (PMSG, hCG and FSH) affect the quality of oocytes and their subsequent ICSI performance similar in mice?

## 1.4 JUSTIFICATION

In Malaysia, the source of goat ovaries from abattoir is limited. Therefore, this will be a major constraint to carry out extensive goat embryo biotechnology studies in this country. As an alternative, it has been suggested that good quality oocytes could be obtained for various assisted reproductive technologies (ART) research studies from does using LOPU procedure (Baldassarre *et al.,* 2004; Wang *et al.,* 2007). Moreover, LOPU-derived oocytes could be obtained from repeated OR cycles of individual healthy does with minor ovarian damage that may not affect the oocytes quality. If the LOPU surgery is carried out properly, there should be minor adhesion particularly when compared to laparotomy procedure which is more traumatic and major injury as well as more physical handling on the reproductive tract. At ABEL laboratory, we have excellent LOPU facilities for goat embryo research. Therefore, this present study took the advantage of the facilities availability and LOPU procedure is dedicated to obtain all the oocytes to be used in ICSI experiment in goat.

This study was initiated by focusing on the OR cycle to determine its effect on oocytes quantity and quality, fertilisation rate and cleavage rate after ICSI procedure. The next phase of this research was to determine the effect of *in vitro* maturation duration on ICSI performance. Since sperm also plays important role in the success of ICSI procedure, an experiment was conducted to evaluate the ICSI performance by using different capacitation chemical treatments. The factors, such as OR cycle, maturation duration and sperm capacitation that were studied in the present project are significant determinants to ensure maximal *in vitro* embryos survival after ICSI procedure. At present, the information on these factors is scarce and controversial; and, therefore, should be clarified and understood before ICSI procedure in goat could be recommended to be used routinely for research laboratory projects or application in goat industries.

In this research, mice were used as model animals before ICSI procedure was carried out in goat. Factors such as the post-hCG duration for recovery the oocytes, the sperm capacitation treatment and sperm morphology were evaluated prior to ICSI procedure proper being carried out in goats. At the same time, using model animals such as mice provided abundance oocytes for researcher to gain skills and expertise.

## 1.5 OBJECTIVES

The general objective of this research was to develop an ICSI procedure for mouse and goat by optimising various factors from sperm and oocyte, such as sperm capacitation chemical treatment, sperm movement criterion, post-hCG administration duration, OR cycle and IVM duration on ICSI performance. Specific objectives were as follows:

a) To determine the effect of sperm capacitation treatment on ICSI performance in mice and goat.

b) To determine the effect of sperm movement criterion on ICSI performance in mice and goat.

c) To determine the effect of post-hCG administration duration on ICSI performance in mice species

d) To determine the effect of OR cycle on ICSI performance in goat.

e) To determine the effect of IVM duration on ICSI performance in goat.

# Chapter 2

## 2.0    REVIEW OF LITERATURE

**Chapter 2**

**2.0    REVIEW OF LITERATURE**

**2.1    BACKGROUND**

Intracytoplasmic sperm injection (ICSI) is widely used in human as an alternative way to overcome the sperm infertility factors in order to obtain offspring (Palermo *et al.,* 1992). This technique is one of the assisted reproductive technologies (ART) that has been carried out in animals. There were first successful reports in ICSI-derived offspring in various mammalian such as described in Table 2.1.

**Table 2.1**: First reports of ICSI-derived live offspring production in various species

| Year | Author | Species |
| --- | --- | --- |
| 1988 | Hosoi *et al.* | Rabbit |
| 1990 | Goto *et al.* | Cattle |
| 1992 | Palermo *et al.* | Human |
| 1995 | Ahmadi *et al.* | Mouse |
| 1996 | Catt *et al.* | Sheep |
| 1998 | Cochran *et al.* | Horse |
| 1998 | Pope *et al.* | Cat |
| 1999 | Hewitson *et al.* | Monkey |

| 2000 | Martin | Pig |
|------|--------|-----|
| 2002 | Hirabayash *et al.* | Rat |
| 2002 | Yamauchi *et al.* | Hamster |
| 2003 | Wang *et al.* | Goat |
| 2003 | Magarey and Mate | Kangaroo |

Source: Adapted from Rahman, (2008a).

Various research and findings were contributed by researchers since the past few decades. These findings helped in improving and increasing the success of ICSI procedure. Other than the ICSI procedure factor, the oocyte and sperm factors played various important roles in order to obtain the optimal ICSI results.

**Table 2.2**: Timeline of selected significant findings of IVM, ICSI and IVC in mice and goat studies regarding the oocyte factors

| Year | Author | Species | Significant event/finding |
|------|--------|---------|---------------------------|
| 1983 | Markert | Mouse | Blastocysts were successfully obtained. |
| 1992 | De Smedt *et al.* | Goat | Maturation rates from follicle sized 2.0-6.0 mm was 86% and 1.0-1.8 mm follicle sized was 24% |
| 1995 | Crozet *et al.* | Goat | Maturation rate from follicle sized 2.0-3.0 mm was 70%, 3.1-5.0 mm was 83% and >5 mm was 97%. |

| 1995 | Ahmadi *et al.* | Mouse | Total number of blastocyst and inner cell mass (ICM) were increased in which sperm was injected 3 hours following injection of $Ca^{2+}$ into oocyte. |
|------|-----------------|-------|------|
| 1995 | Rajikin | Goat | Maturation rate of abattoir-derived oocytes after 20-30 hours in TCM-199 + FCS + BSA + OGS (20%) without hormones obtained from COCs (55.1%) and CFOs (20.0%). Maturation rate after 40-48 hours from COCs (58.5%) and CFOs 57.6%). |
| 1995 | Martino *et al.* | Goat | Maturation rate of abattoir-derived prepubertal COCs in TCM-199 + FBS (10%) for 27 hours with presence (72.0%) or absence of GCs (76.9%). |
| 1996 | Sharma *et al.* | Goat | Maturation rate of abattoir-derived with COCs in TCM-199 + OGS (20%) with IVM duration 32 hours (71.6%), 36 hours (59.7%), 31 hours (55.8%) and 24 hours (50.3%). |
| 1996 | Pawshe *et al.* | Goat | Maturation rate of abattoir derived COCs for 24 hours in IVM media containing FSH, LH and oestradiol using Ham's-12 + OGS(10%) = 49.0%, Ham's-12 + FCS (10%) = 64.8%, TCM-199+OGS (10%) = 62.6%, and TCM-199 + FCS (10%) = 90.0%. |
| 1996 | Burruel *et al.* | Mouse | Oocyte activation and subsequently produced normal offspring could be obtained from oocyte injected by grossly misshapen head. |
| 1996 | Gall *et al.* | Goat | Maturation rate of COCs (hormone stimulated slaughter goat) in TCM-199 + FCS (10%) with IVM duration 20 hours |

| (22.0%) and 27 hours (96.0%); from 2-6 mm follicle sized (3.5%) and 1.0-1.8 mm follicle sized (8.0%). |

| 1996 | Keskintepe *et al.* | Goat | Success to obtain blastocyst and subsequently produced offspring through uterine transfer. |
| 1997 | Keskintepe *et al.* | Goat | Success to obtain cleavage (57.7%), morula (35.5%) and blastocyst (24.4%) from the oocyte injected with broken tail sperm without any activation treatment. |
| 1997 | Yadav *et al.* | Goat | The optimal IVM duration was 30 hours based on chromosomes sequential configuration. |
| 1999 | Malik *et al.* | Goat | Maturation rate of abattoir-derived COCs for 28 hours in different media; TCM-199 + OGS (20%) (63.6%), goat peritoneal fluid (55.6%) and rabbit peritoneal fluid (44.6%). |
| 2000 | Samaké *et al.* | Goat | Maturation rate of COCs (synchronised and superovulated goat) for 24 hours in TCM-199+FBS (10%) through both laparotomy and ovariectomy methods was 100%. |
| 2001 | Rho *et al.* | Goat | Maturation rate of abattoir derived COCs in M-199 + FCS (10%) in 27 hours (73%), 24 hours (55%) and 20 hours (30%). |
| 2002 | Velilla *et al.* | Goat | Maturation rate of abattoir-derived COCs (prepubertal goat) in TCM-199 + FBS (10%) and Hoechst stained after 27 hours (51.05), 20 hours (36.9%) and 15 hours (25.7%). |

| 2002 | Liu *et al.* | Mouse | *In vitro* matured preantral follicles could gave significant higher of 2-cell development rate via ICSI compared with IVF. |
|------|--------------|-------|------|
| 2003 | Baldassarre *et al.* | Goat | LOPU is reliable technique for oocyte recovery and the donors could be repeated used with known health status. |
| 2003 | Koeman *et al.* | Goat | Gonadotrophin-primed prepubertal goat gave higher yield of oocytes than adult but no significant different *in vitro* development through LOPU technique. |
| 2003 | Wang *et al.* | Goat | The embryo development using G1.3-G2.3 medium gave cleavage (74%), morula (15%) and blastocyst (9%), while for mTALP-mKSOM medium, the cleavage (89%), morula (41%) and blastocyst (35%) using Piezo-ICSI with tail cut sperm. |
| 2003 | Lacham-Kaplan and Trounson | Mouse | Activation of oocyte using $SrCl_2$ that has injected by frozen sperm resulted in live offspring. |
| 2004 | Zhou *et al.* | Goat | Oocyte activation after ICSI with ionomycin alone or ionomycin plus 6-DMAP significantly increased the embryo development rate after ICSI. |
| 2004 | Pierson *et al.* | Goat | LOPU can be repeated up to five times at different intervals and seasons with less or no important change in overall response. |

| 2005 | Jimenez-Macedo *et al.* | Goat | Chemical activation (ionomycin plus 6-DMAP) is important and able to produced embryos ups to 8-cell stage in G1.3/G2.3 medium culture. |
|---|---|---|---|
| 2006 | Jimenez-Macedo *et al.* | Goat | The oocyte diameter smaller than 125 μm were unable to develop to blastocyst stage. |
| 2006 | Kharche *et al.* | Goat | Maturation rate of COCs in TCM-199 + 0, 10, 15, 20% OGS for IVM duration between 24 to 27 hours were 28.6%, 61.9%, 72.7%, and 78.6%, respectively. |
| 2007 | Tateno and Kamiguchi | Mouse | The chromosome aberrations in ICSI-derived embryos were found depended on type of medium and incubation time. |
| 2007 | Jimenez-Macedo *et al.* | Goat | Oocytes size has a relationship on yield and quality of blastocyst. ICSI and embryo biopsy give no negative effect on embryo development. |
| 2007 | Katska-Ksiazkiewicz *et al.* | Goat | The selection of COCs based on morphology features might be helped in obtained the competent oocyte before IVM. |
| 2007 | Rahman *et al.* | Goat | LOPU oocyte source shown better maturation and embryo development rate compared with abattoir source. Morula stage of embryos was obtained without using the artificial activation of IVM heterogenous oocytes. |
| 2008 | Abdullah *et al.* | Goat | Optimal yield of oocytes obtained using 60 or 72 hours post FSH/hCG before LOPU. |

| 2009 | Anguita *et al.* | Goat | Oocyte diameter and COC morphology could be influenced the oocyte development competence in prepubertal goat. |
| 2009 | Kharche *et al.* | Goat | The cleavage rate of using media contained fatty acid free albumin was significantly higher compared to unmodified albumin. The supplementation of 20% OGS increased the cleavage rate of oocytes compared to defatted albumin. |
| 2010 | Lv *et al.* | Goat | β-mercaptoethanol supplementation and oocyte selection may influence the progression of prepubertal oocytes to undergo metaphase II, whereas the inhibitory of IVM are caused by high concentration of oestradiol. |
| 2010 | Kong | Goat | Maturation, cleavage and embryo development rate were increased using the oocytes that undergo IVM duration at 22 to 25 hours from LOPU-derived oocytes via ICSI technique and subsequently activated with the combination of $Ca^{2+}$ ionophore and 6-DMAP. |

**Table 2.3**: Timeline of selected significant findings of ICSI in mice and goat studies regarding the sperm factors

| Year | Author | Species | Significant event/finding |
|------|--------|---------|---------------------------|
| 1995 | Lacham-Kaplan and Trounson | Mouse | Acrosome reaction induction from sperm treatment with calcium ionophore was increased the formation of pronuclear in intact oocyte. |
| 1995 | Kimura and Yanagimachi | Mouse | First report of live offspring from sperm injection using the piezo-injector and subsequently undergo embryo transfer. |
| 1996 | Kuretake *et al.* | Mouse | Normal embryo development might be obtained when the sperm heads were separated by sonification with or without Triton X-100 treatment. It shows that sperm with intact structure is not needed. Plasma, acrosome membrane ans tail component is non essential in mouse ICSI. |
| 1998 | Kimura *et al.* | Mouse | The injection of primary spermatocyte would undergo premature chromosome condensation (PCC) during second meiotic division. The breakage of chromosomes was observed prior to first cleavage. |
| 1999 | Palomo *et al.* | Goat | Sperm motility and viability could be improved through swim-up protocol. |
| 1999 | Ogura *et al.* | Mouse | The PCC occurrence in spermatid chromosomes could move safely to opposite poles after oocyte activation. |
| 2001 | Yazawa *et al.* | Mouse | The used of elongated sperm could produce the normal offspring and has suggested that normal oscillation patern of $Ca^{2+}$ is not necessary for normal |

| | | | fertilisation and embryo development. |
|---|---|---|---|
| 2002 | Szczygiel *et al.* | Mouse | Cryopreserved sperm (frozen sperm) was more efficiently used in ICSI procedure compared to IVF to obtain the embryos. |
| 2003 | Wang *et al.* | Goat | First report of ICSI-derived live offspring in goat, procedure was conducted by using the Piezo-ICSI, where injecting the oocyte with tail-cut sperm. |
| 2003 | Lacham-Kaplan and Trounson | Mouse | Fertilisation rate using the cryopreserved sperm was similar with fresh sperm. However, the blastocyst rate was reduced. |
| 2004 | Zhou *et al.* | Goat | Sperm treatment using 0.0005% Triton X-100 before ICSI shows high significant rates of fertilisation and embryo development compared to other concentration and manual immobilisation. |
| 2006 | Ajduk *et al.* | Mouse | Sperm capacitation through acrosome removal before ICSI with Calcium ionophore A23187 but not with Triton X-100 would allow more synchronous chromatin remodeling, delayed the DNA synthesis and lead to produce the good embryo. |
| 2006 | Jimenez-Macedo *et al.* | Goat | Sperm treatment before ICSI with heparin plus $Ca^{2+}$ ionophore and culturing in mSOF media gave 23.7% cleavage, 8.25 morula and 5.1 blastocysts. |

| 2007 | Jimenez-Macedo *et al.* | Goat | Sperm treatment before ICSI with heparin plus $Ca^{2+}$ ionophore and culturing in mSOF media according to oocyte diameter. the oocyte diameter cleavage and blastocyst rate in sized 110-125 μm (51.0% and 14.7%) and sized >135 μm (66.2% and 34.5%, respectively). |

## 2.2 OESTRUS SYNCHRONISATION AND SUPEROVULATION

The main aim of superovulation was to induce multiple ovulations in animals. Superovulation regime was closely related with oestrus synchornisation in ART programme. In goat, the manipulation of either the luteal or the follicular phase of the oestrous cycle could aid for oestrus synchronisation, by extending the luteal phase by using exogenous progesterone or by shortening the phase by prematurely regressing existing corpura lutea (Wildeus, 2000). In mice, the pheromone phenomenon (odour of a male) was to stimulate the oestrous cycle and to synchonise the females in high percentage on the third day of pairing (Whitten, 1956). Superovulation was normally carried out through exogenous gonadotrophin treatment.

### 2.2.1 Oestrus Synchronisation

Classically, corpura lutea (CL) would undergo timely regression in all animals at any stage of oestrous cycle through progesterone or progestagen treatment. Most of the progestagen was built with vaginal pessaries. For examples, the polyurethane sponge impregnated with fluorogestone acetate (FGA), medroxyprogestrone acetate (MAP) or Y-shaped silicone-coated device (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). Other than vaginal pessaries, there were implants impregnated containing highly potent synthetic device, which was inserted under the skin on the upper side of 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 underside of tail (East and Rowe, 1989). In terms of synchronisation efficiency, there was no difference between sponges and subcutaneous implants (Holtz and Sohnrey, 1992).

The subcutaneous implants or CIDR devices were more practically used compared to sponges because of discomfort to the animals and the device may adhere to the vaginal surfaces that made it difficult to withdraw from vagina. Subsequently, a luteolytic dose of prostaglandin or an analogue (cloprostenol) was administered either at the onset (Rubianes and Menchaca, 2003) or at the end or 24 to 48 hours prior end of progestagen treatment. During the progesterone treatment, the progesterone prevented and reduced the formation of dominant follicles (Adam, 1999; De Castro *et al*., 1999; Menchaca and Rubianes, 2001; Simoes *et al.,* 2006).

### 2.2.2   Ovarian Superovulation

Superovulation in animals could help to increase the number of competent oocytes for *in vivo* and *in vitro* embryo production (Malhi *et al*., 2008).   The superovulation treatment commonly combined with oestrous synchronisation through gonadotrophin administration to induce the ovulation in order to release more number of oocytes from the ovaries. The presence or absence of dominant follicle and CL at the time of follicle stimulating hormone (FSH) administration gave an effect on superovulation response.

In goat studies, it was suggested that the number and quality of embryos produced were enhanced by the presence of CL prior to the first dose of FSH injection

(Gonzalez-Bulnes *et al.*, 2002, 2003, 2004, 2005). At the final stage of oestrous cycle (preovulatory), the progesterone level was initially high and decreased after regression of CL (De Castro *et al.*, 1999; Mechaca and Rubianes, 2001) and resulted in increase in oestrogen level with the development of the dominant follicles (De Castro *et al.*, 1999). The rapid development of dominant follicles normally occurred before ovulation (Kojima *et al.*, 2003). A single treatment of FSH plus equine chorionic gonadotrophin (eCG), injected 36 hours before LOPU proved to be as effective as traditional regime with FSH multi-doses in goat (Baldassarre *et al.*, 1996, 2002). Abdullah *et al.* (2008) suggested that prolonging the interval (60 to 72 hours) from ovarian superovulation to LOPU improves oocytes yield, quality and development competence in goat.

However, in the superovulatory response, there are variabilities in the number of follicles and oocytes obtained. Therefore, this may be a major constraint in obtaining successful results in goat IVP programme. The extrinsic factors such as source, batch purity of gonadotrophin and regime of hormone (Wollen *et al.*, 1985; Cognie, 1999); and the intrinsic factors such as breed/strain, age and reproductive status (Baril *et al.,* 1993) were the causes the variability in superovulation responses. The new gonadotrophin preparations and animal management system (Cognie, 1999) may also contribute to this variability.  Thus, to optimise the number of quality of oocytes obtained, it was suggested that application of appropriate hormone stimulation should be seriously considered in any superovulation protocols (Sirard *et al.*, 2006).

### 2.2.2.1  Factors influencing superovulation

The major constraints for superovulatory response being classified either as extrinsic (depending on the treatment protocol used for ovarian stimulation) and intrinsic factors (related to the physiological status of the animal).

### *2.2.2.1.1 Age*

The effect of age on ovarian response after superovulation is commonly ignored. However, it has been shown that superovulatory response variation may occur between animals of different ages due to individual physiological variation (Jainudeen *et al.,* 2000). For example, the optimal age for superovulated female mice was 21 to 42 days could produce large number of oocytes from a particular strain (Hogan *et al.,* 1986; Zudova *et al.,* 2004). The optimal age was related with strain of mice: 25 days for C57BL/6J female (Hogan *et al.,* 1986) and 21 days for BALB/ca (Gates, 1971). According to Hogan *et al.* (1986), at this age, the follicle maturation wave increased the number of follicles capable of responding to FSH. The age difference in the superovulation response was also related to the genetic difference and age at puberty (Gates and Bozarth, 1978). In superovulatory response, the $F_1$ hybrid responded earlier (16-17 days old) and were more responsiveness at  a younger age (22-24 days) compared to the inbred strains of BALB/c (25-30 days) and 129 (28-33 days) mice.

Information on influence of age on superovulatory response in goat is scarce. Younger goats showed low superovulatory response due to high sensitivity to the negative effects of steroids compared to adult goats (Driancourt, 2001; Senger, 2003). The age of goats less than 3 years showed poor superovulatory response (Mahmood *et al.,* 1991).  The poor results could be due to cytoplamic, ultrastructural,metabolic and nuclear abnormalities in prepubertal female oocytes (Armstrong, 2001). However, it

was reported that older female animals were reproductively competent due to decreasing uterine health and oocyte viability (Carnevale *et al.,* 1993, 1997; Morris and Allen, 2002; Morel *et al.,* 2005). The reason for the reduction in the number of oocytes obtained with old age after superovulation was related to ovulation of smaller pre-ovulatory follicles that failed to undergo the final meiotic division and/or maturation before ovulation (Carnevale *et al*., 1999). Also the poor superovulatory response in old animals may be due to a reduction in capability of number of follicles responding to the gonadotrophin treatment (Lerner *et al.,* 1986).

In contrast, previous reports have shown that the age of the animal was not considered to be main factor in superovulatory response (Hasler, 1992) whereby no significant age effects were shown on quantity and quality of oocytes obtained (Katska and Smorg, 1984; Wani *et al.,* 1999; Kong, 2010) as well as no similar effect on the total number of embryos obtained (Donaldson, 1984; Kong, 2010).

### 2.2.2.1.2 Weight and Nutrition

The body weight of animals and nutrition were shown to be related to reproductive performance. The available information regarding these effects on the embryo quality and recovery rate was mainly from superovulation of cattle and sheep.

The optimal yield of oocytes through superovulated mice was between 12.5 and 14 grammes (Hogan *et al.,* 1986). The nutritional and health status of animals may affect the bodyweight of the females. In mice, the low body weight may cause less superovulatory response as reflected in lower yield of oocytes obtained after superovulation  (Hogan *et al.,* 1986).

The hypothalamus-pituitary-ovarian axis was influenced by dietary intake of animals. The follicular growth was affected by the changes in the plane of nutrition (Gutierrez *et al.,* 1997; Gong *et al.,* 2002; Diskin *et al.,* 2003; Mihm and Bleach, 2003) through the 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). Different dietary regimes were shown to change the endocrine signaling pathways. However, the effect of these changes on fertility is unclear. The growth of oocytes and the follicular environment *in vivo* were highly correlated with hormonal and ovarian receptor communication.

The oocyte morphology (O'Callaghan *et al.,* 2000), oocytes development and embryo production were affected by dietary intake. The detriment oocyte quality, embryo mortality and late embryo development *in vivo* (Mantovani *et al.,* 1993; McEvoy *et al.,* 1995; Negrao *et al.,* 1997) and *in vitro* (Papadopaulos *et al.,* 2001) were affected by overfed animals. Overfeeding can lead to reduced pregnancy rates (Parr *et al.,* 1987) and decreased the presence of embryos collected on Day 2 after fertilisation (Creed *et al.,* 1994). On the other hand, during under-nutrition, embryo development was delayed during first two weeks after fertilisation (Parr *et al.,* 1987; Abecia *et al.,* 1995) embryo motality was higher during the first two weeks of pregnancy (Rhind *et al.,* 1989; Abecia *et al.,* 1995).

The variable responses and inconsistent outcomes in relation to nutrition and reproduction in ruminants are complex. For examples, low dietary intake can reduce the ovulation rate in sheep (Smith, 1991) and mice (Hogan *et al.,* 1986), and the dietary supplements with high energy and protein can increase the ovulation rate even though in poor body condition and not supplemented with exogenous gonadotrophins (Downing *et al.,* 1995). The increased dietary intake can enhance the ovarian folliculogenesis in

cattle as well as the superovulatory response (Gutierrez *et al*., 1997). This may be due to the small follicle populations are induced to increase.

The feeding level on oocyte quality is dependent on the body condition of the animals (Adamiak *et al.,* 2005). The high level of dietary intake can be beneficial to oocytes from animals of low body condition but detrimental to oocytes from moderately high body condition animals. The embryo production is influenced by nutrition remains to be fully characterised. It may affect the oocytes development before fertilisation and early embryo development. In order to optimise the oocytes and embryo production, good nutritional management of donor goats for superovulation programme and LOPU is required (Scaramuzzi and Murray, 1994).

### 2.2.2.1.3 Breed or Strain

The superovulatory response to the gonadotrophin is different among the various breeds of animal. This has been reported in goat (Amoah and Gelaye, 1990; Mohd Noor Hisham, 2006), sheep (Torres *et al.,* 1987; Vivanco *et al.,* 1994), cattle (Crister *at al.,* 1979; Holness *et al.,* 1980; Donaldson, 1984), and mice (Gates and Bozarth, 1978; Hogan *et al*., 1986; Spearow, 1998, Zudova *et al.,* 2004, Byers *et al.,* 2006). There is a good relationship between superovulatory response and genetic background of animal, resulting in high ovulation rates (Bindon *et al.,* 1986; Synder, 1986). For examples, the number of ovulated oocytes in A/J mice (5.4±2) was shown to be lower number than C57BL/6J mice (25±1.2) (Byers *et al.,* 2006); the breed with high prolificacy was found to be more sensitive to gonadotrophin (Smith, 1976; Piper *et al.,* 1982) compared to less prolific breed (Kelly *et al.,* 1983; Bindon *et al.,* 1986; Picazo *et al.,* 1996). The differences in superovulatory response in breed were related to differential kinetic behaviour of gonadotrophin or differential follicular dynamics and function in response

to the hormone (Ammoun *et al.,* 2006). For example, the absorption and elimination of FSH in the blood between breeds was different (McNeilly, 1985; Fry *et al.,* 1987) which may be caused either by hormone preparation used or by route of administration or by the inherent variability of hormone elimination (Demoustier *et al.,* 1988; Prakash *et al.,* 1996).

### 2.2.2.1.4 Application of Gonadotrophin

The effect of exogenous hormones on stimulation of goat follicles has been studied (Lambert *et al*., 1986; Amoah and Gelaye, 1990). The common gonadotrophin treatments for superovulation in goats are FSH and eCG. The FSH is more expensive compared to eCG because latter is easily available in local market and requires a single injection (Monniaux *et al*., 1983). However, the FSH is more efficient in superovulatory response than eCG (Armstrong *et al*., 1983; Nuti *et al*., 1987; Mahmood *et al.,* 1991; Nowshari *et al.,* 1992) whereby it contains an appropriate admixture of LH and FSH. Thus, the use of eCG with or without a follow-up with eCG antibodies (Pintado *et al.,* 1998) will not deliver the good response (Saharrae *et al.,* 1998; Cognie, 1999). According to Baldassarre and Karatzas (2004), the anti-eCG antibodies were developed as an immune response to previous treatment in an effort to use of eCG repeatedly without detrimental effect on superovulatory response. Generally, the superovulatory response was maintained by using the FSH repeatedly in goats (cFSH) and sheep (oFSH) (Baril *et al.,* 1993).

A route of administering the hormone will lead to variation in superovulatory response based on the rate of gonadotrophin absorption (Dobbs *et al.,* 1994). The absorption of FSH is faster through intramuscular (i.m.) compared to subcutaneous (s.c.) administration of FSH. According to the previous studies in goat, the

administration through i.m. was found to be highly variable in ovulation rate (Armstrong *et al.,* 1983; Selgrath *et al.,* 1990; Mahmood *et al.,* 1991). The FSH injection through s.c. proved to be l more predictable than i.m., lowed unfertilised ova yield and enhanced fertility (Senthil Kumar *et al.,* 2003; Lehloenya and Greyling, 2009). Even though the s.c route of FSH administration can lead to follicular growth and ovulation, the oocytes obtained do not undergo complete maturation. Generally, two types of gonadotrophin were administered in mice, which were PMSG and hCG, with the recommended dose of PMSG (5 IU) via intraperitoneal (i.p.), even though s.c. administration was suggested to be equally efficient (Hogan *et al.,* 1986). The second injection through i.p. was hCG (5 IU dose) to induce the ovulation. The hCG was suggested to be injected via i.p. because it is crucial that the hCG enter the circulation quickly, before the endogenous LH released (Hogan *et al.,* 1986).

### 2.2.2.1.5 Timing of Gonadotrophin treatment

Previous studies 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), the oocyte pick-up (OPU) was performed 36 hours after hCG administration. In goat oocytes collected 36 hours after FSH and eCG or hCG injection were still at the immature stage and therefore need to undergo IVM for 27 hours before becoming meiotically competent (Baldassarre *et al.*, 2003). Good quality goat oocytes for IVM and embryo production were obtained via LOPU at 60 and 72 hours after FSH and hCG treatment (Abdullah *et al.,* 2008). In a later study, it was claimed that the yield of good quality goat oocytes and embryo production were obtained from 60 hours post-treatment of FSH and CG and underwent the IVM for a duration of  22 to 25 hours (Kong, 2010).

In mice, the time interval of PMSG/hCG injection and the light cycle of the animal house will affect the developmental uniformity and the yield of oocytes that recovered from donor (Hogan *et al.*, 1986). They reported that the optimal yield of oocytes in most mouse strains could be obtained from the time interval of 42-48 hours between injection of PMSG and hCG injection. Other evidence also exists about the time interval between PMSG and hCG administration (Mizoguchi and Dukelow, 1980; Hiller *et al.*, 1985; Edgar *et al.*, 1987; Vergara *et al.*, 1997) as well as the time from hCG injection to oocytes collection for IVP (Hashlamoun and Killian, 1985; Vergara *et al.*, 1997) which may affect the yield of fertilised oocytes.

### 2.2.3 Adverse effect of superovulation

The variation in the viability of embryos was related to the influence from the superovulation protocol (synchronisation-hyperstimulation) (Leyva *et al.*, 1998). The progestagen treatment during synchronisation could contribute to alteration in endocrine (Scaramuzzi *et al.*, 1988) and follicular function (Leyva *et al.*, 1998) by lack of complete suppressive effect on LH secretion (Kojima *et al.*, 1992). The donor with endogenous progesterone from a previous CL may suppress the LH secretion. The deficiencies in pituitary regulation by the progestagen will be higher in the absence of CL. The alteration in follicular function led to the ovulation of oocytes with the abnormalities of their development competence, diminished potential fertility and alteration in the normal process of early embryo development (Greve *et al.*, 1995). The uses of PMSG in superovulation regime treatment may cause a high number of non-ovulated follicles, early regression of CL, short or irregular oestrus cycle and potential risk of embryo expulsion (Amoah and Gelaye, 1990). Superovulation with PMSG-hCG combination has been claimed to lead the number of fragmented, degenerated and

denuded oocytes (Miller and Amstrong, 1981; Walton and Amstrong, 1981; Moor *et al.,* 1985; Lehtonen and Kankondi, 1987). Based on previous studies, it was suggested that the embryonic development abnormalities after superovulation with gonadotrophin was mainly influenced by the effect of hormone treatment on maternal oviductal and uterine environment (Elmazar *et al.,* 1989; Van der Auwera *et al.,* 1999).

## 2.3    RECOVERY OF OOCYTES

In order to sustain and success in *in vitro* production (IVP) programme, continuous supply of competent oocytes is important. Oocytes can be collected from both live and killed donors. Oocyte recovery (OR) from live donor may be done through laparoscopic oocytes pick-up (LOPU), surgical oocytes retrieval (SOR), ovariectomy, laparotomy or transvaginal ultrasound-guided aspiration (TUGA) procedures. The OR from killed donors could be obtained at post-mortem via laparotomy and ovariectomy especially in mice.

### 2.3.1 Laparoscopic oocytes pick-up (LOPU) and Surgical Oocytes Retrieval (SOR)

LOPU was not fully practical until the IVP programme was developed in sheep and goat (Baldassare *et al.,* 1994, 2002; Tervit, 1996; Kuhholzer *et al.,* 1997, Graff *et al,* 1995; Pierson *et al.,* 2004).Even though this technique is less invasive, it involves anaesthesia and requires sophisticated equipment and good technical skill for handling the procedure (Baril *et al.,* 1993; Flores-Foxworth, 1997).  Briefly, the donor was restrained on a standard laparoscopy table under general anaesthesia. The ovarian follicles were aspirated under laparoscopic observation (laparoscopy camera and monitor) using a 20G needle mounted in a plastic pipette connected to a collection tube

and a vacuum line. Normally this procedure was performed to retrieve the immature oocytes. Therefore, most of the oocytes obtained were underwent the IVM procedure.

This procedure normally was performed in short time duration (10 to 20 minutes), depending on the number of follicles to be aspirated (Kuhholzer *et al.,* 1997). Commonly, the number of oocytes retrieved from this procedure was more than 5 oocytes per donor (Baldassarre and Karatzas, 2004). Through this procedure, a good quality and quantity of oocytes from prepubertal, pubertal or ageing animal and this showed that the MOET is not possible to be performed (Koeman *et al.,* 2003; Baldassarre *et al.,* 2007). Therefore, LOPU is an efficient method for the 'reproductive rescue' of valuable donor that has reduced fertility because of ageing factor (Baldassarre *et al.,* 2007). LOPU procedure is less traumatic and results in fewer surgical adhesions than laparotomy, thus this procedure could be repeated for several times without ovarian damage or decrease in the donor fertility (Stangl *et al.,* 1999; Alberio *et al.,* 2002; Baldassarre *et al.,* 2003; Baldassarre and Karatzas, 2004). According to Pierson *et al.* (2004) LOPU could be repeated up to 5 times in the same donor with minimal surgical adhesions and no major changes in overall response, and that donor could become pregnant and kid after insemination or natural mating. Another study reported that LOPU had no detrimental effect on the fertility of donor even repeated up to 20 times (Stangl *et al.,* 1999).

Surgical oocytes retrieval (SOR) was a method to collect the ovulated oocytes, does not require euthanasia and preserve the reproductive potential (Byers *et al.,* 2009). This method application is similar with the laparoscopic surgery; however that is not a practical method to small rodents. Previous traditional technique that had been used need euthanasing the donor mice, removing the oviducts and dissecting cumulus oocytes masses from the ampulae (Fowler *et al.,* 1957; Nagy *et al.,* 2003). A clump of cumulus oocytes complexes (COC) inside the ampulla were become a cumulus oocyte

mass (COM). Prior to SOR procedure, the mice were anaesthetising, 12 to 15 hours after post-hCG injection. After that, the ovary and oviduct were surgically exposed and making an incision (1-2 mm) in the ampulla wall to remove the COM (Byers *et al.,* 2009). Then, the incision part were sealed with a tissue adhesive and returned the oviduct to the body cavity and finally, sealed back the abdominal muscle and skin. Multiple oocytes collection could be made from this procedure and also could be repeated using the same donor (Byers *et al.,* 2009). This procedure are still made the mice breed and produce litters for further breeding programme.

### 2.3.2 Laparotomy and ovariectomy

The oocytes recovery via surgical and laparoscopic methods were expensive and the number of oocytes obtained was also very small (Pawshe *et al.,* 1994). Laparotomy or standard surgery that commonly together with ovariectomy was an invasive oocytes recovery method. This is due to it frequently causing adhesion of ovaries, related with complications and even death (Melican and Gavin, 2008). Usually this both technique were done on slaughtered animals, aged animals that has decrease fertility activity or animal that have been underwent several repeated times of LOPU session (>3) (Rahman, 2008b).

Ovaries obtained from the slaughter animals are the cheapest and could give the large number of oocytes (Agrawal *et al.,* 1995) through aspiration the visible ovarian follicles (Wahid *et al.,* 1992), slicing the ovary (Slavik *et al.,* 1992; Watson *et al.,* 1994) and follicular dissection (Fukui *et al.,* 1988) methods. Ovary slicing was reported as simple and more efficient method compared to follicle aspiration (Martino *et al.,* 1994; Pawshe e*t al.,* 1994). However, it was found that more debris will interfere the number of oocytes recovery. Follicle aspiration was done through puncturing the ovarian surface

by an 18G hypodermic needle and recovery the oocytes through aspiration, was an alternative to slicing method (Mogas *et al.,* 1992; Pawshe *et al.,* 1994). The aspiration method may be attributed to the presence of some follicles embedded deeply within the cortex, that were released by puncturing the ovary and some of the oocytes may be lost during aspiration of the follicles that might be happened in this method. Due to this, the number of oocytes recovered might be lower than slicing method.

## 2.4    IN *VITRO* MATURATION (IVM)

*In vitro* maturation is the one of the essential steps in IVP process in animal assisted reproductive technology activity such as IVF, ICSI and cloning. In mammals, the primary oocytes enter meiosis in early prenatal life and progress to the diplotene stage of prophase 1 (Geminal Vesicle; GV stage). At GV stage the oocytes were remaining arrested until before the time of ovulation. However, the oocytes were able to resume the meiosis spontaneously when removed from the follicles and cultured in vitro (Chang, 1951; Edwards, 1965; Gilchrist and Thompson, 2007). At the same time, the nuclear membrane of oocytes disappears and germinal vesicle breakdown (GVBD) and followed by chromosome decondensation that happened at MI stage. Subsequently, the presence of first polar body shows that the oocytes were at MII stage which was ready for the next IVP process. However, the successful of IVM should be to make sure that the oocytes must undergo synchronically nuclear and cytoplasmic maturation as well.

### 2.4.1   Events of Oocytes Maturation

The process name of the oogenesis was called meiosis. This will be start with GV stage of first meiotic prophase during the growth process (Voronina and Wessel, 2003). Then, the oocytes were underwent GVBD and proceed to metaphase I (MI) stage. The extrusion of first polar body shows that the oocytes were at metaphase II (MII) stage. The oocytes growths were happened in follicle. Therefore, the follicle growth rate was related with the oocytes growth rate. The oocytes acquired the ability to progress to MI in follicles diameter were between 1.0 to 1.8 mm and to during achieving the MII stage the follicles diameter size were more than 2 mm. According to De Smedt *et al.* (1992) meiotic competence of oocytes was obtained in antral follicles diameter size between 0.5 to 3.0 mm in goats.

The pre-ovulatory surge of luteinising hormone (LH) were lead the final phase of oocyte maturation within the ovulatory follicle. This may triggers the resumption of meiosis and its progression to ovulate at MII stage. Once the oocytes were removed from the follicle and in vitro cultured, it may result in spontaneous resumption of meiosis (Pincus and Enzmann, 1935). The stage of follicular development where the oocytes were removed was important in order to development into transferable embryo (Hyttel *et al.,* 1997; Dielman *et al.,* 2002). Therefore, the oocytes developmental competence occurs continuously through folliculogenesis, the influence of follicle size and follicle atresia (Mermillod *et al.,* 1999).

During the growth phase of oocytes, the protein were synthesised and storage, ribosomal as well as heterogenous ribonucleic acid (RNA) were taking place (Crozet *et al.,* 1981). This express that the oocytes ability to be competence not only nuclear maturation but also cytoplasmic changes also occurred to maintain embryo development. The cytoplasmic changes were included protein and RNA storage,

development of calcium regulatory mechanisms, maturation-promoting factor (MPF) activity and mitogen-activated protein kinase (MAPK) changes and redistribution of cellular organelles. MPF was played as important role in regulation of cytoplasmic maturation. MPF activity was visible just before GVBD, increased until MI and followed by a sudden decrese in oocytes releasing the first polar body (Hashimoto and Kishmoto, 1988; Choi *et al.,* 1991; Dekel, 1996; Zernicka-Goetz *et al*., 1997). The MPF activity was increased again at MII as well as remains high until fertilisation activity (Dedieu *et al*., 1996; Eppig, 1996).

## 2.4.2   Nuclear and Ooplasmic Maturation

Oocytes acquire the intrinsic ability to support the subsequent stage of development and reaching activation of the embryonic genome. All of this involves complex and distinct events of nuclear and cytoplasmic maturation.

Nuclear maturation refers to meiosis activity that entails the ability of the oocyte nucleus to progress from GV stage to MII stage. GV chromatin of goat oocytes were classified based on the size of nucleoli and the degree of chromatin condensation; i) GV1: large nucleoli and diffuse chromatin; ii) GV2: medium-sized nucleoli and clumped chromatin; iii) GV3: small nucleoli and clumped chromatin and; iv) GV4: no nucleolus but clumped chromatin. The variation in GV stage of oocytes recoverey from a range antral follicles both at the time collection and after period of IVM (Grupen *et al*., 1997; Nagai *et al.,* 1997; Funahashi *et al.,* 1997).   The asynchronous meiotic progression via IVM will increase the aged oocytes, that have different 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*., 1997).

Cytoplasmic maturation involved in a step-wise manner (Eppig and Schroeder, 1989), requiring complete antral development (Ceconi *et al.,* 1996) and maintenance of functional gap junction between somatic and germ cells (Buccione *et al.,* 1990; Ceconi *et al.,* 1996; Eppig *et al.,* 1996). The process of modifying the oocyte cytoplasmn which are important for fertilisation and pre-implantation the embryo development was describing the cytoplasmic maturation. The cytoplasmic maturation involves: a) protein and mRNA synthesis b) development of calcium regulation c) changes in MPF activity (Masui and Markert, 1971) and d) redistribution of cellular organelles. Thus, these were required to obtained oocytes development competence that fosters embryonic development competence (Bravini-Gandolfi and Gandolfi, 2001; Krisher, 2004; Sirard *et al.,* 2006; Watson, 2007). During the cytoplasmic maturation, cumulus cells was played the important role through increased the number of cumulus cell layers and cumulus-oocyte complex (COC) compactness pre-IVM correlated with improved developmental outcome (Shioya *et al.,* 1988; Abeydeera, 2002).

### 2.4.3 Factors Affecting IVM

The event occurring during oocyte maturation can influence the embryonic development (Rajikin *et al*., 1994, Rajikin, 1995; Teotia *et al.,* 2001). During the maturation process, there were several factor has been reported to be affecting the process, for examples, donor age (Izquierdo et al., 2002), follicle size (Pavlok *et al.,* 1992; Blondin and Sirard, 1995), oocyte diameter (Hyttel *et al.,* 1997), oocyte developmental stage (Hagemann *et al.,* 1999), media composition (Lonergan *et al.,* 1997), hormones (Zuelke and Brackett, 1990) and serum (Avery *et al.,* 1998). Duration of maturation also important factor that influencing the successful of good maturation rate. Otherwise, improper timing of maturation could lead the abnormal chromatin formation (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

The oocytes developmental competence could be affected by donor age from prepubertal does (Izquierdo *et al.,* 2002). The prepubertal oocytes developmental competence was decrease may be due to a deficiency in cytoplasmic maturation bringing to reduced sperm penetration, lack of MPN formation, failure to block polyspermy, failure to cleave, failure to reach or survive the transition from maternal to embryonic genomic expression, and failure during pregnancy (during the preimplantation and postimplantation stage (Armstrong, 2001; Velilla *et al.,* 2004). The prepubertal and adult oocytes was different in term of ultrastructure that related with the kinetics of nuclear maturation including abnormal chromatin and microtubule configurations (Damiani *et al.,* 1996; de Paz *et al.,* 2001), level and activity of histone

H1 kinase reduced (indicative of MPF activity) and MAPK (Damiani *et al.,* 1998; Salamone *et al.,* 2001).

**2.4.3.2 Follicle and oocyte diameter**

The follicle diameter, oocyte diameter, meiotic competence and embryo development in goats were found to have a direct relationship among each other (Crozet *et al.,* 1995, 2000). The developmental potential of oocytes is determined by multifactorial interactions; for examples, the oocyte development with same COC morphology and follicle size with the same grade of cumulus expansion might be different in developmental competency (Han *et al.,* 2006). Oocytes from larger follicles (>5 mm in diameter) produced more blastocysts compared to follicles less than 5 mm diameter (Crozet *et al.,* 1995). The oocytes from follicles less than 3 mm in diameter are not fully meiotically competent, and this may lead a limited ability to support embryo development following IVM (Abeydeera, 2002).

Generally, the oocyte diameter shows the level of oocyte growth, where the size of oocyte is increasing when it undergoes intensive synthesis of RNA during maturation (Crozet *et al.,* 1981; Lazzari *et al.,* 1994; Lonergan *et al.,* 1994). The smaller oocytes tend to follow the abnormal path of meiotic maturation and may cause disturbances in maturation process (Lechniak *et al.,* 2002). The meiotic oocyte competence has been categorised based on oocyte diameter for both adult (De Smedt *et al.,* 1992) and prepubertal (Martino *et al.,* 1995) goats as incompetent oocytes (< 110 µm), partially competent oocytes (110-125 µm) and competent oocytes (125-135 µm). It has been demonstrated that once the oocyte growth were increased, its ability to develop to the blastocyst stage *in vitro* were also increased until the optimum rates at a maximum diameter of 135 µm (Arlotto *et al.,* 1995; Fair *et al.,* 1995; Harada *et al.,* 1997).

The degree of expansion of cumulus cells can be used as a morphological indicator of quality of oocytes for IVM and embryo development (Ball *et al.,* 1983; Chen *et al.,* 1993; Qian *et al.,* 2003; Han *et al.,* 2006). The better quality oocyte is characterised by more layers of cumulus cells (CC) surround the oocyte (Blondin and Sirard, 1995; Zuerner *et al*., 2003; Warriach and Cohan, 2004; Yuan *et al.,* 2005; Rahman *et al.,* 2007). It has been observed that high maturation and embryo development rate were obtained using the oocytes with complete layers of CC (> 5 layers of CC) compared with when using the cumulus free occytes (CFO) or having less than one layer of CC.

### 2.4.3.3  IVM culture media

The goat oocytes are maturing in buffered TCM 199 supplemented with L-glutamine, hormones (FSH and oestradiol-17β) and serum (10-20%) (Mogas *et al.,* 1997). Most of the IVM culture media are supplemented with gonadotrophin and oestradiol-17β that can improve the maturation rate significantly (Keskintepe *et al.,* 1994; Izquierdo *et al.,* 1998; Dhruba and Majumdar, 2002).  The presence of gonadotrophin in IVM medium may enhance the oocyte quality and developmental potential by possible alteration of metabolic process (Brackett and Zuelke, 1993).  The oestradiol may enhance the synthesis of presumed male pronucleus growth factors and stimulating DNA polymerase β, where involves in ooplasmic maturation. Previous research has shown that the maturation of oocytes was increased in the medium in presence of oestradiol-17β (Pawshe and Totey, 2003).

Serum contains unidentified growth factors, hormones and peptides which may support the growth and development of oocytes (Kane, 1985). Fatty acid also contains in serum for energy substrates for embryo development (Kane, 1979). In goat, the

concentration of heat-activated serum in IVM media is 10 to 20%. It provides nutrients to cells in COC and prevents zona pelucida (ZP) hardening in sheep (Wani, 2002). In goats, different types of serum were used in IVM medium including homologous and heterologous oestrus goat serum (OGS) (Agrawal *et al.,* 1995; Sharma *et al.,* 1996; Malik *et al.*, 1999), steer serum (SS) (Rodriguez-Gonzalez et *al.,* 2003; Urdaneta *et al.,* 2003b; Jimenez-Macedo *et al.*, 2005; Jimenez-Macedo *et al.,* 2006a; Jimenez-Macedo *et al.,* 2007), foetal calf serum (FCS) (Crozet *et al.,* 1999; Gall *et al.,* 1996; Rho *et al.,* 2001) and foetal bovine serum (FBS) (Martino *et al.,* 1995; Keskintepe *et al.,* 1997; Crozet *et al.,* 2000; Samaké *et al.,* 2000; Mayor *et al.,* 2001; Velilla *et al.,* 2002).

Follicular fluid (FF) from non-atretic or gonadotrophin-stimulated large follicles (>4 mm) has beneficial effect on goat maturation (Cognie *et al.,* 2003) and it can be used as a supplement in maturation medium. This beneficial effect may be due to the presence of growth factors, hormones and intra-ovarian peptides in FF physiological (Cognie *et al.,* 2004). Other than FF, the follicular cells also have been supplemented in goat IVM culture media (Teotia *et al.,* 2001; Dhruba and Majumdar 2002; Jimenez-Macedo *et al.*, 2005). These cells were energised by the granulose cells (GC) that interacted with COC during the IVM (Crister *et al.,* 1986). They initiated the protein and/or polypeptide synthesis that provided the cytoplasm competent to assume normal cooperation with the male genome (Thibault *et al.,* 1987). The GC cells may delay maturation for a few hours (Teotia *et al.,* 1997), however, it will improve the ooplasmic maturation and thus will increase the maturation rate. GC secretion may lead to the synthesis of oocyte glutathione (GSH) which may be involved in male pronuclear formation (Calvin *et al.,* 1986) and in early development. The GSH level was increased during maturation and decreased during the fertilisation and embryo development (Yoshida *et al.,* 1993). The addition of cysteamine increased the GSH concentration and improved the embryo development during IVM and IVC by protect the cells from

culture oxidative stress (De Matos *et al.,* 1995; Luvoni *et al.,* 1996; De Matos and Furnus, 2000).

In order to prevent bacterial and fungal contamination, the antibiotics were generally added in IVM medium. Penicillin and streptomycin have been added in IVM medium (Zhou *et al.,* 2000). Gentamicin has a wider range of pH stability compared to penicillin or streptomycin, and it can maintain the biological activity in presence of serum (Schafer *et al.,* 1972). The same authors suggested that gentamicin was preferred antibiotic to prevent bacterial contamination of goat oocytes in maturation medium.

### 2.4.3.4 IVM duration

The IVM duration is the critical point of the successful of oocytes maturation. The inappropriate duration of maturation may lead the abnormal chromatin (Dominko and First, 1997), oocyte aging (Hunter 1989; Hunter and Greve, 1997) and reduced development (Marstan and Chang, 1964). According to the previous studies, the appropriate time for goat oocytes to reach the 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). On the other hand, 24 hours of IVM duration has been found as the best time for goat maturation *in vitro* (Younis *et al.,* 1991). The latest research also found by Kong (2010) that the optimal maturation rate of LOPU derived oocytes was found when oocytes underwent maturation between 22 and 25 hours. For goat abattoir-oocytes, the optimal duration of IVM was 32 to 30 hours, respectively, in a study on the basis of sequential configuration of chromosomes (Sharma *et al.,* 1996 and Yadav *et al.,* 1997).

It has been reported that variation in the timing of oocyte maturation process *in vitro* could be due to oocyte quality which is related to the age of donor goats (Izquierdo

*et al.,* 2002; Koeman *et al.,* 2003). The increase in abnormal oocytes was probably due to pH variation, osmolarity or accumulation of toxic materials in the media during the later stages of maturation (Shea *et al.,* 1976; McGaughey, 1977; Deb and Goswami, 1990). Therefore, the variable in duration of oocyte maturation may be influenced by the medium used, serum percentage, medium concentration and the culture system (Baldassarre *et al.*, 1996; Samaké *et al.,* 2000; Teotia *et al.,* 2001; Bormann *et al.,* 2003; Cognie *et al.*, 2003).

## 2.5     INTRACYTOPLASMIC SPERM INJECTION (ICSI)

Intracytoplasmic sperm injection (ICSI) is widely used in animals and humans as one of the assisted reproductive techniques (ART) in which a sperm is mechanically injected in the cytoplasmn of an oocyte (Ming-Wen and Llyod, 2006). ICSI technique could overcome the fertility problem by enabling the use of sperm with functional defects and even immature sperm cells to produce normal embryos and offspring.

ICSI technique was initiated in sea urchin (Hiramoto, 1962), followed by mouse (Lin, 1966). The first of live offspring obtained from ICSI technique in domestic animals were reported in several animals, such as in cattle (Goto *et al.,* 1990), sheep (Catt *et al.,* 1996), pig (Martin, 2000) and goats (Wang *et al.,* 2003).

### 2.5.1  Factors Affecting the Outcome of ICSI

ICSI technique requires skill and experience of the performer in order to obtain optimal results. However, this procedure also depends on many other factors, including the microtools, sperm, oocytes, medium used and the ICSI protocol. These factors will be described in the following subsections based on human, mouse, goat and cattle ICSI.

### 2.5.1.1  Microtools

The microtools required in ICSI are ICSI microneedle and holding microneedle. The functions of ICSI microneedle are for sperm catching, sperm immobilising and inserting the sperm into the ooplasma of oocytes. The holding microneedle is used for oocytes holding and manipulation during the ICSI procedure. Both microneedles are mainly made from borosilicate and Pyrex glass. The glass physical properties may influence the setting or programme of the instrument that used in microtools preparation (Joris *et al.,* 1998). The size of capillaries of glass is important to make both microtools (Payne, 1995). The inner and outer diameter of capillaries of ICSI microneedle and holding microneedle was shown to affect the success of embryo development (Tocharus *et al.,* 1996). The ICSI microneedle must be bevelled and sharp and it can either be fitted with a sharp spike or without the spike (Catt *et al.,* 1995). The bevel angel of ICSI microneedle can vary from 28° to 30° (Hamberger *et al.,* 1995; Palermo *et al.,* 1995) and about 45° to 50° (Van Steirteghem *et al.,* 1993). The outer (OD) and inner diameter (ID) of the microneedles can also affect the success of ICSI-derived embryo development (Tocharus *et al.,* 1996). The size of microneedle (OD and ID) should be just large enough with the sperm head to enter and aspirated into the ICSI microneedle. This may prevent the additional mechanical force and to increase the volume of injection solution. The wider inner diameter may result extra distruption of the ooplasm

and arrest development (Tocharus, *et al.,* 1996). The narrow inner diameter may result in difficulty in sperm movement during aspiration and injection (Payne, 1995). It was suggested that the better ID of ICSI microneedle should be varied for different species of animals: human (6-7 µm) (Joris *et al.,* 1998), mouse (7-9 µm) (Ahmadi *et al.,* 1995) and goat (6 to 7 µm)  (Wang *et al.,* 2003).

## 2.5.1.2 General ICSI procedure

The conventional ICSI procedure includes several steps which are sperm catching, sperm immobilisation, oolemma breakage and sperm injection into oolemma. The ICSI procedure requires the use of micromanipulator and inverted microscope. It has been suggested that oocytes should not be exposed to outside environment during micromanipulation for more than 10 minutes (Keskinetepe *et al.,* 1997*)*. In order to maintain proper temperature similar to $CO_2$ incubator, the specimens (oocytes and sperm) are allowed to warm using a transparent stage warmer that is fixed onto the ICSI microscope. There is an optimal temperature requirement for species of animals, for examples, mouse 37.5°C and goat 38.5°C.

Sperm immobilisation could be considered as oocyte activation. Sperm immobilisation was performed on motile sperm before injecting the sperm into the oocyte oolemma. There were several methods of sperm immobilisation include aspirating the sperm in and out of ICSI microneedle (Redgment *et al.,* 1994; Kimura and Yanagimachi, 1995; Yanagida *et al.,*  1999), squeezing the sperm tail using the ICSI microneedle (Fishel *et al.,* 1995; Van den Bergh *et al.,* 1995; Vanderzwalmen *et al.,* 1996) and piezo-pulse applied to mid-piece of the sperm (Kimura and Yanagimachi, 1995; Yanagida *et al.,* 1999). Sperm immobilisation would disrupt the sperm plasma membrane and lead the release of a soluble factor with subsequent intracellular $Ca^{2+}$

oscillation (Dozortsev *et al.,* 1997). This also may lead to sperm nuclear decondensation (Tesarik and Kopecny, 1989; Montag *et al.,* 1992), resumption of meiosis, second polar body extrusion, pronuclear formation and exocytosis (Stice and Robl, 1990; Swann, 1990), DNA synthesis and first mitotic cleavage (Schultz and Kopf, 1995).

Prior to inject the sperm into the oocytes, the MII stage oocyte was held with the position of polar body position at 6 or 12 o'clock to avoid damage to meiotic spindle (Nagy *et al.,* 1995; Joris *et al.,* 1998). The disruption of spindle may cause of oocyte aneuploidy or maturation arrest, thus the cytoskeletal integrity of oocyte may influence the fate of the embryos. However, polar body position is not a reliable guide to the location of metaphase plate (Palermo *et al.,* 1995).

During injection of sperm, one of the technical failures was not injecting the sperm into the oocyte cytoplasmn. This could be due to unsuccessful breaking the oocyte membrane during aspiration of ooplasm into the ICSI microneedle. Therefore, once the sperm was placed next to the membrane and the oolema returned to the original position, the sperm was pushed out into perivitelline space or trapped inside a sac formed by the membrane (Esfandiari *et al.,* 2005). The sperm may stick to the tip or within of ICSI microneedle and pulled out upon withdrawal the microneedle from the cytoplasm. The use of inappropriate size of microneedle for examples, too large or not sharp enough may result in faulty ICSI procedure. In addition, over aspiration of ooplasm may degenerate the oocyte after ICSI. It has been postulated that the cytockelatal structure could be disturbed during ICSI procedure (sperm injection process) and lead to irregular chromosome segregation (Macas *et al.,* 1996; Rosenbusch and Sterzik, 1996).

### 2.5.1.3  Sperm factor

Sperm factor is responsible for initiating the oocyte activation, where the sperm components can trigger oocyte activation in the absence of sperm-oocyte plasma membrane interaction (William, 2002). Other than deficiency of sperm activating factor, the lack of ooplasmic factor could trigger sperm chromatin decondensation (Van Blerkom *et al.,* 1994; Yanagida *et al.,* 1999). The mutual interactions of factors among ooplasm and sperm sub-membrane components initiated the decondensation of sperm nucleus and oocyte activation (Perry *et al.,* 1999). Insufficient and inappropriate capacitation of sperm before ICSI may obstruct the release of sperm factors for take action for oocyte activation (Stricker, 1999) and/or lead in block of sperm head decondensation. It was reported that there are several to  destabilise the sperm membrane before ICSI, such as freezing and thawing (Perreault *et al.,* 1988; Catt and Rhodes, 1995), immobilisation by crushing the sperm tail with microneedle (Keskintepe *et al.,* 1997) or laser shot (Montag *et al.,* 2000) and sonification for  removing the tail and acrosome of the sperm (Goto, 1993; Keefer, 1989).

Sperm capacitation treatments were carried out to increase sperm membrane permeabilisation, acrosome reaction and sperm head condensation following ICSI using by various chemicals. The strategy of treatments is removing the sperm membrane that may improve male nuclear formation and facilitate the sperm-borne oocyte activating factor available to cytoplasm of oocyte. This could be beneficial for embryo development of sperm-injected oocytes (Rho *et al.,* 1998; Wei and Fukui, 1999; Suttner *et al*., 2000). The examples of chemicals that have been used for sperm capacitation were heparin (Keefer *et al.,* 1990; Chen and Seidel, 1997; Wei and Fukui, 1999), caffeine (Gato, 1990; Iwasaki and Li, 1994; Wei and Fukui, 1999), theophylline (Hishinuma and Sekine, 2004) and $Ca^{2+}$ ionophore (Chen and Seidel, 1999; Wei and Fukui, 1999; Rahman, 2008a). Sperm treatment with Triton X-100 (non-ionic detergent)

could have oocyte fertilisation with same efficiency as intact sperm head (Kuretake *et al.,* 1996). The combination sperm treatment between Triton X-100 and dithiothreitol (DTT) was reported to cause paternal chromosome damage and should be avoided in ICSI (Szczygiel and Ward, 2002). However, the combination treatment using heparin and ionomycin can improve the fertilisation and embryo development (Wang *et al.,* 2002; Urdaneta *et al.,* 2004).

Sperm morphology alone cannot be used as an indicator for the successful of ICSI derived embryo development. The abnormalities, such as amorphous, round and elongated heads are related with chromosome defects, however, there was no increase in chromosome aberrations were found in sperm with large or small heads compared to normal morphology (Burruel *et al*., 1996; Ohta *et al.,* 2009). This shows that the abnormal sperm may not be detrimental to embryonic development and further growth as it may not affect the necessary genome and organelles. Thus, the abnormal sperm morphology is not necessary reflect a genetic abnormality of the gamete compared inability to cross physiological barriers (Ben-Yosef and Shalgi, 2001).

Sperm motility and sperm vitality are correlated with the ICSI performance. The sperm is considered dead when the sperm plasma membrane are severely damaged and lost. The immotile sperm that does not fertilise oocyte is obviously dead as cells (Goto, 1997). However, according to Nijs *et al.* (1996) the immotile sperm shows some motility after 2 to 3 hours of incubation. The dead sperm DNA may have already started the degeneration process (Goto, 1997). It was reported that normal offspring were produced from killed and dead sperm by using frozen-thawed sperm without cryoprotectant (Goto *et al.,* 1990; Hatakeyama e*t al.,* 1994; Hoshi *et al.,* 1994; Wakayama *et al.,* 1998). These could show that the sperm were dead as cells but the nuclei were still alive for further development process (Goto, 1997). Even though research has shown that the immotile sperm has the possibility to fertilise the oocyte, in

clinical situation, the motile sperm should be selected for ICSI to get a better chance of choosing normal DNA compared the degenerated DNA from immotile sperm.

**2.5.1.4 Oocytes factors**

Effect of oocyte morphology on the quality of ICSI-derived embryos has been controversial especially using the abnormal morphology oocyte. Some researchers claimed that no correlation between oocyte morphology, fertilisation rate and embryo quality (De Sutter *et al.,* 1996; Balaban *et al.,* 1998). However, other researchers found that the oocyte morphology influenced the fertilisation rates and embryo quality following ICSI. The dysmorphic oocyte could undergo normal fertilisation but did not have same developmental potential (Sehral *et al.,* 1997) or less competent (Rahman, 2008a) than normal oocytes. Abnormal oocytes or oocyte dysmorphism can exhibit in different forms such as excessive granularity, vacuolarisation, fragmented polar body and refractile bodies.

The *in vitro* maturation (IVM) culture condition could be affected by unsuitable culture condition (Geshi *et al.,* 1999). This inappropriate condition may lead to glutathione depletion in oocytes block decondensation of male pronuclear (MPN) during fertilisation (Sirard *et al.,* 2006). The duration of IVM may also affect the fertilisation rates after ICSI (Garcia-Rosello *et al.,* 2006; Kong, 2010). For examples, oocytes that undergo IVM with duration time at 22 to 25 hours were more survival compared to the longer conventional IVM duration at 27 to 32 hours (Kong, 2010). These shows that the delayed injection of ICSI may contribute to oocyte aging, failure of MPN formation and reduce the fertilisation rate (Zheng *et al.,* 2004).

The flexibility or elasticity aspect of oocyte oolemma is important to ensure the success of ICSI. A high elasticity of oolemma can damage the oocyte once the insertion

of ICSI microneedle into the oocyte. The less elasticity of oolemma can allow the ICSI microneedles penetrate easily into the oolemma, but if it becomes more elastic it is difficult to penetrate the oocyte. In order to minimise the oolema damage during ICSI, the ICSI microoneedle is designed with a sharp spike at the tip of the needle (Nagy *et al.,* 1995; Palermo *et al.,* 1996). The high elasticity of zona pellucida and oolemma of some species, for example the mice, would result in injection difficulty. This may be due to low viscosity of the ooplasm (Kimura and Yanagimachi, 1995). In conventional ICSI, even though using the sharp tip of ICSI microneedle, the zona pellucida was found to be easy to break; however, the oolemma was hard to break even the tip of microneedle reached the cortex opposite side of oocyte (Kimura and Yanagimachi, 1995). This may cause the oocyte to burst during ICSI. Therefore, to overcome this difficulty, Piezo-driven was widely used in mouse ICSI which was introduced by Kimura and Yanagimachi (1995). Using this device, the efficiency of ICSI was improved not only in mice (Kimura and Yanagimachi, 1995) but also in other species such as cattle (Katayose *et al.,* 1999), human (Huang *et al.,* 1996) horse (Choi *et al.,* 2002) and goat (Wang *et al.,* 2003). This device was found to give less traumatic to the oocytes than the conventional method, where the oolemma breakage can be performed using completely flattened tips together with applying the piezo-electric effect. However, most of the ICSI procedures are still performed conventionally in which optimal results in fertilisation rate and embryo development could be achieved (Zhou *et al.,* 2004; Jimenez-Macedo *et al.,* 2005, 2006, 2007; Rahman, 2008a, b; Kong, 2010; Ainul Bahiyah, 2010).

**2.5.1.5 Role of medium used in ICSI**

In common ICSI medium contains hepes buffer to maintain pH of 7.2 to 7.4 outside the $CO_2$ incubator. This ICSI medium functions when oocytes are manipulated during ICSI procedure. The ICSI medium could be used from the basic culture media, such as modified synthetic oviductal fluid (mSOF) (Takahashi and First, 1992), modified defined medium (mDM) or BO (Brackett and Oliphant, 1975; Younis *et al.,* 1991) and tissue culture medium 199 (TCM-199) (Lonergan *et al.,* 1994).

The oocyte are used for ICSI have to be free from cumulus cells (CC) by using hyaluronidase. However, the amount of concentration of hyaluronidase should be reduced from 760 IU/ml to 160 IU/ml, because oocyte activation was observed before injection procedure (Palermo *et al.,* 1992).The lower concentration of hyaluronidase (80 IU/ml) was shown to increase fertilisation rate and the yield of activated oocyte was decreased drastically (Joris *et al.,* 1998).

Polyvinylpyrrolidone (PVP) is a large weight polymer (molecular weight 360,000), has been widely used in ICSI to increase viscosity of sperm solution that facilitate sperm handling, control of ooplasmic aspiration and sperm injection procedure, prevent adhesion of sperm to wall of injection microneedle (Uehara and Yanagimachi, 1976) and allow clear observation of sperm motility patterns (Cohen *et al.,* 1994). PVP also has been reported can affect the acrosomal status of sperm and enhance pronuclear formation in ICSI (Kato and Nagao, 2009). However, the exposure of sperm to the PVP can cause submicroscopic changes in structure and the nucleus becomes damaged (Strehler *et al.,* 1998). This may be due to breakdown of sperm membrane, thus the nucleus tends to decondense frequently (Strehler *et al.,* 1998). It was found the oocyte that was exposed to PVP may cause delaying between sperm injection and the beginning of calcium oscillation after ICSI, and may suppress the

embryonic development (Dozortsev *et al.,* 1995a,b). Even though some studies have shown the adverse effect of PVP in ICSI, there was a report on the success of ICSI pregnancy in human with using 10% (w/v) concentration of PVP for manipulation and sperm injection process (Palermo *et al.,* 1992). Therefore, PVP is still being used for ICSI in facilitating the sperm motility with lower concentration (~10%, w/v) in mouse and goat.

### 2.5.2   Oocyte Activation Following ICSI

The lower oocyte fertilised and developed to embryos is probably due to insufficient of oocyte activation and sperm capacitation that make the oocyte fail to fertilise and develop to embryo. Proper oocyte activation may cause the oocyte to form two pronucleai, cleaved and developed normally into embryo (Nakagawa *et al.,* 2001). The artificial oocyte activation has been studied not only for understanding the mechanism of oocyte activation (Schuetz, 1975) but also for studying parthenogenesis (Steinhardt *et al.,* 1974) and nuclear transplant (Wakayama *et al.,* 1998). There are several types of artificial oocyte activation, depending on species, involving chemical activation, mechanical activation and electrostimulation activation.

### 2.5.2.1 Chemical activation

Chemical activation for artificial oocyte activation has been used as single chemical activator such as ethanol (Hamano *et al.,* 1999), $Ca^{2+}$ ionophore (Goto *et al.,* 1990; Probst and Rath, 2003; Rahman, 2008a), ionomycin (Rho *et al.,* 1998); or combined with more than one chemical such as, ionomycin and 6-dimethilaminopurine (6-DMAP) (Fulka *et al.,* 1991; Rho *et al.,* 1998; Chung *et al.,* 2000; Kong, 2010). The initiation and propagation of oocyte activation is mainly performed by calcium in intracellular signaling function. There is a variable pattern of intracellular $Ca^{2+}$ increase in oocyte, depending on various chemicals used. For examples, calcium ionophore A23187, ionomycin and ethanol induce only one increase in intracellular $Ca^{2+}$ for single treatment. However, the MPF was incomplete to inactivate due to reaccumulation of cyclin B (Liu and Yang, 1999) and lead the oocyte to arrest again at MII stage (Liu and Yang, 1996; Rho *et al*, 1998; Chung *et al.,* 2000). Therefore, these chemicals were accompanied with other chemicals to obtain the efficient activation process, such as cycloheximide (CHX) which performed as a protein synthesis inhibitor (Galli *et al.,* 2003) or 6-DMAP as a protein kinase inhibitor (Rho *et al.,* 1998; Ock *et al.,* 2003; Oikawa *et al.,* 2005, Kong, 2010). These combinations of chemicals can make the MPF in activation (directly or indirectly) without changing the intercellular calcium. The histone kinase inhibitor and the prevention of MPF reaccumulation can improve the efficiency of oocyte activation (Susko-Parish *et al.,* 1994) .Strontium prepared in combination of cytochalacin B was effectively used in mouse oocyte activation. This treatment induced the $Ca^{2+}$ transient in oocyte activation (Kono *et al.,* 1996) and mediated through inositol triphosphate receptor (Zhang *et al.,* 2005).

### 2.5.2.2 Mechanical activation

The injection of sperm into oocyte during ICSI was found to lower the MPF activity of oocyte after ICSI. The MPF could not maintain at low level and has a shortage of cyclin B that promoted progression towards early cleavage was inhibited in the ooplasm by temporary elevation (Fujinami *et al.,* 2004). Other mechanical activation, for example using using the Piezo-drive would increase the oocyte activation and cleavage rates. This has been shown in previous research in goat (Wang *et al.,* 2003), cows (Katayose *et al.,* 1999; Wei and Fukui, 2002) and mouse (Kimura and Yanagimachi, 1995).

### 2.5.2.3 Electrostimulation

Electrostimulation is a device that can be used for oocyte activation. Oocytes were placed between parallel electrodes plates, electric field will generate by direct current voltage. This process would cause protein to be charged in lipid bilayer of the cell membrane to move and pores were formed in the membrane (Zimmerman and Vienken, 1982). Electrostimulation was not only been used for ICSI but also in other studies such as parthenogenesis and animal embryo cloning (Yanagida *et al.,* 2008). The activation oocytes occurred when the extracellular $Ca^{2+}$ transient elevating the interior $Ca^{2+}$ concentration through the pores during generating the electric field. The process will takes  about 10-40 minutes (37°C) for pore to repair and will take longer duration if the temperature is lower (Bates *et al.,* 1987). This method was successfully applied to produced embryo development via ICSI in various species such as mouse (Sasagawa and Yanagimachi 1996; Yanagida *et al.,* 1999), pig (Probst and Rath, 2003; Yoo *et al.,* 2011), cattle (Hwang *et al.,* 2000).

## 2.6    *IN VITRO* CULTURE (IVC)

First report of *in vitro* culture (IVC) of mammalian embryos was made by Schenk (1880). The development of IVC system and media was paralleled with the development of *in vitro* production (IVP) technologies. There are numerous factors or stressors affecting the performance of IVC (Lane, 2001; Lane and Gardner, 2005). These stressors are the inappropriate of media formulation and supplement, problem in culture system, technical issue or improper of quality control and quality assurance management during handling the cultivation (Gardner, 2004). These stressors tend to bring the cell or embryo to change its morphology (Pollard and Leibo, 1994; Crosier *et al.,* 2000, 2001; Abe and Hoshi, 2003), metabolism (Khurana and Niemann, 2000; Thompson, 2000; Houghton and Leese, 2004), cell proliferation and apoptosis (Knijn *et al.,* 2002; Gjorret *et al.,* 2003), transcriptome (Wrenzycki *et al.,* 2005; Fabian *et al.,* 2005; Corcoran *et al.,* 2006; Sagirkaya *et al.,* 2006) and proteome (Katz-Jaffe *et al*., 2005). In addition, the *in vitro* embryonic development  is affected by various factors such as, embryo density during culture (Lane and Gardner, 1992), co-culture with somatic cells (Smith *et al.,* 1992), growth factors (Grupen *et al.,* 1997) supplementation with antioxidants (Kitagawa *et al.,* 2004) and oxygen tension (Karja *et al.,* 2004; Booth *et al.,* 2005).

### 2.6.1   *In Vitro* Culture Media

In earlier stages in developing the optimal media, most of the studies were conducted in mice. There are two types of medium used in embryo culture work which are biological and chemical defined media. In the pioneer experiments, a mixture of egg yolk with inorganic salts (biological defined medium) without culturing in $CO_2$ atmosphere was proved to be able to obtain the blastocysts from 8-cell stage mouse embryos

(Hammond, 1949). Chemical defined medium is an artificial mixture of chemical components that can replace or similar to the natural microenvironment of cells after they are explanted. The first chemical defined medium was described by Whitten (1956). It is widely used compared to biological medium due to its availability to be used any time in any laboratory, and free of enzyme activities that can interfere with the responses being studied.

The basal culture medium composition for embryos is an important factor for development process. There were different types of chemical semi-defined and defined media as a basal culture medium. For examples, the Synthetic Oviductal Fluid (SOF) (Tervit *et al.,* 1972; Krisher *et al.,* 1999), CR1aa and CR2 (Rosenkrans and First, 1991), Chatot-Ziomek-Bavister (CZB) (Chatot *et al.,* 1989; 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).

Carbohydrate is important for energy that placed by $Na^+$ and $K^+$-ATPase required for blastoceol formation (Thompson, 1996). Pyruvate and/or L-lactate are not a compulsory requirement but it can enhance the embryo development. The pyruvate is used for the early cleavage stage nutrient where this chemical are involved in sequential media. The sequential medium was developed to respond to the different level requirements of nutrient to different stages of embryo development (Bavister, 1995; Gardner and Lane, 1997; Pool, 2004). In sequential medium, during earlier cleavage stage when the low levels oxygen is consumed, pyruvate is added in the medium. Then, during the post-compaction period, the new same medium is used where the pyruvate is switched to glucose to allow the embryo development process meets the increased energy demand for blastulation, differentiation and growth (Donnay *et al.,* 1999; Thompson, 2000; Houghton and Leese, 2004; Lopes *et al.,* 2007). Furthermore, through sequential medium, the byproduct accumulation, such as ammonium and serum and this

may be reduced the risk of large offspring syndrome (Van Wagtendonke Leeum *et al.,* 2000).

Serum albumin supplementation and oestrus serum (OS) are used as protein supplement in IVC media. The serum albumin supplementation is good for preventing embryos from adhering to the Petri dish surface and chelates the metal cations. The OGS is used as protein source to provide the undefined beneficial growth factors, peptides, hormones (Phua, 2006). Lipid and fatty acids were found to not be important requirement in IVC medium. It has been found that the embryos were successfully cultured by using the lipid-free medium (Seidel *et al.,* 1991; Bavister *et al.,* 1992).

The toxic heavy metal and free radical production may remove and prevent from IVC medium by using the antioxidant. The ethylene diamine tetraacetic acid (EDTA) could be used for this purpose (Nasr-Esfahani *et al.,* 1992). The free oxygen radical was found to lead blocking in embryo development process (Legge and Sellens, 1991). Other antioxidants could be used are thioredoxin and superoxide dismutase (Nonogaki *et al.,* 1991), catalase (Nasr-Esfahani and Johnson, 1992) and vitamin (C and E) (Vermeiden and Bast, 1995).

### 2.6.2 *In Vitro* Culture System

The culture system referring the culture environment may influence the embryo development and gene expression (Harvey *et al.,* 2004; Fisher-Brown *et al.,* 2005; Gyu-Jin *et al.,* 2007).The physical nature of embryo culture, temperature, osmolarity, oxygen concentration, degree of humidification, oil used and embryo density are varied among laboratories.

Embryos have varying capabilities to develop depending on the genetic background (Hansen, 2007; Thompson *et al.,* 2007) and the sperm used (Palma and Sinowatz, 2004; Fischer-Brown *et al.,* 2005) in any particular culture system. The low atmospheric oxygen concentration level was found to be reflected the rate of embryo development in many mammalian species (Hooper *et al.,* 2001; Orsi and Leese, 2001). The embryo oxygen requirement changes with development level. The post-compaction may need less oxygen concentration compared to at pre-compaction embryos, based on ATP generation from oxidative phosphorylation decrease during the compaction begins (Thompson *et al.,* 1996). The low oxygen (5%) was found to increase the embryo development rates in mouse (Hooper *et al.,* 2001; Orsi and Leese, 2001) and also in goat (Keskintepe *et al.,* 1997; Wang *et al.,* 2003; Jimenez-Macedo *et al.,* 2007).

The osmolarity, temperature and pH of media have been to be important in embryo development environment process. The osmolarity of media was kept at 270 to 280 mOsm/kg (Tervit *et al.,* 1972; Younis *et al.,* 1991) or 280 to 300 mOsm/kg (Brackett and Oliphant, 1975; Takahashi and First, 1992). The temperatures of media were different between species, where the mouse temperature is 37.5°C and for the goat is 38.5°C in the $CO_2$ incubator. The pH of IVC medium was maintained at range 7.2 to 7.4 both for mouse and goat embryo cultivation.

There are various types of oil can be used to overlay the microdroplet of medium, such as mineral oil, paraffin oil or silicone oil. The light mineral oil or paraffin oils are commonly used for this purpose because they are less toxic and clear in appearance compared to silicone oil. The used of oil is to prevent evaporation and gas diffusion, thus can stabilise the pH, temperature and osmolarity of medium (microenvironment surrounding the samples).

**Chapter 3**

**3.0 MATERIALS AND METHODS**

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## 3.1 INTRODUCTION

This study was conducted to produce viable embryos through intracytoplasmic sperm injection (ICSI) technique with special reference to sperm capacitation, *in vitro* maturation duration, post-hCG duration and oocytes recovery cycle. Three species of experimental animal were used which were mice, pigs and goats. Mice and pigs were used as preliminary studies before conducting the goat experiments.

The animal management, medium preparation and protocol were slightly different among the species of animals used. Mouse and goat experiments were conducted at the Nuclear Transfer and Reprogramming Laboratory (NaTuRe), Institute of Research Management and Monitoring (IPPP). All media and reagents were prepared in Embryo Micromanipulation Laboratory (EMiL), Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia. The experiments were conducted from February 2010 to May 2011.

## 3.2 EXPERIMENTAL ANIMALS

### 3.2.1 Mice

The mice were bred in the Animal House, ISB Mini Livestock Farm, University of Malaya. Mice were kept in clean cages and were separated according to gender or kept in mating cages (male: female ratio of 3:5). They were fed with commercial pellet and

clean drinking water was made available to the animals *ad libitum*. The cage was layered by dry saw-dust and cleaned at least once a week.

The mice were bred at room temperature (25°C) with natural light: dark cycle (12 hours: 12 hours). Both male and female mice were bred from same strain (ICR inbred; Appendix Figure 2.1). The date of birth was noted for identify the age and growth development of the offspring. The newborn offspring were separated from the mother after 1 month old (4 weeks). After separation from mother, the pups were straightly separated by gender and the date of birth was noted. The separated female mice could be ready to use for experiments when they reached 8 to 10 weeks old and for the male at 10 to 12 weeks old.

### 3.2.2 Goats

The experimental goats were sourced from ISB Mini Farm, University of Malaya. Female goats were selected as oocytes donors which were under went oestrus synchronisation and superovulation. The experimental goats that have been used consisted of Boer crossbred and Local Mix breed. The age of the animals ranged from 12 to 42 months old.

The frozen semen was obtained from Jermasia buck was used for ICSI procedure in goats. The semen were cryopreserved at University of Malaya before used in this study. The frozen semen were kept in 0.5 ml French straws and stored in the liquid nitrogen storage tank at -196$^{\circ}$C.

## 3.3    MATERIALS

Materials used in the present study included various equipment, chemicals, reagents, consumables and disposables. These are briefly described in the following sections.

### 3.3.1   Equipment

The equipment that have been used in the present study were listed with model number, manufacturer's and supplier's name in Appendix Table 1.1. The commonly used equipment included autoclave, $CO_2$ incubator, centrifuge, flushing and aspiration system, laparoscopic system, fluorescent microscope, inverted microscope with micromanipulator, stereomicroscope, laminar flow work station, liquid nitrogen tank, micropipette microforge, micropipette micropuller, micropipette grinder, osmometer, pH meter, stage warmer, surgical set, ultrapure water system and waterbath.

### 3.3.2   Chemicals and Reagents

Analytical grade laboratory and cell tested chemicals and reagents were used in preparation of the solutions and media. The chemicals and reagents were mainly purchased 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 are listed in Appendix Table 1.2. For media, the pH was adjusted to 7.2 to 7.4 and the osmolarity to 280 to 300 mOsm/kg (Tervit *et al.,* 1972; Brackett and Oliphant, 1975; Younis *et al.,* 1991; Takahashi and First, 1992).

### 3.3.3 Disposables and Consumables

A list of disposables and consumables with manufacturer's name and catalogue number used in this study is tabulated in Appendix Table 1.3.

### 3.4 METHODOLOGY

### 3.4.1 General Maintenance for a Good IVP Environment

In order to obtain a good IVP environment, all laboratory activities and facilities adhered to strict cleanliness regimes and sterile techniques were followed throughout all experimental procedures. The laboratory users were reminded to be responsible to cleanliness and hygienic environment in order to optimise the outcomes of the experiments. It is very important to minimise the potential infection or contamination while handling and culturing the sperm, oocytes and embryos.

Most of the embryo works were carried out using the $CO_2$ (5%) incubator in humidified air to maintain the correct physiological pH (pH range 7.3 to 7.4) with the temperature of $38.5\degree C$ (for goat samples) or $37\degree C$ (for mouse samples). In order to maintain the sterility and ideal environment for the embryos, oocytes or sperm, the incubator was cleaned monthly. The cleaning process involved wiping the inside wall, doors and racks with sterile reverse osmosis (RO) water using the sterile towels or tissues. The tray and the RO water contained in it which was to provide humidity were sterilised and changed according to the scheduled time. The $CO_2$ (5%) incubator should be monitored regularly and the LED display of temperature was checked with independent reading. For better precaution steps, the repeated opening and closing of the $CO_2$ (5%) incubator must be kept to the minimum because it may affect the stability of the oocyte or embryo environment.

The washable glassware and non-disposable items were rinsed vigorously with tap water to remove any debris. Blood, sediment items and tissues, for examples, were rinsed once or twice before being soaked in a diluted detergent (7x®-PF) for a few minutes. Then, they were cleaned using sponge after which were rinsed vigorously five times with tap water followed by reverse osmosis (RO) water. After that, they were either wrapped with aluminum foil or packed in autoclavable disposable bags. All the items were sterilised by autoclaving them at $121^{\circ}$C for 20 minutes. Later, all items were dried in an oven at $60^{\circ}$C. The waterbath should be cleaned and the water changed using RO water at least once a week.

Before conducting the experiments or preparing the medium, the general cleanliness could be achieved by applying hand sanitiser or washing with diluted detergent and spraying with 70% alcohol. Equipment such as laminar flow and microscope should be wiped with 70% alcohol using cleaned and dried tissues. The work station should be sterilised with 70% alcohol before commencing the experiment. The residual traces of alcohol were allowed to evaporate for at least 20 minutes before starting the laboratory activities. Subsequently, all glassware, culture dishes and other consumables ought to be UV sterilised by placing them inside the laminar flow for 15-30 minutes before conducting the experiments. However, the media, chemicals and reagents should not be exposed to UV light.

### 3.4.2 Preparation of Stock Solutions and Media

All stock solutions and working media were prepared 'in-house' instead of purchasing commercially. In the process of preparing the media, the toxic contaminants in the culture medium ingredients, including water, were a major concern. Therefore, in a way to obtain the good media, water source should be from the ultrapure water purification system; with treatments of particulate filtration, activated carbon filtration, RO and electrode ionisation (EDI), ultraviolet oxidation system, followed by Milli-Q PF Plus purification (18.2 MΩ-cm). All the prepared media finally would be filtered with a membrane filter (0.22 µm) to discard trace particles as well as to prevent bacterial contamination.

Normally, the fresh culture media were prepared weekly or fortnightly. Preparation of different culture media requires accurate but time consuming measurements. Due to these, it is convenient to prepare media from a series of stock solutions (Nagy *et al.,* 2003). The media were prepared into two types which were stock solutions and working media. All fundamental stock solutions prepared were filter-sterilised using syringe filter (0.22 µm pore sizes) aliquot in microcentrifuge tubes or bottles and stored in refrigerator (2-8$^{°}$C) or freezer (-20$^{°}$C) as appropriate. All the media were prepared under laminar air flow work station.

In present study, two species of animals were used, namely goat and mouse. Consequently, most of the stock solutions and working media used were different as the culture conditions for the two species were different. Thus, in this study, the media preparation was prepared depending on the experimental animals.

### 3.4.2.1  Medium preparation for goat experiments

In general, the goat experiments involved the following techniques such as LOPU, IVM, sperm preparation, ICSI, activation and IVC. The detailed preparations of media are given below.

### 3.4.2.1.1  *Preparation of heparinised saline solution*

The heparinised saline solution was used for preventing blood clotting during the surgery (LOPU) procedure. The medium was prepared by weighing the NaCl (9.00 g) and heparin (0.05 g) using a digital balance and dissolved in one litre ultrapure Milli-Q water by stirring gently. Then, the saline was sterilised by autoclaving ($120^{o}$C) and kept for 3 months in the refrigerator ($4^{°}$C) for further use.

**Table 3.1**: Composition of heparinised saline solution

| Chemical (catalogue no.) | Concentration | Quantity/ 1000 ml |
|---|---|---|
| NaCl (S5886) | 0.9 (w/v) | 9.00 g |
| Heparin (H0777) | 0.0 mg/ml | 0.05 g |

### 3.4.2.1.2  *Preparation of flushing medium*

Flushing medium was prepared during aspirating or collecting the oocytes from the ovarian follicle during oocyte retrieval via LOPU technique. Generally, a microvolumes of fluid was flushed into the ovarian follicle and then would be aspirated with the oocyte together with fluid from the follicle. The aspirated contents (3 ml) were then collected in a sterile round-bottom test tube which was pre-warmed by test tube

heating system. Then, the collected fluid was searched for the oocytes under stereomicroscope. Typically, this medium was prepared 12 hours before oocytes retrieval. The flushing medium consisted of diphosphate-buffered saline (DPBS) supplemented with penicillin G, streptomycin sulfate, heparin and polyvinylpyrrolidone (PVP).

**Table 3.2**: Composition of flushing medium

| Chemical components (catalogue no.) | Quantity/ 1000 ml |
|---|---|
| DPBS tablets (BR0014G) | 10 tablets |
| Penicillin G (P779) | Penicillin G was dissolved in DPBS prior to use |
| Streptomycin sulphate (S1277) | Streptomycin sulphate was dissolved in DPBS prior to use |
| Heparin (H0777) | Heparin (*mg) was dissolved in DPBS solution prior to use |
| PVP (PVP360) | PVP was dissolved in DPBS prior to use |

*1 mg of heparin contains 156 IU.

### 3.4.2.1.3 *Preparation of blood and heat-inactivated serum*

The blood was collected from the goat during the oestrus time to obtain the serum which was known as oestrus goat serum (OGS). This serum is traditionally supplemented in oocyte or embryo culture media to provide additional unidentified beneficial growth factors, hormones and peptides. Prior to using the serum, it was heat-inactivated to inactivate potentially harmful components. The blood samples were collected aseptically via jugular vein from oestrus goat using Vacutainer® tubes

(without heparin) with a needle (21G) and a venojector holder. The collected blood was left to clot for 30 to 60 minutes in a laminar flow at room temperature (25$^{\circ}$C) to obtain the serum from the clot blood. Then, the blood was centrifuged (500 $x$ $g$, 10 minutes, 25$^{\circ}$C). The supernatant (serum) was aspirated gently into conical centrifuge tube (15 ml). Later, the serum was heat in-activated in waterbath for 30 minutes in 56$^{\circ}$C to destroy components that might lead to cell lysis by antibody binding. After heat-inactivation, the serum was cooled at the room temperature (25$^{\circ}$C) and it was centrifuged again (500 $x$ $g$, 10 minutes, 25$^{\circ}$C) to sediment residue erythrocytes. The OGS sterilised using sterile syringe filter (0.22 µm pore size) and aliquot in sterile microcentrifuge tubes (1.5 ml) and stored with a maximum shelf life of 6 months in the freezer (-20$^{\circ}$C). The OGS should be warmed (room temperature) immediately prior to use.

### 3.4.2.1.4    *Preparation of IVM medium*

### 3.4.2.1.4 (a) Preparation of IVM stocks solution

The IVM medium were prepared from the IVM stock and equilibrated inside the $CO_2$ incubator (5%) at least 3 hours before use. Prior to preparation of the IVM solution, several stocks solution should be prepared. All the stock solutions should be kept in cryotubes which were covered with aluminium foil.

**Table 3.3**: List of stock solutions for IVM medium

| Stock solution | Chemical composition | Method of preparation | Storage temperature and shelf life |
|---|---|---|---|
| TCM-Py | Sodium pyruvate (P4562) Cystein (C2529) TCM 199 (11150-059) | Weigh 0.0022 g of sodium pyruvate and 0.0085 of cystein and dissolved with 1 ml of TCM 199 in 1.5 ml microcentrifuge tube. Slowly vortex the components for around 10 sec and filter with sterile syringe filter. | $4^{o}$C for 2-3 days |
| bFSH | Folltropin V$^{®}$ (L032-B053) | Dissolve 0.0050 g of the powder to 1 ml of solution provided with the powder. Gently dissolve and filter with sterile syringe filter. | $4^{o}$C for 6 months |
| Gentamycin | Gentamycin (G3632) DPBS solution | Weigh and add 1 g of gentamycin to 20 ml of DPBS solution. Gently dissolve and filter with sterile syringe filter. | $4^{o}$C for 6 months |
| Oestradiol 17β (E$_2$) | Oestradiol 17β (E8875) Ethanol | Weigh and add 0.001 g of oestradiol in 1 ml ethanol (already sterile filtered before used). | $4^{o}$C for 6 months |
| Oestrus goat serum (OGS) | - | Preparation of OGS was similar as stated in 3.4.2.1.3. | $-20^{o}$C for 6 months |

*3.4.2.1.4 (b) Preparation of IVM working solution*

The IVM working solution was freshly prepared and equilibrated inside the $CO_2$ (5%) incubator at least 3 hours before being used. In order to make the IVM working solution, all stock solutions were warmed under room temperature ($25^{o}$C) inside a laminar flow. Stock solutions should be avoided from being exposed to the light to prevent the oxidation. Table 3.4 shows the volume that was needed for preparation of the IVM working solution. All the solutions were mixed, except the oestradiol. Then, the medium was sterilised using the sterile syringe filter (0.22 μm; Milipore). Finally,

oestradiol was added into the sterile medium and later, the solutions were gently mixed. Prior to equilibration, a droplet form of IVM working solution were made and covered with the mineral oil (M8410) and kept inside the $CO_2$ (5%) incubator.

**Table 3.4**: List of components for IVM working solution

| Stock solution | Amount of volume/ 10 ml |
| --- | --- |
| TCM-199 with Earle's salt, L-glutamine and sodium pyruvate (11150-059) | 8.90 ml |
| TCM-Pyruvate | 100.00 µl |
| bFSH | 10.00 µl |
| Gentamycin | 5.00 µl |
| (Oestrus goat serum) OGS | 1.00 ml |
| Oestradiol 17β ($E_2$) | 10.00 µl |

*3.4.2.1.5    Preparation of other solutions*

A number of stock solutions and other solutions were prepared to be used in sperm preparation, oocyte denudation or activation medium.  These are as follows:

### 3.4.2.1.5(a) Preparation of heparin stocks

Heparin was used in the sperm capacitation medium to aid in sperm capacitation activity. The heparin stock solution was prepared by dissolving heparin (5 mg) in ultrapure water (5 ml). The solution was mixed properly and sterilised using syringe filter (0.22 µm). Then, the solution was aliquot (100 µl) in microcentrifuge tube (0.1 ml) and stored for six months in the freezer (-20$^{\circ}$C).

**Table 3.5**: Composition of heparin stock solution

| Chemical (catalogue no.) | Concentration | Quantity/ 5 ml | Storage temperature and shelf life |
|---|---|---|---|
| Heparin (H0777) | 1 mg/ml | 5.00 mg | -20$^{\circ}$C for 6 months |
| Ultrapure water | - | 5.00 ml | |

### 3.4.2.1.5(b) Preparation of hyaluronidase solution

Hyaluronidase solution was used to aid in COCs denudation after maturation. Hyaluronidase solution (0.1%) was used for this study. 0.01 g of IV-S hyaluronidase from bovine testes was dissolved into TCM 199 with Hepes (5 ml) stock solution medium. Then, the mixed solution was stirred until dissolved properly. Additional TCM 199 with Hepes (5 ml) was added and stirred again until the powder was dissolved properly. The prepared solution was filter-sterilised by syringe sterile filter (0.22 mm), aliquot into microcentrifuge tube (100 µl) and stored for 3 months in the freezer (-20$^{\circ}$C). The storage hyaluronidase (0.1%) was warming before used.

**Table 3.6**: Composition of hyaluronidase solution (0.1%)

| Chemical (Catalogue no.) | Concentration | Quantity/ 10 ml | Storage temperature and shelf life |
|---|---|---|---|
| IV-S Hyaluronidase from bovine testes (H4272) | 0.1 % | 0.01 g | -20$^{\circ}$C for 3 months |
| TCM 199 with Hepes | 1x | 10.00 ml | |

### 3.4.2.1.5(c) Preparation of calcium ionophore solution

Oocyte activation was done using double activation with calcium ionophore (CaI) and 6-dimethylaminopurine (6-DMAP). The CaI stock A (5 mM) was prepared by dissolving calcium ionophore (10 mg) with DMSO (38.2 ml) and stored at -20$^{\circ}$C. Aliquot of stock A (10 µl) into microcentrifuge tube (1 ml).

**Table 3.7** Composition of calcium ionophore stock A solution

| Stock | Chemical (Catalogue no.) | Concentration | Quantity/ 38.2 ml | Storage temperature and shelf life |
|---|---|---|---|---|
| A | Ca$^{2+}$ ionophore (A23187) | 5 mM | 10.00 mg | -20$^{\circ}$C for 3 months, aliquot 10 µl |

At least 3 hours before used, IVC medium (KSOM; 990 µl) was added into stock A (10 µl) to become 5 µM of the solution concentration. After preparing the CaI working solution, the solution was sterilised by using the syringe filter (0.22µm) and kept inside the CO$_2$ (5%) incubator for calibration.

**Table 3.8**: Composition of calcium ionophore (5 µM) working solution

| Chemical | Concentration | Quantity/ 100µl | Storage temperature and shelf life |
|---|---|---|---|
| Stock A | 5 µM | 10.00 µl | Freshly prepared, calibrated 3 hours before used |

*3.4.2.1.5(d) Preparation of 6- dimethylaminopurine (6-DMAP) solution*

6-DMAP was used together with CaI to activate the goat oocytes following ICSI. Stock A with the concentration (0.2 M) was obtained by dissolving the 6-DMAP (0.25 g) into the ultrapure water (7.7 ml). This stock A could be kept for 3 months, and aliquot (10 µl) into each microcentrifuge tube (100 µl).

**Table 3.9**: Composition of 6-DMAP stock A solution

| Stock | Chemical (Catalogue no.) | Concentration | Quantity/ 7.7 ml | Storage temperature and shelf life |
|---|---|---|---|---|
| A | 6- dimethylaminopurine (D2629) | 0.2 M | 0.25 g | -20$^{°}$C for 3 months, aliquot 10 µl |

In order to make 6-DMAP working solution, stock A was added with IVC medium (KSOM; 990 µl) at least 3 hours before used to obtain the final concentration (2 mM). After preparing the working solution, the solution was sterilised by using the syringe filter (0.22 µm) and kept inside the $CO_2$ (5%) incubator for calibration.

**Table 3.10**: Composition of 6-DMAP (5 µM) working solution

| Chemical (Catalogue no.) | Concentration | Quantity/ 100 µl | Storage temperature and shelf life |
|---|---|---|---|
| Stock A | 5 µM | 10 .00 µl | Freshly prepared, calibrated 3 hours before used |
| KSOM | 1x | 990.00 µl | |

### 3.4.2.1.5(e) Preparation of TCM-199 with Hepes supplemented with FBS

The TCM-199 with hepes which is supplemented with OGS (10%) was used during handling the oocytes or embryo outside the $CO_2$ (5%) incubator especially during washing or ICSI procedures. Both TCM199 with hepes and OGS (10%) were mixed, filter sterilisation (0.22 µm) and calibrated inside the $CO_2$ (5%) incubator at least 3 hours prior used. The media could be kept for 1 week under $4^{\circ}C$.

**Table 3.11** : Composition of  TCM-199 with Hepes

| Chemical (Catalogue no.) | Concentration | Quantity/ 100µl | Storage temperature and shelf life |
|---|---|---|---|
| TCM-199 with Hepes | 1x | 270.00 µl | Freshly prepared, calibrated 3 hours before used |
| Foetal bovine serum (FBS) | 1x | 30.00 µl | |

### 3.4.2.1.5(*f*)  *Preparation of sperm-TALP (sp-TALP) solution*

The sp-TALP was functioned for sperm handling medium. The medium consisted of NaCl, KCL, NaHPOC$_3$, NaH$_2$PO4, Na Lactate (60% syrup), CaCl$_2$.2H$_2$O, MgCl$_2$.6H$_2$O, HEPES C$_8$H$_{17}$O$_4$SNa and Phenol Red. The concentration and quantity of each component of the medium is shown in Table 3.12. The concentration of the stock solution is 10x (100 ml) by weighing and dissolving all component, except Na Lactate (60% syrup) with ultrapure water (40 ml) in Schott bottle (100 ml). The mixture of chemical was stirred slowly and then Na Lactate (60% syrup) was added carefully and slowly because it was in viscous liquid form. Then, the ultrapure water was top-up until 100 ml, and all components were slowly stirred again until they were well mixed (approximately about 10 to 15 minutes). After that, the prepared medium was filter sterilised using syringe filter (0.22 µm) and kept inside a new sterile bottle under 4$^\circ$C for 3 months before used.

**Table 3.12**: Composition for sp-TALP stock solution

| Chemical | Catalogue no. | Quantity/ 100 ml |
|---|---|---|
| NaCl | S5886 | 0.5840 |
| KCl | P5405 | 0.0023 |
| NaHCO3 | S 5761 | 0.2100 |
| NaH2PO4 | S5011 | 0.0035 |
| Na Lactate (60% syrup) | L7900 | 368 µl |
| CaCl2.2H2O | C3881 | 0.0310 |
| MgCl2.6H2O | M2393 | 0.0080 |
| HEPES C$_8$H$_{17}$N$_2$O$_4$SNa | H3784 | 0.1190 |
| Phenol red | P3532 | 0.0010 |

The sp-TALP working solution was freshly prepared at least 3 hours before used and already calibrated inside the $CO_2$ (5%) incubator. The non-calibrated prepared medium could be stored for a week before used.

**Table 3.13**: Composition for sp-TALP working solution

| Chemical | Catalogue no. | Quantity/ 10 ml |
|---|---|---|
| Sp-TALP stock | - | 10.00 ml |
| Sodium pyruvate | P4562 | 0.0011 g |
| BSA-FAF | A6003 | 0.06 g |
| Gentamycin stock solution | - | 5.00 μl |

### 3.4.2.1.5 (g)   *Preparation of sperm capacitation*

In the present study, there was two types of chemicals were used for capacitation which were heparin and theophylline. Both chemicals were widely used for capacitation in other species especially in cattle.

Preparation of sperm capacitation medium using heparin was by warming the heparin stock (refer section 3.4.2.1.5(a)). Heparin stock (20 μl) was added into sp-TALP working solution (1.88 ml). This solution is freshly prepared and need to be $CO_2$ incubated (5%) at least 3 hours before used.

Preparation of sperm capacitation medium using theophylline was by adding theophylline (0.009 g) into sp-TALP working solution (10.00 ml). The medium also need to be $CO_2$ incubated (5%) at least 3 hours before used.

**Table 3.14**: Composition for sperm capacitation using heparin

| Chemical | Catalogue no. | Quantity/ 2 ml |
|---|---|---|
| Heparin stock | - | 200 μl |
| Sp-TALP | - | 1.80 ml |

**Table 3.15**: Composition for sperm capacitation using theophylline

| Chemical | Catalogue no. | Quantity/ 10 ml |
|---|---|---|
| Theophylline | T1633 | 0.009g |
| Sp-TALP | - | 10.00 ml |

### 3.4.2.1.5 (h)  *Preparation of potassium simplex optimised medium (KSOM)*

The KSOM medium is widely used for embryo culture system in domestic animals. This KSOM stock solution consists of NaCl, KCL, $KH_2PO_4$, $MgSO_4$, Na lactate (60% syrup), Na pyruvate, D-Glucose, NaHCO3, $CaCl_2.2H_2O$, L-Glutamine and EDTA. All the chemicals components were weighed as described for sp-TALP preparation. Ultrapure water (40 ml) was added to the components in Schott bottle (100 ml) and stirred. Then, Na lactate (60% syrup) was added slowly until fully mixed. Finally, the medium was topped-up until 100 ml level and sterilised by syringe filter (0.2 μm pore size). The prepared medium was kept inside refrigerator (4°C) for 3 months shelf life before used in various experiments.

**Table 3.16**: Composition for KSOM stock solution

| Chemical | Catalogue no. | Quantity/ 100 ml |
|---|---|---|
| NaCl | S5886 | 0.5553 |
| KCL | P5405 | 0.0186 |
| $KH_2PO_4$ | P5655 | 0.0048 |
| $MgSO_4$ | M7506 | 0.0024 |
| Na lactate (60% syrup) | L7900 | 186 µl |
| Na pyruvate | P4562 | 0.0022 |
| D-Glucose | G6152 | 0.0036 |
| NaHCO3 | S5761 | 0.2101 |
| $CaCl_2.2H_2O$ | C7902 | 0.0251 |
| L-Glutamine | G3126 | 0.0146 |
| EDTA | E9884 | 0.0004 |

The working solution was prepared and calibrated inside the $CO_2$ incubator (5%) at least 3 hours before used. The prepared medium could be used within 1 week.

**Table 3.17**: Composition for KSOM working solution

| Chemical | Catalogue no. | Quantity/10 ml |
|---|---|---|
| Stock solution | - | 9.85 ml |
| BSA | A6003 | 0.04 g |
| MEM | M7145 | 50.00 µl |
| BME | B6766 | 100.00 µl |

### 3.4.2.2 Medium preparation for mouse experiments

In mouse experiments generally involved the following techniques, such as sperm preparation, ICSI, activation and IVC. The detailed preparation of medium is given below.

### 3.4.2.2.1 Toyoda, Yokohama and Hosi's medium (TYH)

TYH medium was used for sperm preparation and capacitation. The medium is a modification of Krebs-Ringer bicarbonate medium by Toyoda *et. al.* (1971). The stock solution for this medium containing several components as follows:

**Table 3.18**: Composition for TYH stock solution

| Chemicals | Catalogue no. | Quantity/100 ml |
|---|---|---|
| NaCl | S5886 | 0.5140 |
| KCl | P5405 | 0.0358 |
| $CaCl_2.2H_2O$ | C7902 | 0.0251 |
| $KH_2PO_4$ | P5655 | 0.0162 |
| $MgSO_4.7H_2O$ | M8150 | 0.0293 |
| $NaHCO_3$ | S5761 | 0.2108 |
| Glucose | G7021 | 0.1000 |
| Natrium pyruvate | P4562 | 0.0055 |
| Penicillin-G | P7794 | 0.0075 |
| Streptomycin | S1277 | 0.0050 |
| L-Glutamine | G3126 | 0.0146 |
| Taurine | T7146 | 0.0125 |
| Phenol red | P3532 | 0.0010 |
| $Na_2EDTA$ | E4884 | 100.00 μl |

The $Na_2EDTA$ stock solution (10 mM) was prepared before added into the TYH stock solution. Milli-Q was added to dissolve the chemicals and make it 100 ml level. The stock solution was sterilised using sterile filter (0.22 μm pore size) and kept in $4^0C$ for 3 months.

BSA (3%) was added into the stock medium (10 ml) prior to use. This solution could be kept for 7 days in $4^{°}C$. The working solution need to calibrate into $CO_2$ incubator at least 3 hours before used.

**Table 3.19**: Composition for TYH working solution

| Chemical | Catalogue no. | Quantity/ 10 ml |
|---|---|---|
| TYH stock solution | - | 10.00 ml |
| BSA | A6003 | 0.03 g |

### 3.4.2.2.2 *Preparation of modified hepes Whitten's medium (HWM)*

The function of HWM medium was for handling oocytes or embryos outside the incubator. Usually this medium was used during oocytes collection, washing and ICSI. Similar to TYH medium, HWM medium was prepared in stock solution before being used (working solution). All chemicals involved were dissolved in Milli-Q water (top-up until 100 ml). HWM medium consists of chemicals as follows:

**Table 3.20**: Composition for HWM stock solution

| Chemicals | Catalogue no. | Quantity/100 ml |
|---|---|---|
| NaCl | S5886 | 0.5140 |
| KCl | P5405 | 0.0356 |
| $KH_2PO_4$ | P5655 | 0.0162 |
| $MgSO_4.7H_2O$ | M8150 | 0.0294 |
| $NaHCO_3$ | S5761 | 0.0304 |
| Na Hepes | H3784 | 0.6508 |
| Glucose | G7021 | 0.1000 |
| Calcium lactate | 2376 | 0.0338 |
| Lactic acid | L7900 | 370.00 µl |
| Natrium pyruvate | P4562 | 0.0029 |
| Penicillin-G | P7794 | 0.0075 |
| Streptomycin | S1277 | 0.0050 |
| Phenol red | P3532 | 0.0010 |

BSA (3%) was added into the stock medium (10 ml) prior to use. This solution could be kept for 7 days in $4^{\circ}$C. The working solution was kept warm inside water bath ($37^0$C) before used.

**Table 3.21**: Composition for HWM  working solution

| Chemical | Catalogue no. | Quantity/ 10 ml |
|---|---|---|
| HWM  stock solution | - | 10 ml |
| BSA | A6003 | 0.03 g |

### 3.4.2.2.3    *Preparation of modified Whitten's medium (WM)*

WM medium was used in culturing the oocytes or embryos (IVC).  This medium was minor modified based on Whitten (1971). The stock solution has been prepared before being used (working solution). All chemicals for stock were weighed and dissolved with Milli-Q. Then, the stock solution was sterilised filter and kept for 3 months in a refrigerator ($4^{\circ}$C). All the chemicals involved are tabulated as shown below:

**Table 3.22**: Composition for WM stock solution

| Chemicals | Catalogue no. | Quantity/100 ml |
|---|---|---|
| NaCl | S5886 | 0.5140 |
| KCl | P5405 | 0.0356 |
| $KH_2PO_4$ | P5655 | 0.0162 |
| $MgSO_4.7H_2O$ | M8150 | 0.0294 |
| $NaHCO_3$ | S5761 | 0.1900 |
| Glucose | G7021 | 0.1000 |
| Calcium lactate | 2376 | 0.0338 |
| Lactic acid | L4263 | 370 µl |
| Natrium pyruvate | P3662 | 0.0029 |
| Penicillin | P7794 | 0.0075 |
| Streptomycin | S1277 | 0.0050 |
| L-Glutamine | G3126 | 0.0146 |
| Taurine | T7146 | 0.0125 |
| Phenol red | P3532 | 0.0010 |
| $Na_2EDTA$ | - | 100 µl |

BSA (3%) was added into the stock medium (10 ml) prior to use. This solution could be kept for 7 days in a refrigerator ($4^0$C). The working solution was calibrated by placing it inside a $CO_2$ (5%) incubator for at least 3 hours before used.

**Table 3.23**: Composition for WM working solution

| Chemical | Catalogue no. | Quantity/ 10ml |
|---|---|---|
| WM stock solution | - | 10.00 ml |
| BSA | A6003 | 0.03 g |

### *3.4.2.2.3    Preparation of other solutions*

Generally, the other solutions that need were for sperm capacitation, oocyte denudation and activation.

### *3.4.2.2.3 (a) Preparation of sperm capacitation medium*

The preparation of sperm capacitation medium using heparin and theophylline was the same as with goat experiments (Refer to Section *3*.4.2.1.5 (g)), except the dissolving medium was using TYH medium (for mouse experiments) instead of using sp-TALP (for goat experiments).

**Table 3.24**: Composition for sperm capacitation using Heparin

| Chemical | Catalogue no. | Quantity/ 2 ml |
|---|---|---|
| Heparin stock | - | 200 µl |
| TYH working solution | - | 1.80 ml |

**Table 3.25**: Composition for sperm capacitation using Theophylline

| Chemical | Catalogue no. | Quantity/ 10 ml |
|---|---|---|
| Theophylline | T1633 | 0.009 g |
| TYH working solution | - | 10.00 ml |

### 3.4.2.2.3 (b) Preparation of Hyaluronidase (0.1%)

The preparation of hyaluronidase (0.1%) was similar to that for goat preparation medium (Refer to Section 3.4.2.1.5 (b)). The difference was the hyaluronidase was dissolved with HWM medium (for mouse experiments).

**Table 3.26**: Composition of  Hyaluronidase solution (0.1%)

| Chemical (Catalogue no.) | Concentration | Quantity/ 10 ml | Storage temperature and shelf life |
|---|---|---|---|
| IV-S Hyaluronidase from bovine testes (H4272) | 0.1 % | 0.01 g | $-20^{\circ}$C for 3 months |
| HWM working solution | 1x | 10.00 ml | |

The medium was incubated for at least 3 hours inside the $CO_2$ (5%) incubator before being used.

### 3.4.2.2.3 (c) Preparation of Strontium chloride (0.02 mM)

**Table 3.27**: Composition for Strontium chloride stock

| Chemical (Catalogue no.) | Quantity/ 10 ml | Storage temperature and shelf life |
|---|---|---|
| Strontium chloride (439665) | 0.027 g | |
| HWM working solution | 1.00 ml | $-20^{\circ}$C for 3 months |

**Table 3.28**: Composition for Cytochalasin B (CB)

| Chemical (Catalogue no.) | Quantity/ 10 ml | Storage temperature and shelf life |
|---|---|---|
| Cytochalasin B (C6762) | 1.00 mg | |
| DMSO (D5879) | 2.00 ml | $-20^{\circ}$C for 3 months |

**Table 3.29**: Composition for  Chatot, Ziamek and Bavier medium (CZB)

| Chemical | Catalogue no. | Quantity/ 100 ml | Storage temperature and shelf life |
|---|---|---|---|
| NaCl | S5886 | 0.479 | |
| KCl | P5405 | 0.036 | |
| $KH_2PO_4$ | P5655 | 0.016 | |
| Lactic acid | L4263 | 0.530 ml | |
| $MgSO_4.7H_2O$ | M8150 | 0.029 | $4^{\circ}$C for 3 months |
| $NaHCO_3$ | S5761 | 0.210 | |
| Disodium lactate | - | 0.004 | |
| Glucose | G6152 | 0.100 | |
| Phenol red | P3532 | 0.001 | |
| BSA | A7906 | 0.500 | |

Strontium chloride (0.02 mM) was used as mouse oocyte activation. In order to prepared this medium. The stock of CB and CZB were prepared as have been describe in Table 3.28 and 3.29, respectively. The activation solution was prepared (Table 3.30) on the day of experiment and should be incubated by $CO_2$ incubator at least 3 hours before used.

**Table 3.30**: Composition for Strontium chloride activation medium

| Chemical | Catalogue no. | Quantity/ 2 ml | Storage temperature and shelf life |
|---|---|---|---|
| Strontium chloride | S5886 | 10.00 µl | |
| CB | P5405 | 1.00 µl | Freshly prepared |
| CZB with $Ca^{2+}$ free | - | 89.00 µl | |

### 3.4.2    Preparation of Microtools

Mouth pick-up pipette and microneedles are mirotools were used for handling the oocytes and embryos. The mouth pick-up pipette was used mainly for picking up, transfer the oocytes or embryos during the experiments. This pipette was in-house made in the laboratory using the rubber tube, cotton, and glass pipette with desired inner diameter size (200-500 µm).

The microtools were accessories in ICSI procedure, which consists of ICSI injecting micropipette and holding micropipette. Both micropipettes were in-house preparation using the specific equipment such as micropuller, microforge and microgrinder.

### 3.4.2.1    Preparation of mouth pick-up pipette

Using glass Pasteur pipette, a mouth pick-up pipette was prepared by exposing the burner flame in the middle of the glass until it became soft. Then, by holding at both ends of the glass, immediately withdrawn from the heat and pulled the glass in opposite direction. This technique will give the desired inner diameter size (200–500 µm) in the middle of the glass. Generally, 2 types of inner diameter size were prepared which were for embryos or denuded oocytes (200-300 µm) and COCs (300-500 µm).

After the desired inner diameter size was determined, the glass was broken using a diamond stone. In a way to obtain a neat break, the pulled portion of the glass was scribed with the diamond stone and snapped at the scribed portion. The tip of the pulled glass was fire-polished by quickly exposed to the flame to achieve a smooth edge of the glass tip. The step is important to avoid any jagged glass end that may potentially cause damage to the zona pellucida during oocytes or embryos handling.

The prepared pipette was rinsed with Milli-Q water and alcohol (70%) for several times (approximately 3x). The pipette was autoclaved (20 minutes; $120^{o}C$) and dried inside the oven ($60^{o}C$). Before conducting the experiment, the pipette should be sterilised with other lab-wares under ultraviolet (UV) light for 30 minutes inside a laminar flow cabinet.

### 3.4.2.2 Preparation of microneedles

Microneedles are important for manipulation of oocytes or sperm during ICSI procedure. They were prepared using the borosilicate capillaries using appropriate equipment.

The preparation of both microneedles (ICSI and holding micropipette) were initiating by pulling the borosilicate capillaries using a horizontal micropuller (P-97, Sutter Instrument, USA). This equipment was programmed with the quantity of heat, pull speed and strength based on previous established and stored programmes.

The pulled capillary was attached with microforge to make the capillary in desired inner and outer size. The inner size of ICSI micropipette (8-10 µm: goat and 4-8 µm: mice) of pulled glass was aimed to be identify at this level. In order to obtain the desired size, the capillary was place in horizontal. A small bead of the glass was placed

on top of the heat element; this is to ensure the glass bead attached with the filament itself would not come into direct contact with the capillary. The glass bead would glow dull red colour when the heat control adjuster was on at certain level (less than 0.5 marked unit). The capillary was lowered continuously as the glass melted until a bead approximately 8-10 µm diameters has formed; and the heat switched off and bring a way from the needle. The heat shock was applied through this step to achieve the desired inner diameter and neat break on the tip of capillary. Next, the tip of capillary was ground to make a bevelled edge with microgrinder at $45^o$ desired angle for approximately 2-3 minutes. The capillary was washed away with hydrofluoric acid (10%; 5 seconds) and rinsed with Milli-Q water (10 seconds). The specialty of ICSI micropipette is a spike. It was made by applying a small, gentle and quickly touch on the heated glass bead (same heat level during breaking the capillary).

The holding micropipette was prepared by scoring the pulled capillary with an ampoule cutter and breaking it (outer diameter: 150-180 µm). The capillary was placing in vertical position where the tip of the edge would face the glass bead. Through heating the glass bead (no more than 3 unit marked), the tip would be fire-polished until to the desired inner diameter (25-30 µm).

Finally, both ICSI and holding micropipette would go through the bending process by placing the capillary in horizontal position and apply the heat until they were bent at 25-30 degrees with microforge to allow a horizontal displacement on the microscope stage.

All prepared ICSI and holding micropipette were rinsed with alcohol (70%), kept in needle holder, dried into oven $60^oC$ and finally they were sterilised under UV light for 30 minutes inside the laminar flow cabinet.

### 3.4.3    Preparation of Experimental Animals

The protocols for preparing the experimental animals are described below:

### 3.4.3.1    Preparation of donor goats

The donor goats were used to provide constant oocyte samples for various experiments. The selected donor goats underwent oestrus synchronisation, superovulation and laparoscopic oocyte pick-up (LOPU). The same goat would be used for another surgery with at least 3 months of intervals surgery cycles using the same procedure. Figure 3.1 shows a summary of schematic presentation of processes involved in oestrus synchronisation, superovulation and LOPU in donor goats.

| Oestrus synchronisation | Superovulation | LOPU |
|---|---|---|

| Day-0<br>Time: 0900 hr<br>CIDR insertion | Day-13<br>Time: 0900 hr<br>Estrumate ® (i.m) | Day-16<br>Time: 1730 hr<br>Off-feed | Day-17<br>Time: 0800 hr<br>LOPU |

Day-14
Time: 1600 hr (Folligon ® (i.m.);
Time: 2100 hr (CIDR Removal; Ovidrel® (i.m.)
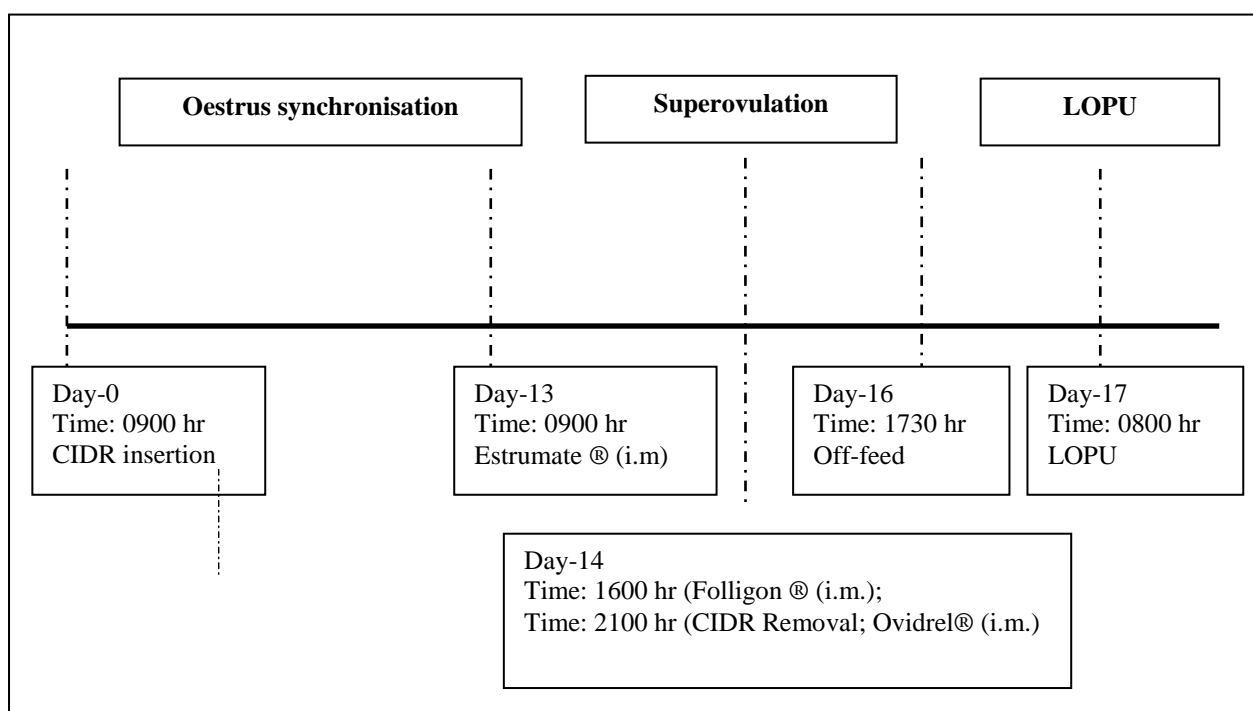
**Figure 3.1**: A schematic represent the summary of process involved in donor goat.

### 3.4.3.1 .1    *Oestrus synchronisation and superovulation*

Prior to oocytes retrieval, the goat preparation was initiated by oestrous synchronisation procedure. This procedure is important for manipulating the oestrous cycle of donor goats. Therefore, it will assist in terms of plan the date and time of oocyte retrieval during LOPU.   Controlled Intravaginal Drug Release (CIDR$^®$, 0.3 g progesterone) device was used for this purpose. CIDR was inserted into the vagina with the assist of a sterile CIDR applicator and a veterinary obstetrical lubricant (K-Y Jelly) for 14 days (at 0900 hr on Day-0). CIDR is made from 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 will unfold in 'T' like formation that aids in retention. In this study, the donor goats with CIDR insertion were monitored daily to confirm that it had not been inadvertently dropped from the vagina. A luteolytic treatment of Cloprostenol (Estrumate$^®$, 125 µg) was administered intramuscularly (at 0900 hr on Day-13) to regress the corpus luteum in order to lower the blood progesterone level, thus initiation of pro-oestrus and eventually resulted in a rapid surge of oestradiol for a  onset of oestrus.

Superovulation was the subsequent procedure carried out following oestrus synchronisation to induce the multiple growths of follicles in the ovaries. The effect of this procedure would make the surface of ovary with visible fluid-filled 'pimple-like' structures. The protrusion of the 'pimple-like' structures indicates the follicles were growing in the ovaries. Therefore, in this study, pregnant mare's serum gonadothrophin (PMSG; Folligon$^®$; 1500 IU) was administrated intramuscary at 1600 hr on Day-14 before the CIDR was being removed. Human chorionic gonadotrophin (hCG; Ovidrel$^®$; 250 IU) was administered intramuscularly at 2100 hr on Day-14, upon the removal of CIDR. The onset of oestrus behaviour such as twitching of tail, visible of vaginal secretion and eagerness to be mounted on by teaser buck was observed after

approximately 48 hours of the PMSG administration. The presence of oestrus indicates superstimulation of ovaries, thus facilitating the subsequent LOPU procedure to be carried out.

### 3.4.3.1 .2 Laparoscopic oocytes pick-up (LOPU) for oocytes retrieval

LOPU is a microsurgical procedure for oocytes retrieval. In the beginning, the donor goat should be sedated and anaesthetised, surgical instruments and accessories were disinfected. The oocytes were retrieved by aspiration of follicular contents from the 'pimple-like' protrusions on the surface of ovary under laparoscopic observation. During the process of oocytes retrieval, the follicular fluids were collected in collecting tubes (3 ml) and passed to the embryologists to search for oocytes under microscope. After the completion of oocytes retrieval, the donor goat underwent post-surgical treatment.

### 3.4.3.1 .2 (a) Sedation and anaesthetisation of donor goat

A day before performing the LOPU, the donor goat was deprived from food and water (1730 hr on Day-16). The next day, prior to LOPU surgery (at 0800 hr on Day-17), the donor goat was sedated and anaesthetised with xylazine hydrochloride (0.22 mg/ kg of body weight), followed by ketamine hydrochloride (11 mg/ kg of body weight) through intramuscular injection. During the period of LOPU surgery, the donor goat was maintained under anaesthesia with injection of ketamine hydrochloride (0.1 mg/ kg of body weight) at the interval of approximately 30 minutes or as required.

### 3.4.3.1 .2 (b)   Preparation of disinfection in LOPU procedure

LOPU procedure should be done in a well organised and free from contamination room. In order to avoid errors and to ensure the operation of LOPU runs smoothly, all equipment and surgical instruments should be in well organised, functional and disinfected to facilitate the surgery to be carried out under hygienic condition (Appendix Figure 2.5). A day before the surgery, non-autoclavable surgical instruments were disinfected by immersing completely in Gigasept® solution (10% for 10 minutes) and subsequently rinsed in sterile autoclaved distilled water before arranging them on a table-cum-trolley. The autoclavable surgical instruments were autoclaved and dried completely in the oven ($56^{\circ}$C) prior to use for surgery. To avoid any contamination during handling the instruments, the outer wrapping of the surgical pack was opened and unfolded carefully without touching the sterilised instruments inside. All the surgical instruments were assemble and arranged orderly on a sterile surgical table-cum-trolley which was already laid with a sterile drape.

The donor goat was immobilised after anaesthetised, then she was placed on a clean small ruminant retaining cradle. The cradle was sprayed with alcohol (70%) before the donor goat being placed on it.  The cradle was set at $45^{\circ}$ angles with the head of the goat lowered to facilitate the LOPU procedure. For disinfection procedure, the abdominal area of donor goat was disinfected with diluted Hibiscrub (10%) using clean gauzes and the hair shaved.  Next, the bare skin was wiped with undiluted Hibiscrub and subsequently with weak iodine solution. After wiping, the abdominal area was covered with a sterile drape with an opening that revealed the disinfected bare skin and was ready for oocyte retrieval procedure. The drape was positioned without the fabric dragging across a non-sterile surface and secured in a place with towel clamps at four corners of the surgical site.

The light system which was lens and probe and the entire length of the fibre optic cable were disinfected with alcohol (70%) before performed the white balance.

### 3.4.3.1 .2 (c)   *Retrieval and searching of oocyte*

The fibre optic cable was connecting the light probe to the light system. The CCD camera and monitor was connected to the light system after the light system was turned on. A small incision was made on the abdominal area and trocar connected to a $CO_2$ tank via the $CO_2$ insufflators was inserted into the incision to insert the $CO_2$ into the abdominal cavity. Three small incisions (3-5 mm) were made once the peritoneum was expended in order to facilitate visualisation of the reproductive tract.   The three incisions were made: i) near the umbilicus to facilitate insertion of trocar for passing the laparoscope, ii) on the right side of lower-ventral abdomen were inserted into each trocar, respectively, iii) on the left side of the abdominal cavity for directly inserted oocyte retrieval needle.

The collecting tubes for collecting the follicle-fluid were pre-warmed (38.5$^{\circ}$C) in a test tube heating system, the collection medium was filled in a sterile luer slip syringe (50 ml) also being pre-warmed (38.5$^{\circ}$C) and placed horizontally in the aspiration system. During the aspiration process, the tube was connected with the tube of oocyte retrieval needle. Prior to use, the oocyte retrieval needle has been sterilised by exposing the UV light (30 minutes) and rinsed with the collecting medium.

The ovaries was visualised and identified through gentle manipulation of uterine horns using the grasper. The ovaries were exposed by pulling the fimbria in different directions using the grasper. Thus, the follicles visible on the surface of ovaries were punctured and fluids were aspirated by aspiration needle which was assisting of

aspiration system. The fluids were filled the collecting tube (1-3 ml) and passed to the embryologist for oocyte searching.

The oocyte searching was done at another area of sterile room known as embryology room. Here, the stereomicroscope used for oocyte searching was pre-warmed (38.5°C) before searching of the oocyte. Once the embryologist obtained the collected follicle fluid tube, the fluids were dispensed into sterile petri dish (90 mm) for isolation and evaluation under 20x and 40x magnifications. The oocytes were rinsed using flushing medium (2x) and IVM medium (allocated droplet for rinsed; 2x) before cultured into real IVM cultured droplet according to oocytes grades.

After oocytes retrieval, the ovary was rinsed with warm heparinised (38.5°C) physiological saline through one of the trocar aid in order to reduce adhesion following oocytes aspiration. Then, the incision parts on the abdomen were sutured and finally the donor goat was removed from the cradle. The sutured incision area was sprayed with antiseptic and insecticide containing cyphenothrin.

### 3.4.3.1 .2 (d)   Post-surgery management

In order to prevent possible post-surgical infection, the donor goat was administered with oxytetracycline (20 mg/ kg body weight) via i.m. injection once in four days within the duration of 2 weeks.

All surgical instruments were physical cleaned with diluted 7X®-PF solution and rinsed 5 times with running tap-water followed by two times with RO water. Then, they were drained dry. The aspiration needle was disinfected with alcohol (70%) at the outer surface area and the opening parts were flushed with the alcohol (70%) using syringe (20 ml) attached to a needle (18 G), drained dry and packed in surgical bag. The

autoclavable instruments were packed in an autoclave bag and pasted with autoclave sticker (recorded the date of sterilisation). Later, the instruments were autoclaved for 20 minutes at 120°C. While, for the non-autoclavable surgical instruments, after cleaning, they were dried and packed individually in a clean transparent bag before proceeding to UV sterilisation (30 minutes).

The floor of surgery room was swept and mopped with disinfectant agent such as Dettol® disinfectant liquid. The cradle for animal, aspiration system and light probe was wiped with alcohol (70%) and kept properly and safely.

### 3.4.3.1 .3    *Ovariectomy*

After third or fourth cycle of LOPU, the donor goat underwent ovariectomy. Before ovariectomy, the goat underwent oestrus synchronisation and superovulation as well as anaesthetisation and disinfection similar with those of LOPU. Ovariectomy procedure was initiated by making vertical abdominal incision (4 to 6 cm). A tunnel, lateral to the skin incision was made using blunt dissection to help the muscles of the posterior abdominal wall separated that facilitated abdominal cavity entrance. A warm heparinised (38.5°C) physiological saline was administered (approximately to 200 ml) into the abdomen to prevent dryness. Due to location of ovaries in a fat pad beneath the muscles, the periovarian fat was gently grasped to lift and easy to place the ovary on the skin using forceps. The fallopian tube and cranial-most part of the uterine horn distal to ovary was crushed using a small and curved haemostatic forceps. This step needs to be carefully carried out not to crush or contact the ovary. Then, the ovary was removed by cutting above the clamped area and the uterine horn was inserted back carefully into the abdomen.  The incisions part was sutured and subsequently, sprayed the area with antiseptic and insecticide containing cyphenothrin. The donor goat was administered

with oxytetracycline (20 mg/ kg body weight) via i.m. injection once in 4 days within the duration of 2 weeks.

The cut ovary was passed to embryologists in a petri dish (35 mm) containing the flushing medium for slicing to search and recover the COCs. The ovaries were rinsed with saline again to remove the blood. Then, under the laminar flow, the ovaries were freed from the surrounding tissues and overlying bursa using surgical scissors and forceps. The ovaries was sliced individually on another petri dish (90 mm) that contained the flushing medium, the checker-board incisions were made along the whole ovarian surface using a quarter sections of antiseptic stainless steel razor blade (wiped with 70% alcohol) held by a sterile haemostat. During the slicing, the media used were pre-warmed (38.5°C) by placing the medium on a stage warmer. Then, the sliced ovaries were dipped completely into another flushing medium to ensure all the possible COCs could be recovered.

The follicular fluids that recovered out from the slicing procedure were assembled on the petri dish (90 mm). Hence, the COCs would be examined under the stereomicroscope (magnification at 20x to 40x). The found COCs were picked up and wash with another flushing medium microdroplets (3x) and IVM microdroplets (2x). The COCs were graded similar done for recovered COCs from LOPU before cultured into IVM medium.

### 3.4.3.2 Preparation of mice

The preparations of mice for experiments were involved superovulation of female, male management, oocytes and sperm recovery.

### 3.4.3.2 .1 *Superovulation of female and male management*

The 8 to 12 weeks of matured female mice were superovulated via intraperitoneal injection (i.p.) of PMSG (5 IU in 0.1 ml) at 1700 to 1800 hours. Approximately 48 hours later, the hCG (5 IU in 0.1 ml) was administered to the same female mice. During the injection, the mouse was held firmly by twisting the tail around the little finger. The hormone was administered using the sterile disposable needle (26G) carefully to avoid the internal organs.

The male mice was kept with enough fed and water and never been used in mating programme (separated with female). This is due to help in obtained the good quality of sperm.

### 3.4.3.2 .2 *Oocytes recovery*

In order to recovery the oocytes, microdroplets of warmed HWM medium (100 µl/ microdroplet) were made. The female mice were scarified by cervical dislocation and the abdominal cavity was cut open (Appendix Figure 2.2). The oviducts were cut and placed into the HWM medium microdoplet. The first or second droplet was used for rinsed the oviduct to discard the debris such as blood and fur. In another microdroplet, the oviduct was held with fine forceps while ampullae region was punctured with a sterile disposable needle (18-G).The COCs was released by gently drawn with the

needle into the microdroplet. The COCs was placed to another washing dish which was containing HWM medium and ready to be denuded by hyaluronidase solution (0.1%).

In this study, the oocytes recovery was done at 13 to 15 hours and 16 to 18 hours post- hCG administration.

### 3.4.3.2 .2 *Sperm recovery*

The TYH medium was prepared in a sterile 4-well culture dish (400 µl) which had been added by sperm capacitation solution (theophylline or heparin). The medium was equilibrated overnight in $CO_2$ (5%) incubator at 37°C.

The 10 to 15 weeks old matured male mice were sacarified by cervical dislocation. The caudal epididymis was held firmly with blunt forceps and was slit with a pair of fine dissecting scissors. Prior to recovery the epididymal sperm, the fine forceps was rinsed by TYH medium and then collect the sperm followed by placing them into the TYH microdroplet. The sperm suspension was then incubated for 60 minutes in $CO_2$ (5%) incubator at 37°C.

### 3.5    TECHNIQUES AND PROTOCOLS

Generally, after underwent animal preparation (goats and mice), medium preparation, oocytes and sperm recovery, the next in *vitro* production (IVP) activities were IVM (for goat experiment), ICSI, activation and IVC procedure. All of these procedures were done in the embryology room. In this study, the activities such as ICSI, activation and IVC procedure were slightly similar, but the differences were found in medium types

using and period cultivation. Therefore, the author would describe the protocols based on the types of activities.

### 3.5.1 *In Vitro* Maturation of Goat Oocytes
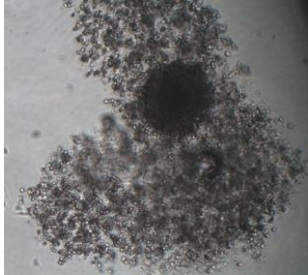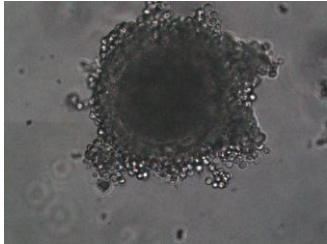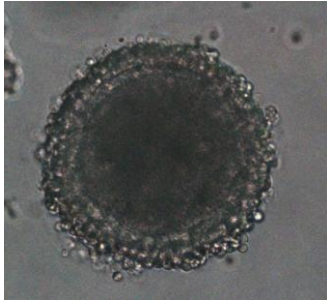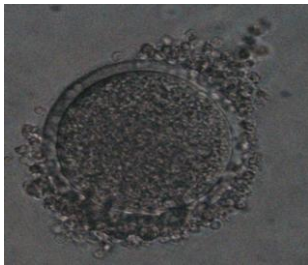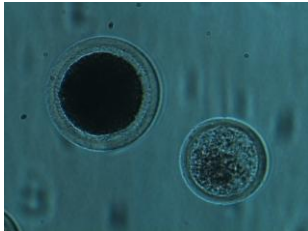
The IVM microdroplets (80 µl) were prepared and overlaid with light mineral oil in a petri dish 35 mm) and labeled with author's initial, date and type of experiment before incubated into the $CO_2$ (5%) incubator for at least 3 hours before used. This is important to allow temperature and gas equilibration.

The stereomicroscope that used for oocytes searching should be warmed by attaching with the stage warmer (38.5°C). After searching the COCs during the LOPU session, the COCs were washed (2x in flushing medium and 2x in IVM medium microdroplets) to remove the debris. Subsequently, the COCs was placed into another IVM microdroplet (10–15 COCs/ 80 µl). The COCs were graded and cultured in separate microdroplets based on cumulus cell (CC) layers. The grades of COCs was done as described by Rahman *et al.* (2007) which shows in Table 3.29.

After grading, the COCs were cultured into the IVM microdroplet inside the incubator environment (38.5°C in humidified atmosphere with 5% of $CO_2$). The duration ranging period for the IVM was depending on the experiment treatment ( i: 18-21 hours and ii: 22-25 hours).

At the end of IVM duration, the COCs layers were expended and need to proceed for the nest procedure which is called denudation to score the matured oocytes with the presence of the polar body.

**Table 3.31**: Grading of the COCs according to the CC layers and morphology

| Characteristics COCs and CFOs | Morphology | Grade | Figure |
|---|---|---|---|
| COCs with more than 5 complete layers of CCs | Homogenous finely granulated ooplasm and normal morphological features | A |  |
| COCs with 3-5 complete layers of CCs | Homogenous finely granulated ooplasm and normal morphological features | B |  |
| COCs with 1-2 complete layers of CCs or COCs with 3-5 partially with CC layers | Homogenous finely granulated ooplasm and normal morphological features | C |  |
| CFOs or oocyte with incomplete layer of CCs (1-2 layers) | Homogenous finely granulated ooplasm and normal morphological features | D |  |
| Oocytes without the CCs layer or apoptotic in jelly-like CC layers or very small oocytes | Degenerating or abnormal, size, shape and heterogenous ooplasm oocytes | E |  |

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

### 3.5.2 Intracytoplasmic Sperm Injection (ICSI)

Prior to ICSI procedure, the, oocyte and sperm preparation, ICSI dish, and microcapilaries alignment need to be prepared. Both of goat and mice experiments preparation for ICSI was same unless the differences would be state in the procedure below.

### 3.5.2.1 Preparation of oocytes for ICSI

The COCs were harvested from the IVM culture (for goat) and recovered from the oviduct (for mice) were denuded by repeated pipping in TCM-199 with hepes (goat) or HWM (mouse) containing hyaluronidase (0.1%, 100 µl). Generally, the hyaluronidase microdroplets were made on the petri dish (60 mm) covered with mineral oil if the number of COCs is less than 10, especially for goat samples. While, if the number of COCs is higher, the denudation was conducted in 100 µl microcentrifuge tube, especially in mice samples. This is due to facilitate the denudation activity. In order to remove the CC layers, the oocytes were applied with repeated pippetting (in and out aspiration using mouth pick-up pipette or micropipettor; 100 µl). For the mouth pick-up pipette, the opening size of Pasteur pipette was about 250 µm and 200 µm in diameter, respectively, under a stereomicroscope.

The denuded oocytes were washed via 3 microdroplets of hepes containing medium (depends on experimental animal). Then, the oocytes were identified for maturation under the inverted microscope. The oocytes presence with clear first polar body (PB-1) were considered as matured which was at metaphase II (MII) stage and meiotic competent. Thus, the oocytes were transferred to another dish and washed through 3 microdroplets (50 µl) of equilibrated TCM-199 with FBS (10%) and incubated in the $CO_2$ (5%) incubator at 38.5°C (for goats) or  were transferred to

another dish and washed through 3 microdroplets (50 µl) of equilibrated WM and incubated in the $CO_2$ (5%) incubator at 37°C (for mice) until begin the ICSI.

### 3.5.2.2 Preparation of sperm for ICSI

The sperm source and preparation for goats and mice were different as described below:

#### 3.5.2.2.1 *Sperm preparation for ICSI in goat*

As been mentioned in earlier part, the goat sperm source was obtained from the frozen-thawed sperm. The preparation for sperm was performed 80 minutes before initiating the ICSI experiment. A straw of frozen sperm was withdrawn from the liquid nitrogen storage tank and left outside briefly at normal temperature (25°C, 1 minute) followed by thawing in a water bath (37°C, 1 minute). The straw was taken out from the water bath and disinfected with alcohol (70%) and allowed it to dry.

The equilibrated sperm wash medium (sp-TALP) was poured (3 ml) into a centrifuge conical tube (15 ml size). Then, the end of straw (sealed end) was cut and the thawed sperm were inserted into the sp-TALP (3 ml). The remaining of sperm in the straw was released into the sp-TALP by cutting another end of straw (cotton-plugged end) and gently passed it through the medium. Before mixing the sperm and medium, a drop of thawed sperm was placed on the slide, in order to examine the movement characteristics of the sperm under the inverted microscope.

The centrifuge conical tube (mixed sperm and medium) was tightly capped and then, the tube was centrifuged (200 x $g$, 15 minutes). After done centrifugation, the supernatant was discarded and the pellet of the sperm was loosen by gently pipetting

using micropipettor before placed it (100-200 µl) at the bottom of the centrifuge conical tube with fresh capacitation medium (2 ml). The capacitation of sperm was carried out using a medium containing heparin or theophylline depending on the experiment. This procedure was taken 60 minutes inside the $CO_2$ (5%) incubator. The end of sperm capacitation process, the three quarter (volume) of supernatant was discarded. The remaining supernatant containing pellet of sperm was gently loosen and withdrawn into the ICSI dish for ICSI procedure. The remaining pellet sperm were kept inside the $CO_2$ (5%) incubator.

### 3.5.2.2.2        *Sperm preparation for ICSI in mice*

After the sperm capacitation incubation (60 minutes) (refer to 3.4.3.2.2), the sperm suspension was withdrawn to examine the sperm mortality and progressive under the inverted microscope before preparing for ICSI on the ICSI dish.

### 3.5.2.3        **Preparation of ICSI dish**

The ICSI dish was prepared using the lid of Petri dish (35 mm). The Petri dish was divided into 3 parts as shown in Figure 3.2. The top fraction was the sperm suspension microdroplet (5 µl), centre fraction was the PVP microdroplet (10%, 3-5 µl) and bottom fraction was the oocyte microdroplet (5 µl).
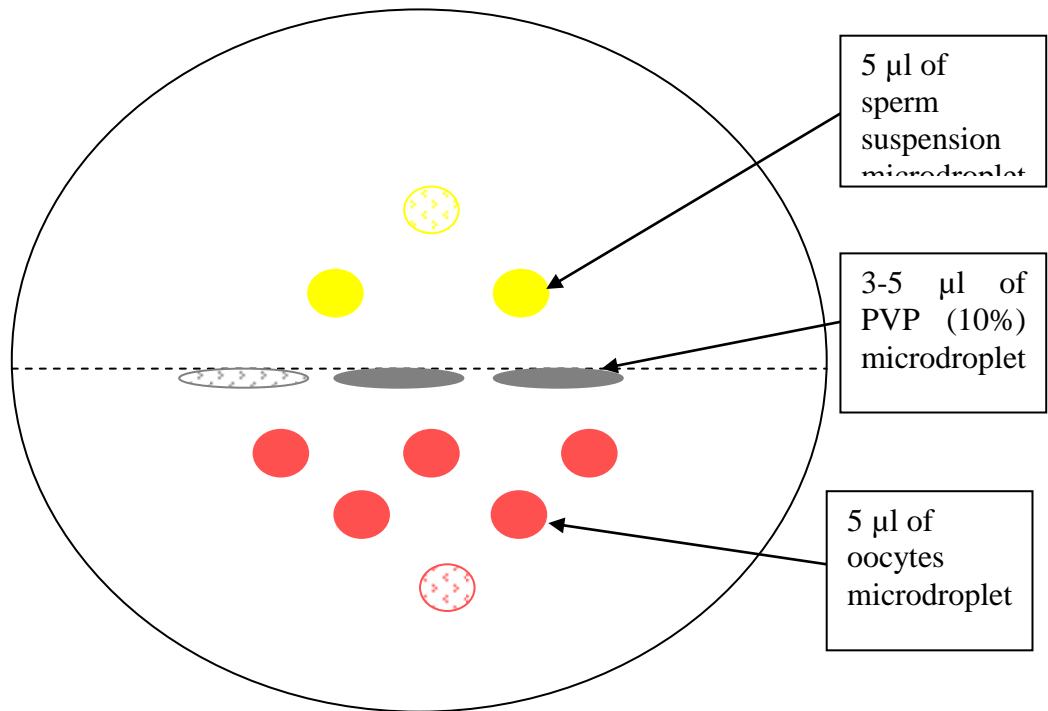
**Figure 3.2**: The diagram represents the microdroplet position preparation for ICSI dish where i) ⬤ is sperm suspension microdroplet, ii) ⬤ is oocyte microdroplet, iii) ⬤ is PVP (10%) microdroplet. The patterned microdroplets are made to standby for microneedles cleaning during ICSI.

The sperm suspension and PVP (10%) microdroplets were prepared in flattened form. This was to ensure the sperm were clear visually and immobilised during ICSI procedure. The sperm swimming space could be reduced by applying this technique. While the oocyte microdroplets were prepared in round shape form without flattened for easy to manipulate during sperm injection. Each type of microdroplet was used for standby for microneedles cleaning during the ICSI procedure. All microdroplets were overlaid with mineral oil for preventing the evaporation and kept in warm (38.5°C for goat samples and 37°C for mice samples).

### 3.5.2.4 Preparation of microneedles alignment

Two microneedles which is ICSI microneedle and holding microneedle were aligned using a micromanipulator. The micromanipulator was attached with the inverted microscope and the warmer stage for placing the ICSI dish. All knobs (X-, Y- and Z-control) and the syringes (3 ml) were adjusted to the centre of the scale.

Alignment of microneedles was done over a Petri dish cover (35 mm). Two flattened microdroplets were prepared containing ICSI medium (for oocyte microdroplet; 2-3 µl) and PVP (10%, 2-3 µl) overlaid with mineral oil at the centre position. ICSI medium was slightly different depending on experimental experiment (refer to Sections 3.4.2.1.5 (e) and 3.4.2.2.2)

The holding microneedle was attached to the holder and adjusts to the centre of the microscope's visual and brought it down using adjusting the knob to make it inserted into the mineral oil. The mineral oil was allowed to insert into the holding microneedle for equilibration to enhance the control within the needle before placed into the ICSI medium and PVP microdroplet. Due to this, the visual of microneedle also could be easy to see and the outline of microneedle was sharply focused to by adjusting the knob. After that, the ICSI microneedle went through the same procedure as that of the holding microneedle. Once both microneedles were equilibrated, they were aligned to make their tips parallel to the microscope stage under the low magnification (4x objective). The ICSI microneedle the tip was carefully touched to the holding microneedle or slowly inserted into the holding microneedle. This step was played for a few times to ensure the microneedles alignment is in good condition. Again, both microneedles were checked under 10x magnifications to assure the accurate alignment and parallel.

The ICSI microneedle was left into the PVP for a few times to allow equilibration to establish the capillary action. The X-, Y- and Z- movement along the bottom of the dish was checked also a smooth and gentle aspiration and expulsion also being checked. While holding microneedle in underwent equilibration, movement, aspiration and expulsion actions in capillary using the ICSI medium were performed.

### 3.5.2.5        Procedure of ICSI

The capacitated sperm were placed (1-2 µl) into the sperm suspension microdroplet and the MII oocytes into the ICSI medium (oocyte microdroplet). The sperm were inserted on the left side of sperm microdroplet. This would allow the sperm to swim towards the right side of the microdroplets for a few minutes. After sperm insertion and mixing, if the microdroplets of the sperm were not flattened, 1-2 µl of microdroplets was gently aspirated out to reduce the volume of medium. These would help the experimenter to aspirate the sperm because the sperm seem like queuing at the edge of the microdroplets. However, enough number of sperm was left for the ICSI procedure. For oocyte microdroplet, the number of oocyte per microdroplet was not more than 5 oocytes.

ICSI was performed on warmer stage (38.5°C for goats experiment and 37°C for mice experiment) of the microscope with magnification of 10x and 20x objectives, respectively. ICSI procedure was started by aspirated the sperm or they would swim by themselves into the ICSI microneedle. Generally, the sperm stayed at the lowered of the edge of sperm microdroplet. Therefore, the aspiration of sperm could be occurred by faced the ICSI microneedle at this point.

Then, the sperm in the ICSI microneedle were focused and drawn into the PVP (10%) microdroplet. Here, the sperm movement was being slowed and easy to perform

the immobilisation through kinked the sperm tail using the spike of ICSI microneedle. The sperm could be assumed immobilised once the sperm was stopped moving.

Once the sperm was immobilised, a single sperm was aspirated the tail first into the ICSI microneedle again. Then, the MII oocyte was held by holding microneedle through firmly aspirated with the position of the polar body was at 6 or 12 o'clock. This could be achieved by rotating the oocyte at the tip of holding needle. Then, the sperm inside the ICSI microneedle was brought closed to the tip (20 μm). The ICSI microneedle was slowly advanced through the zona pelucida and into the ooplasm at 3 o'clock position. A small amount of ooplasm (1-2 pl) was vigorously sucked in and out but gently break the oolemma. A sudden flux of ooplasm into the ICSI microneedle confirmed the oolemma breakage and would be easy for sperm injection.

A single sperm was inserted into the ooplasm with the minimum amount of PVP (<5 pl). After sperm injection, the ICSI microneedle was gently withdrawn from the oocyte. Then, the oocyte was released from the holding microneedle. The injected oocyte was allocated at the bottom of microdroplet. The non-injected oocytes would be maintained at the top of the microdroplet. Once all 5 oocytes were injected with the sperm, the oocytes were placed into calibrated droplet of TCM-199 with OGS (10%) (for goats) and WM (for mice) and kept into $CO_2$ (5%) incubator before being treated with activation medium. The next batch of oocyte for ICSI would be inserted to another newly prepared oocyte microdroplet (ICSI medium).

### 3.5.3 Chemical Activation

All injected oocytes were incubated into TCM-199 with OGS (10%) (for goats) and WM (for mice) for at least 30 minutes before being activated with specific chemicals. The activation chemicals used were various depending on the experimental animals.

#### 3.5.3.1 Chemical activation for goat oocytes

In goat experiment, the chemicals that had been used were calcium ionophore and 6-DMAP. Microdroplets of activation medium were prepared (80 µl) with overlaid by mineral oil inside the Petri dish (35 mm) and should be equilibrated inside the $CO_2$ (5%) incubator at least 3 hours before being used. All injected oocytes were activated in calcium ionophore (5 µm) inside the $CO_2$ (5%) incubator for 5 minutes. After that the oocytes were washed 3x in 6-DMAP microdroplets to rinsed out the calcium ionophore medium before culture in 6-DMAP for another 4 to 5 hours also inside the $CO_2$ (5%) incubator.

#### 3.5.3.2 Chemical activation for mouse oocytes

For mice experiment, the chemicals used for activation were strontium hexahydrate containing cytochalasin B and CZB calcium free medium. The mice oocytes were cultured into this medium for 3 hours.

### 3.5.4  *In Vitro* Culture  (IVC)

Culture medium for IVC was various depends on experimental animal, where the goat sample was using the KSOM while the mice sample was using the WM medium. The microdroplets of IVC (80 µm) was overlaid with mineral oil and equilibrated inside the $CO_2$ (5%)  incubator Both animal samples were wash with the respective IVC medium (3x) before being cultured into the IVC medium. The culture was incubated at 38.5°C (goat) and 37°C (mice) and humidified air for 9 days. The embryos were evaluated at Day 2, 5, 7 and 9 for embryonic development.

### 3.5.5  ICSI-derived Embryos using Hoechst Staining

In order to ensure the embryonic development stage, the cleaved embryos were stained with Hoechst. The number of nuclei in the blastomeres was determined using epifluorescence microscope.

Generally, a few droplets of PBS (-) (100 µl) and fixative solution (100 µl) were made and overlaid with mineral oil on the Petri dish (35 mm or 60 mm). The cleaved embryos were washed in PBS (-) (5x) followed by in fixative solution (3x) on heated stage of stereomicroscope. Then, the embryos were placed to the last droplet of fixative solution for 5 minutes at 25°C.  Four small drops of Vaseline-wax were placed on the centre of the glass slide. After 5 minutes, the embryos were transferred on the slide and mounted with the coverslip. The excessive fixative solution was slowly aspirated out from the embryos before mounted with coverslip. The coverslip was sealed with adhesive (cutex), labeled on the side of slide and kept in refrigerator (4°C) before being examined under epifluorescence microscope.

## 3.6 EXPERIMENTAL DESIGN

The main objective of this project was an attempt to investigate the effects of selected sperm and oocytes factors on ICSI performance in mice and goats.

### 3.6.1 Effects of Sperm Factors on ICSI Performance in Mice (Experiment 1)

The objectives of this experiment were (i) to study the effect of sperm capacitation chemicals on embryo development, (ii) to investigate the effect of sperm movement characteristics using different sperm capacitation chemicals on embryo development. Subsequently, the optimal of sperm capacitation chemicals and sperm movement were identified based on the embryo development. The collected sperm from epididymis were capacitated for 60 minutes either using Heparin (50 µg/ ml) or Theophylline (9 µg/ ml) as sperm capacitation chemicals. The sperm movement criteria (Rapid and Slow) on ICSI performance were also compared. Rapid sperm was scored by rapid (motile) movement and the Slow sperm was scored by locally motile movement (Giwercman *et al.,* 2003). ICSI procedure and oocytes activation were carried out for both factors to determine the optimal ICSI-derived embryos' developmental competence. The parameters such as oocytes maturation rate, embryo cleavage rate and embryo develomental rate between different grades of oocytes were also evaluated.

### 3.6.2 Effects of Oocyte Factors on ICSI Performance in Mice (Experiment 2)

The female mice were superovulated with PMSG and hCG in order to obtain COCs used in this experiment. The durations of post-hCG administration (13-15 hours vs. 16-18 hours) on embryo development were investigated. The number of matured oocytes was determined by removing the cumulus cells (CCs) layer using the hyaluronidase

(0.1%). The matured oocytes (MII meiotic stage) with the presence of first polar body were selected for ICSI. The post-ICSI oocytes were activated and cultured inside the $CO_2$ (5%) incubator at 37.5°C for 9 days. The parameters such as maturation rate, cleavage rate and embryo development rate were determined.

### 3.6.3 Effects of Sperm Factors on ICSI Performance in Goat (Experiment 3)

The objectives of this experiment were (i) to compare the effect of sperm capacitation chemicals on embryo development, and (ii) to evaluate the effect of sperm movement characteristic on embryo development. Subsequently, the optimal sperm capacitation chemicals treatment and, sperm movement on ICSI performance were investigated.

The frozen sperm of Jermasia male goat were thawed (37.5°C) and underwent for sperm washing 15 minutes by centrifugation (200 x $g$). Prior to washing, the sperm motility was checked. After washing, the sperm pellet was isolated and followed by sperm capacitation (60 minutes), using sperm capacitation medium that consisted of either Heparin (50 µg/ ml) or Theophylline (9 µg/ ml) as the sperm capacitation chemicals. The sperm movement criteria (Rapid and Slow) on ICSI performance were also compared. Rapid sperm was scored by rapid (motile) movement and the Slow sperm was scored by locally motile movement (Giwercman *et al.,* 2003). ICSI procedure and oocytes activation were carried out for both factors. The parameters such as oocytes maturation rate, embryo cleavage rate and different embryo development rate between different grades of oocytes were investigated.

### 3.6.4   Effects of Oocyte Factors on ICSI Performance in Goat (Experiment 4)

The aims of this experiment were (i) to investigate the effects of LOPU cycle on yield of oocytes and (ii) to evaluate the effects of IVM duration on maturation rate. A total of 16 goats underwent at least three cycles of LOPU procedure for retrieval of the oocytes.

The goats were oestrus synchronised and superovulated before LOPU surgery was carried out (Section 3.4.3.1.1).   The number of oocytes retrieved and maturation rate performance from first, second and third cycles of LOPU were investigated. The retrieval oocytes (COCs) from LOPU were cultured into IVM medium for duration between 18 to 21 hours and 22 to 25 hours. The COCs were categorised according to different grades (A, B, C, D and E). ICSI procedure was carried out only in Grades A, B and C oocytes. Briefly, oocytes were removed from CCs using hyaluronidase (0.1%) to help in visual examination of matured oocytes. Only the matured oocytes (MII meiotic stage) were selected for ICSI procedure. The parameters such as number of retrieved oocytes, maturation rate and grades of oocytes were determined for the factors studied in these experiments.

## 3.7    STATISTICAL ANALYSIS

The effects of mouse sperm capacitation chemicals and sperm movement characteristics (Experiment 1); mouse post-hCG duration (Experiment 2); goat sperm capacitation chemicals and sperm movement characteristics (Experiment 3); goats oocytes retrieval cycle (LOPU cycle), quality of oocytes and maturation duration (Experiment 4) were analysed by using one-way analysis of variance (ANOVA). The differences among the means obtained were determined usingDuncan's Multiple range Test (DMRT). The analyses were performed by using SPSS statistical analysis programme. The data obtained from various experiments were presented as mean plus or minus standard error of means (mean±SEM) and at 5% significant level.

**MICE**

**EXPERIMENT 2**
Post-hcg administration:
  i)    13-15 hr
  ii)   16-18 hr
→ Embryo developmental rate

Oocyte and sperm recovery

**EXPERIMENT 1**
Sperm capacitation chemicals treatment (1 hr):
  i)    Heparin (50 µg/ ml)
  ii)   Theophylline (9 µg/ ml)
→ Embryo developmental rate

ICSI using different sperm movement criterion:
  i)    Rapid
  ii)   Slow
→ Embryo developmental rate

Oocytes activation [$SrCl_2$ + CB + CZB-$Ca^{2+}$] (3 hr)]
→ Embryo staining

**GOATS**

**EXPERIMENT 4**
LOPU performed:
  i)    OR cycle 1
  ii)   OR cycle 2
  iii)  OR cycle 3
→ Embryo developmental rate

IVM duration:
  i)    18-21 hr
  ii)   22-25 hr
→ Embryo developmental rate

**EXPERIMENT 3**
Sperm capacitation chemicals treatment (1 hr):
  i)    Heparin (50 µg/ ml)
  ii)   Theophylline (9 µg/ ml)
→ Embryo developmental rate

ICSI using different sperm movement criterion:
  i)    Rapid
  ii)   Slow
→ Embryo developmental rate

Oocytes activation → Embryo staining

**Preparation of donor goat for LOPU:**

a) Day 0 (0900 hr)
   CIDR in

b) Day 13 (0900 hr)
   Estrumate® (0.5 ml; 125 µg)

c) Day 14 (1600 hr)
   PMSG (150 IU)

Day 14 (2100 hr)
   CIDR removal and Ovidrel® (250 IU)

d) Day 16 (1730 hr)
   Off-feed and water

e) Day 17 (0800 hr)

**Figure 3.3**: The represented diagram showed the flow chart of experimental design in this study.

**Chapter 4**

**4.0 RESULTS**

## Chapter 4

## 4.0 RESULTS

## 4.1 EFFECT OF SPERM CAPACITATION ON ICSI PERFORMANCE IN MICE (EXPERIMENT 1)

As shown in Table 4.1, a total of 3077 cumulus oocytes complex (COC) were recovered from 120 donor mice. Out of these, 73.16% of oocytes were in MII stage (presence of polar body), 16.67% of oocytes were absent of polar body and 10.14% were in dysmorphic form (fragmented, disintegrated or degenerated).

Tables 4.2 show the embryonic development after ICSI for different sperm capacitation chemical treatments and sperm movements. Even though insignificance (P>0.05), generally the Heparin group gave higher cleavage rates than Theophylline group for all the developmental stages regardless of the sperm movement. Similarly, the Rapid group gave higher cleavage rates than Slow group regardless of the sperm capacitation chemical treatment (P>0.05). However, when disregard sperm movement (Table 4.3), the Heparin group gave significantly higher (P<0.05) cleavage rates than theophylline group at 2- and 4-cell stages (with values of 64.67±1.69% versus 60.50±1.21% and 50.55±2.01% versus 45.09±1.60%, respectively). As for sperm movement, when disregard sperm capacitation chemical treatment (Table 4.4), there was no significant difference (P>0.05) in the cleavage rate at all stages of development (2-cell to morula stage).

**Table 4.1**: Number and percentages of oocytes obtained through oviduct oocyte retrieval in mice

| *Total no. of mice | **Total no. of ovaries | Total no. of oocytes per ovary | Percent of oocytes with polar body | Percent of oocytes without polar body | Percent of dysmorphic oocytes |
|---|---|---|---|---|---|
| 120 | 224 | 13.74 (n=3077) | 73.16 (n=2252) | 16.67 (n=513) | 10.14 (n=312) |

\* Total number of mice used for studies
\*\* Total number of ovaries was based on ovaries that contained oocyte
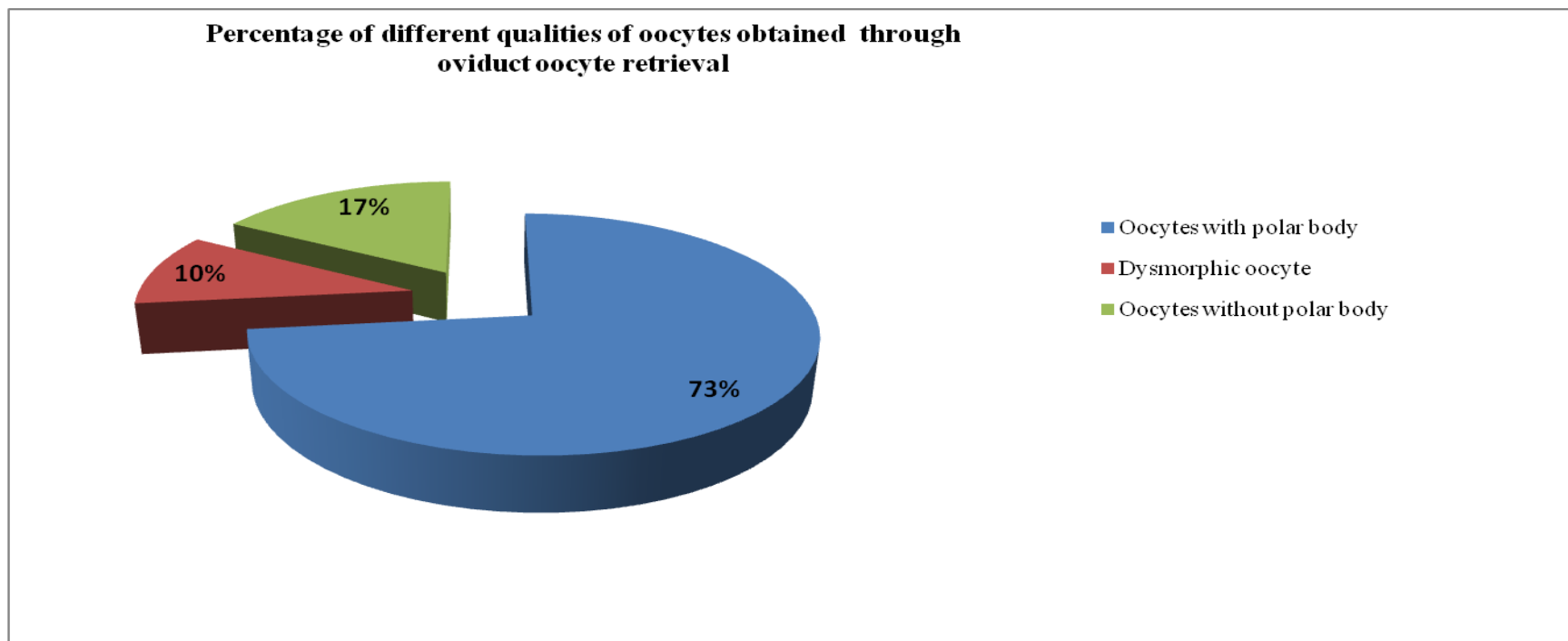


**Figure 4.1**: Percentage of oocytes obtained in mice.

**Table 4.2**: Cleavage rate (mean ± SEM) of ICSI derived embryos for different sperm capacitation chemical treatments and sperm movements in mice

| Sperm capacitation chemical treatment | Sperm movement | *No. of injected oocyte | Cleavage rate (%) | | | |
|---|---|---|---|---|---|---|
| | | | 2-cell | 4-cell | 8-cell | Morula |
| Heparin | Rapid | 617 | $65.43 \pm 1.90^{az}$ (n=418) | $52.32 \pm 1.96^{ay}$ (n=334) | $33.78 \pm 2.31^{ax}$ (n=215) | $12.61 \pm 1.51^{aw}$ (n=94) |
| | Slow | 547 | $63.86 \pm 2.88^{az}$ (n=367) | $48.67 \pm 3.59^{ay}$ (n=287) | $29.83 \pm 3.36^{ax}$ (n=174) | $10.78 \pm 1.75^{aw}$ (n=78) |
| | Average (Total) | 1164 | $64.67 \pm 1.69^{z}$ (n=785) | $50.55 \pm 2.01^{y}$ (n=621) | $31.86 \pm 2.02^{x}$ (n=389) | $11.72 \pm 1.15^{w}$ (n=172) |
| Theophylline | Rapid | 526 | $61.39 \pm 1.37^{az}$ (n=328) | $45.87 \pm 1.86^{ay}$ (n=249) | $31.42 \pm 2.32^{ax}$ (n=168) | $13.86 \pm 1.55^{aw}$ (n=79) |
| | Slow | 562 | $59.51 \pm 2.06^{az}$ (n=344) | $44.23 \pm 2.72^{ay}$ (n=260) | $28.43 \pm 3.05^{ax}$ (n=175) | $11.91 \pm 1.62^{aw}$ (n=76) |
| | Average (Total) | 1088 | $60.50 \pm 1.21^{z}$ (n=672) | $45.09 \pm 1.60^{y}$ (n=509) | $30.00 \pm 1.88^{x}$ (n=343) | $12.93 \pm 1.12^{w}$ (n=155) |

* No. of injected oocytes was based on total no. of oocyte used for ICSI (MII stage)

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

[wxyz] Mean value within row with diferent superscripts were significantly different (P<0.05

**Table 4.3**: Cleavage rate (mean ± SEM) of ICSI derived embryos for different sperm capacitation chemical treatments in mice

| Sperm capacitation chemical treatment | *No. of injected oocyte | Cleavage rate (%) | | | |
|---|---|---|---|---|---|
| | | 2-cell | 4-cell | 8-cell | Morula |
| Heparin | 1164 | $64.67\pm1.69^{bz}$ (n=785) | $50.55\pm2.01^{by}$ (n=621) | $31.86\pm2.02^{ax}$ (n=389) | $11.72\pm1.15^{aw}$ (n=172) |
| Theophylline | 1088 | $60.50\pm1.21^{az}$ (n=672) | $45.09\pm1.60^{ay}$ (n=509) | $30.00\pm1.88^{ax}$ (n=343) | $12.93\pm1.12^{aw}$ (n=155) |
| Average (Total) | 2252 | $62.56\pm1.05^{z}$ (n=1457) | $47.78\pm1.31^{y}$ (n=1130) | $30.92\pm1.37^{x}$ (n=732) | $12.33\pm0.80^{w}$ (n=327) |

[*] Number of injected oocytes was based on total no. of oocyte used for ICSI (MII stage)
[ab] Mean values within a column with different superscripts were significantly different (P<0.05)
[wxyz] Mean value within row with different superscripts were significantly different (P<0.05)

**Table 4.4**: Cleavage rate (mean ± SEM) of ICSI derived embryos for different sperm movements in mice

| Sperm capacitation chemical treatment | *No. of injected oocyte | Cleavage rate (%) | | | |
|---|---|---|---|---|---|
| | | 2-cell | 4-cell | 8-cell | Morula |
| Rapid | 1143 | $63.36\pm1.91^{az}$ (n=746) | $49.02\pm1.43^{ay}$ (n=583) | $32.57\pm1.63^{ax}$ (n=383) | $13.25\pm1.07^{aw}$ (n=173) |
| Slow | 1109 | $61.68\pm1.78^{az}$ (n=711) | $46.45\pm2.25^{ay}$ (n=547) | $29.13\pm2.24^{ax}$ (n=349) | $11.34\pm1.18^{aw}$ (n=154) |
| Average (Total) | 2252 | $62.56\pm1.05^{z}$ (n=1457) | $47.78\pm1.31^{y}$ (n=1130) | $30.92\pm1.37^{x}$ (n=732) | $12.33\pm0.80^{w}$ (n=327) |

*  Number of injected oocytes was based on total no. of oocyte used for ICSI (MII stage)
a  Mean values within a column with same superscripts were not  significantly different (P>0.05)
wxyz  Mean value within row with different superscripts were significantly different (P<0.05)

## 4.2 EFFECT OF OOCYTE FACTORS ON ICSI PERFORMANCE IN MICE (EXPERIMENT 2)

The effects of post-hCG administration durations (13-15 hours and 16-18 hours) on the oocyte retrieval were evaluated in this experiment (Table 4.5). A total of 224 ovaries were obtained from 120 donor mice with total numbers of oocytes per ovary of 13.91±0.61 (1557) and 13.57±0.54 (1520) for 13-15 hours and 16-18 hours durations, respectively. There were significant differences between the post-hCG administration duration for percent for polar body presence and percent of polar body absence with values of 75.61±1.64% versus 67.37±1.50% and 12.50±1.55% versus 21.82±1.49%, respectively. However, there were no significance differences for the percent dysmorphic oocytes with the values of 11.88±1.16% versus 10.76±1.24%, respectively.

Table 4.6 shows the cleavage rates of ICSI derived embryos for different post-hCG administration durations. The cleavage rates at 2-, 4-, 8-cell and morula stages were significant higher ($P<0.05$) at 13-15 hours compared to 16-18 hours duration (67.95±1.06% versus 57.02±1.33%, 53.51±1.13% versus 41.90±1.98%, 39.40±1.01% versus 22.21±1.62%, and 14.83±1.09% versus 9.77±1.02%, in respectively). The cleavage rates were decreased in all cases from early to the later stages of embryonic development significantly ($P<0.05$).

**Table 4.5**: Number (mean ± SEM) and percent (mean ± SEM) of oocytes obtained through oviduct oocytes retrieval in mice

| Post-hCG duration | *Total no. of mice | **Total no. of ovaries | Total no. of oocytes per ovary | Percent of oocytes with polar body | Percent of oocytes without polar body | Percent of dysmorphic oocytes |
|---|---|---|---|---|---|---|
| 13-15 hours | 60 | 112 | $13.91\pm0.61^{a}$ (n=1557) | $75.61\pm1.64^{bz}$ (n=1208) | $12.50\pm1.55^{ay}$ (n=177) | $11.88\pm1.16^{ay}$ (n=173) |
| 16-18 hours | 60 | 112 | $13.57\pm0.54^{a}$ (n=1520) | $67.37\pm1.50^{az}$ (n=1044) | $21.82\pm1.49^{by}$ (n=336) | $10.76\pm1.24^{ax}$ (n=139) |
| Average (Total) | (n=120) | (n=224) | $13.74\pm0.41$ (n=3077) | $71.49\pm1.14^{z}$ (n=2252) | $17.16\pm1.12^{y}$ (n=513) | $11.32\pm0.85^{x}$ (n=312) |

\* Total no. of mice used for studies
\*\* Total no. of ovaries was based on ovaries that contained oocyte
[ab] Mean values within a column with different superscripts were significantly different (P<0.05)
[xyz] Mean value within row with different superscripts were significantly different (P<0.05)

**Table 4.6**: Cleavage rate (mean ± SEM) of ICSI derived embryos for different post-hCG duration in mice

| Post-hCG duration | *No. of injected oocyte | Cleavage rate (%) | | | |
|---|---|---|---|---|---|
| | | 2-cell | 4-cell | 8-cell | Morula |
| 13-15 hours | 1208 | $67.95 \pm 1.06^{bz}$ (n=836) | $53.51 \pm 1.13^{by}$ (n=662) | $39.40 \pm 1.01^{bx}$ (n=481) | $14.83 \pm 1.09^{bw}$ (n=200) |
| 16-18 hours | 1044 | $57.02 \pm 1.33^{az}$ (n=621) | $41.90 \pm 1.98^{ay}$ (n=468) | $22.21 \pm 1.62^{ax}$ (n=251) | $9.77 \pm 1.02^{aw}$ (n=127) |
| Average (Total) | 1126 (n=2252) | $62.56 \pm 1.05^{z}$ (n=1457) | $47.78 \pm 1.31^{y}$ (n=1130) | $30.92 \pm 1.37^{x}$ (n=732) | $12.33 \pm 0.80^{w}$ (n=327) |

* Number of injected oocytes was based on total no. of oocyte used for ICSI (MII stage)
ab Mean value within a column with different superscripts were significantly different ($P<0.05$)
wxyz Mean value within row with different superscripts were significantly different ($P<0.05$)
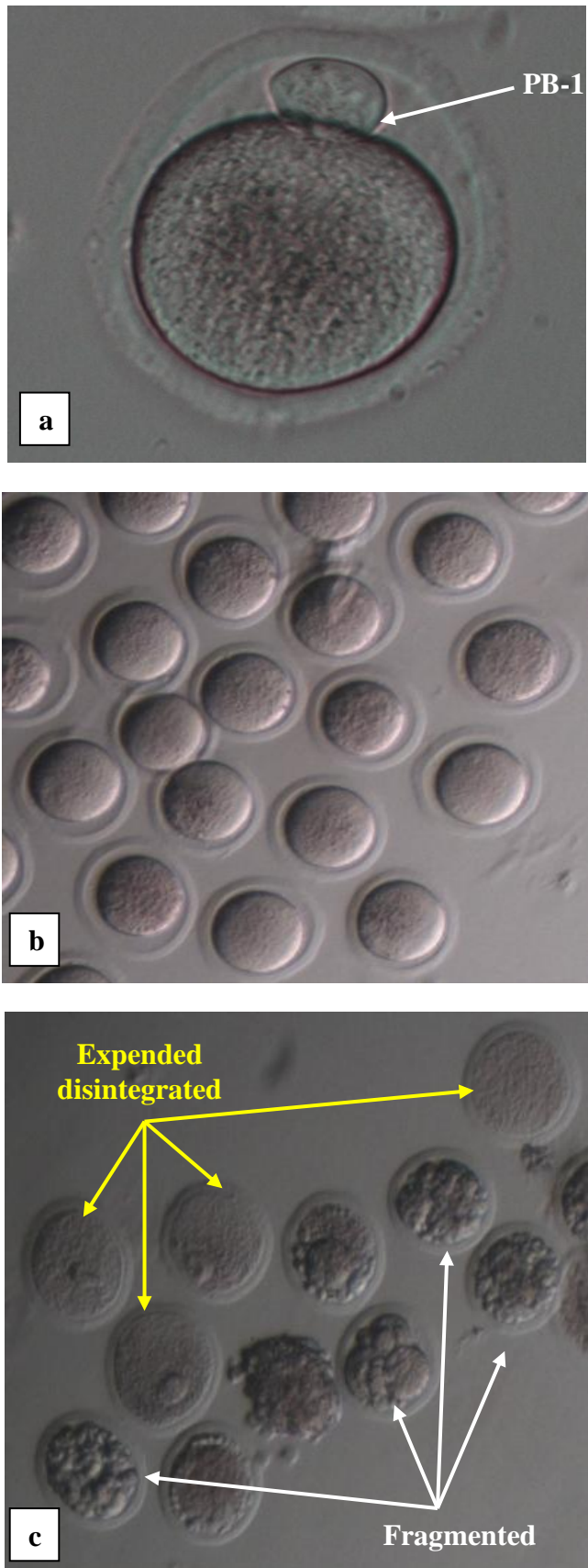
**Figure 4.2**: Morphology of different oocyte quality obtained after oviduct oocyte retrieval in mice. (a) Present of polar body, (b) Absent of polar body and (c) Dysmorphic oocytes.
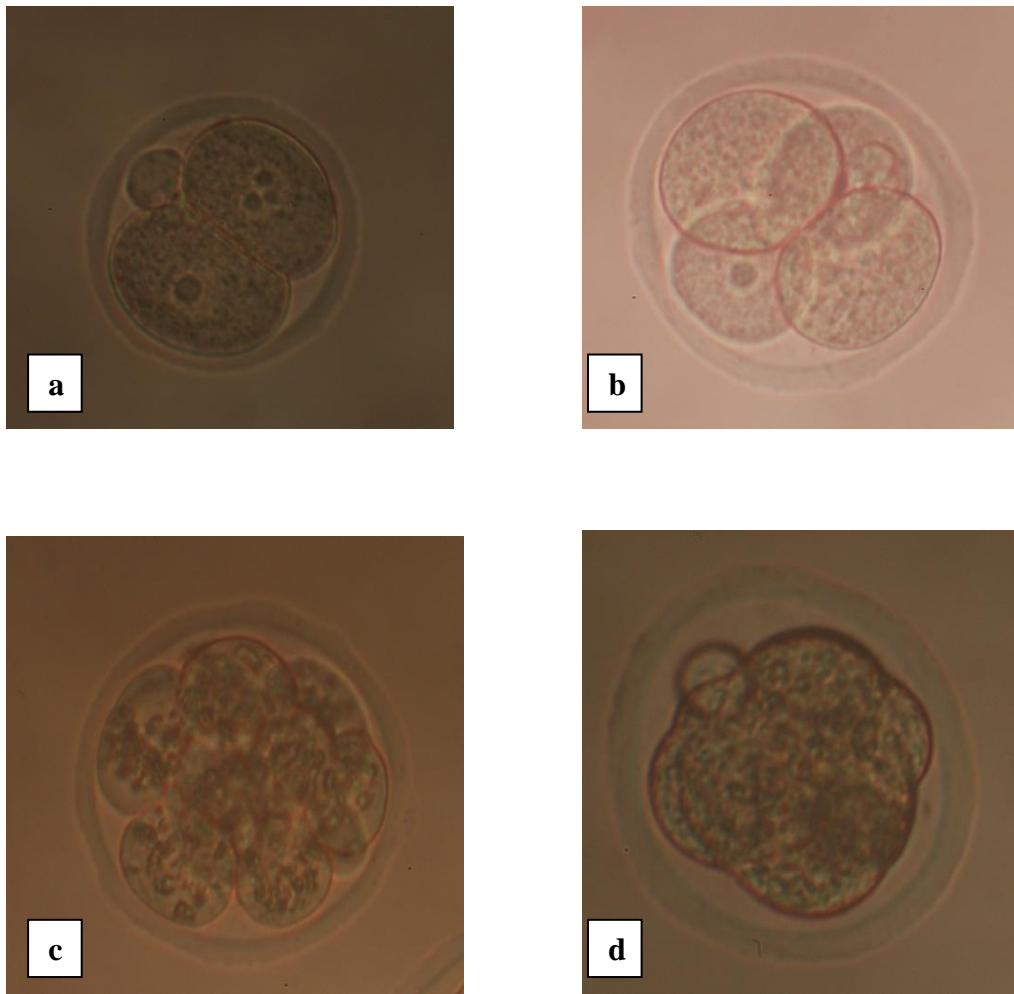
**Figure 4.3**: Morphology of different embryonic development stage in mice. (a) 2-cell embryo, (b) 4-cell embryo, (c) 8-cell embryo and (d) Morula.

## 4.3 EFFECTS OF SPERM FACTORS ON ICSI PERFORMANCE IN GOAT (EXPERIMENT 3)

This experiment was designed to evaluate the effect of two sperm factors (sperm capacitation chemicals treatment and sperm movement) on ICSI derived embryo developmental competency.

A total of 445 cumulus oocytes complexes (COC) from Grades A, B, C, D and E were retrieved from 64 goat ovaries through LOPU procedure (Table 4.7). The respective percent oocytes recovered were 28.93±3.45%, 20.40±2.98%, 29.25±3.45%, 15.41±3.08% and 6.02±2.07%. Correspondingly, the numbers of oocytes per ovary were 1.89±0.23, 1.44±0.19, 2.40±0.34, 0.98±0.22 and 0.28±0.08, respectively. Table 4.8 shows the maturation rates of oocytes after LOPU according to grades of oocytes. Percent oocytes matured for Grades A, B, C, D, and E were 96.35±2.11%, 87.59±4.69%, 84.40±2.78%, 51.09±9.59% and 4.17±4.17%, respectively. Correspondingly, numbers of matured oocytes per ovary were 1.80±0.22, 1.27±0.17, 2.00±0.30, 0.59±0.16 and 0.02±0.02, respectively. Grades D and E of oocytes were omitted in this experiment due to the significantly lower maturation rates obtained compared with Grades A, B and C.

Table 4.9 shows the embryonic development after ICSI for different sperm capacitation chemical treatments and sperm movements. Even though insignificance (P>0.05), generally in Heparin group, Grade A oocytes oocytes group at 2-, 4- and 8-cell stage of the Slow group gave higher embryonic development cleavage rates than Rapid group. In morula, the Rapid group was higher than Slow group. Grade B group, showed higher embryonic development rate at 2-cell stage from Slow group. Meanwhile, the Rapid group showed higher embryonic development cleavage rate at 4- and 8-cell stages. The Rapid group of Grade C oocytes showed higher cleavage rates for

all stages of embryonic development stages (2-, 4-, 8-cell and morula stages). When combined the grades, only in 4-cell stage embryonic development showed higher cleavage rate for Rapid group. The rest of embryonic cleavage rate (2-, 8- and morula cell stage) were highly showed in Slow group.

In the Theophylline group, the Grade A oocytes group gave significantly higher when using Rapid compared to the Slow sperm movement at 2-, 4-, and 8-cell stage (with the values of 95.83±4.17% versus 63.27±9.10%, 83.33±12.60% versus 47.96±9.92% and 77.08±12.96% versus 20.15±8.52%, respectively). In Group B oocytes group, only at 2-cell stage showed significantly different when Rapid group was compared with Slow sperm movement group (84.38±5.98% versus 50.00±11.89%). The Grade C oocyte group gave no significant difference (P>0.05) for all development rates. The combined grades oocytes group (A, B and C) also gave significant higher using Rapid than Slow sperm movement group at all developmental stage (2-, 4-, 8- and morula cell stage) (85.42±4.17% versus 54.35±6.20%, 71.39±6.49% versus 42.28±6.31%, 57.43±7.63% versus 21.63±5.34% and 10.76±5.03% versus 0.88±0.62%, respectively).

When disregard the sperm movement (Table 4.10), generally the Theophylline group gave higher cleavage rates than Heparin group. However, both sperm capacitation chemicals showed no significant difference (P>0.05) in cleavage rate at all developmental stages. As for sperm movement, when disregard sperm capacitation chemical treatment (Table 4.11), there was no significant difference (P>0.05) at 2-cell stage cleavage rate. However, at other stages of development (4-, 8- and morula cell stage), the Rapid group gave significantly higher (P<0.05) than Slow group, where the respective values were 56.62±4.69% versus 41.80±4.49%, 39.51±4.70% versus 24.24±4.05% and 9.21±2.85% versus 2.24±0.94%.

**Table 4.7**: Number (mean ± SEM) of oocytes obtained after LOPU according to grades of goat oocytes

| Grade | Total no. of oocytes recovered | *No. of oocytes per ovary | Percent oocytes recovered |
|---|---|---|---|
| A | 119 | $1.89\pm0.23^{cd}$ | $28.93\pm3.45^{c}$ |
| B | 92 | $1.44\pm0.19^{bc}$ | $20.40\pm2.98^{bc}$ |
| C | 153 | $2.40\pm0.34^{d}$ | $29.25\pm3.45^{c}$ |
| D | 63 | $0.98\pm0.22^{b}$ | $15.41\pm3.08^{b}$ |
| E | 18 | $0.28\pm0.08^{a}$ | $6.02\pm2.07^{a}$ |
| Average (Total) | 89 (n=445) | $1.40\pm0.11$ | $20.00\pm1.44$ (100%) |

\* Number of oocytes was based on 64 ovaries for all grades of oocytes
[abcd] Mean values within a column with different superscripts were significantly different (P>0.05)

**Table 4.8**: Maturation rates (mean ± SEM) of oocyte after LOPU according to grades of goat oocytes

| Grade | Total no. of oocytes per ovary | Total no. of oocytes matured | *No. of oocytes matured per ovary | Percent of oocytes matured |
|---|---|---|---|---|
| A | 1.89±0.23$^{cd}$ (n=119) | 115 | 1.80±0.22$^{cd}$ | 96.35±2.11$^c$ |
| B | 1.44±0.19$^{bc}$ (n=92) | 81 | 1.27±0.17$^c$ | 87.59±4.69$^c$ |
| C | 2.40±0.34$^d$ (n=153) | 128 | 2.00±0.30$^d$ | 84.40±2.78$^c$ |
| D | 0.98±0.22$^b$ (n=63) | 38 | 0.59±0.16$^b$ | 51.09±9.59$^b$ |
| E | 0.28±0.08$^a$ (n=18) | 1 | 0.02±0.02$^a$ | 4.17±4.17$^a$ |
| Average (Total) | 1.40±0.11 (n=445) | 72.5 (n=363) | 1.13±0.10 | 64.72±5.89 (100%) |

\* Total number of oocytes matured per ovary was based on 64 ovaries for all grades of oocytes

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

**Table 4.9**: Cleavage rate (mean ± SEM) of ICSI derived embryos for different sperm capacitation chemicals treatments in goats

| Sperm capacitation chemical treatment | Grade of oocyte | Sperm movement | *No. of injected oocyte | Cleavage rate (%) | | | |
|---|---|---|---|---|---|---|---|
| | | | | 2-cell | 4-cell | 8-cell | Morula |
| Heparin | A | Rapid | 40 | $62.89 \pm 9.41^{az}$ (n=28) | $49.56 \pm 9.48^{ayz}$ (n=24) | $30.78 \pm 7.97^{axy}$ (n=15) | $14.56 \pm 7.09^{ax}$ (n=6) |
| | | Slow | 30 | $84.67 \pm 6.80^{az}$ (n=23) | $55.50 \pm 13.43^{ayz}$ (n=18) | $43.50 \pm 13.04^{ay}$ (n=16) | $8.25 \pm 5.14^{ax}$ (n=3) |
| | Average (Total) | | 35 (n=70) | $71.60 \pm 6.53^{z}$ (n=51) | $51.93 \pm 7.67^{y}$ (n=42) | $35.87 \pm 7.03^{y}$ (n=31) | $12.03 \pm 4.69^{x}$ (n=9) |
| | B | Rapid | 13 | $52.38 \pm 15.61^{az}$ (n=7) | $47.62 \pm 15.61^{az}$ (n=6) | $29.76 \pm 13.83^{ayz}$ (n=4) | $0.00 \pm 0.00^{y}$ (n=0) |
| | | Slow | 25 | $70.17 \pm 10.24^{az}$ (n=16) | $43.50 \pm 10.98^{ayz}$ (n=12) | $26.67 \pm 11.71^{axy}$ (n=6) | $0.00 \pm 0.00^{x}$ (n=0) |
| | Average (Total) | | 19 (n=38) | $62.84 \pm 8.78^{z}$ (n=23) | $45.20 \pm 8.82^{yz}$ (n=18) | $27.94 \pm 8.66^{y}$ (n=10) | $0.00 \pm 0.00^{x}$ (n=0) |
| | C | Rapid | 41 | $63.18 \pm 8.70^{az}$ (n=26) | $41.16 \pm 9.11^{ay}$ (n=20) | $20.25 \pm 6.51^{ax}$ (n=13) | $4.81 \pm 2.71^{ax}$ (n=4) |
| | | Slow | 35 | $45.58 \pm 10.31^{az}$ (n=19) | $26.26 \pm 8.18^{ayz}$ (n=11) | $12.84 \pm 5.53^{axy}$ (n=7) | $3.12 \pm 2.13^{ax}$ (n=2) |
| | Average (Total) | | 38 (n=76) | $54.76 \pm 6.81^{z}$ (n=45) | $34.03 \pm 6.22^{y}$ (n=31) | $16.70 \pm 4.28^{x}$ (n=20) | $4.00 \pm 1.71^{x}$ (n=6) |
| | Combines grades | Rapid | 94 | $60.83 \pm 5.93^{az}$ (n=61) | $46.19 \pm 6.01^{ay}$ (n=50) | $26.85 \pm 4.98^{ax}$ (n=32) | $8.12 \pm 3.37^{aw}$ (n=10) |
| | | Slow | 90 | $66.12 \pm 6.01^{az}$ (n=58) | $41.25 \pm 6.47^{ay}$ (n=41) | $27.19 \pm 6.22^{ay}$ (n=29) | $3.77 \pm 1.86^{ax}$ (n=5) |
| | Average (Total) | | 92 (n=184) | $63.35 \pm 4.20^{z}$ (n=119) | $43.84 \pm 4.38^{y}$ (n=91) | $27.01 \pm 3.91^{x}$ (n=61) | $6.04 \pm 1.98^{w}$ (n=15) |

Continued

Continued

| Sperm capacitation chemical treatment | Grade of oocyte | Sperm movement | *No. of injected oocyte | Cleavage rate (%) | | | |
|---|---|---|---|---|---|---|---|
| | | | | 2-cell | 4-cell | 8-cell | Morula |
| Theophylline | A | Rapid | 14 | $95.83\pm4.17^{bz}$ (n=13) | $83.33\pm12.60^{bz}$ (n=10) | $77.08\pm12.96^{bz}$ (n=9) | $18.75\pm13.15^{ay}$ (n=2) |
| | | Slow | 31 | $63.27\pm9.10^{az}$ (n=21) | $47.96\pm9.92^{az}$ (n=16) | $20.15\pm8.52^{ay}$ (n=8) | $1.02\pm1.02^{ay}$ (n=1) |
| | Average (Total) | | 22.5 (n=45) | $75.11\pm6.81^{z}$ (n=34) | $60.82\pm8.47^{yz}$ (n=26) | $40.86\pm9.20^{y}$ (n=17) | $7.47\pm4.98^{x}$ (n=3) |
| | B | Rapid | 20 | $84.38\pm5.98^{bz}$ (n=16) | $63.54\pm11.78^{az}$ (n=13) | $59.38\pm12.34^{az}$ (n=12) | $7.29\pm4.84^{ay}$ (n=2) |
| | | Slow | 21 | $50.00\pm11.89^{az}$ (n=11) | $28.03\pm11.91^{ayz}$ (n=6) | $25.00\pm12.15^{ayz}$ (n=5) | $0.00\pm0.00^{ay}$ (n=0) |
| | Average (Total) | | 20.5 (n=41) | $64.47\pm8.21^{z}$ (n=27) | $42.98\pm9.24^{yz}$ (n=19) | $39.47\pm9.41^{y}$ (n=17) | $3.07\pm2.13^{x}$ (n=2) |
| | C | Rapid | 31 | $76.04\pm9.50^{az}$ (n=19) | $67.29\pm9.25^{az}$ (n=16) | $35.83\pm11.46^{ay}$ (n=8) | $6.25\pm6.25^{ax}$ (n=2) |
| | | Slow | 23 | $46.67\pm12.12^{az}$ (n=12) | $50.00\pm10.83^{az}$ (n=12) | $20.00\pm6.94^{ay}$ (n=6) | $1.67\pm1.67^{ay}$ (n=1) |
| | Average (Total) | | 27 (n=54) | $59.72\pm8.5^{z}$ (n=31) | $57.69\pm7.38^{z}$ (n=28) | $27.04\pm6.47^{y}$ (n=14) | $3.70\pm2.88^{x}$ (n=3) |
| | Combines grades | Rapid | 65 | $85.42\pm4.17^{bz}$ (n=48) | $71.39\pm6.49^{byz}$ (n=39) | $57.43\pm7.63^{by}$ (n=29) | $10.76\pm5.03^{bx}$ (n=6) |
| | | Slow | 75 | $54.35\pm6.20^{az}$ (n=44) | $42.28\pm6.31^{az}$ (n=34) | $21.63\pm5.34^{ay}$ (n=19) | $0.88\pm0.62^{ax}$ (n=2) |
| | Average (Total) | | 70 (n=140) | $66.99\pm4.49^{z}$ (n=92) | $54.12\pm4.91^{y}$ (n=73) | $36.19\pm4.97^{x}$ (n=48) | $4.90\pm2.15^{w}$ (n=8) |

*    Number of injected oocyte was based on MII stage oocyte that used for ICSI, in respectively grade
ab   Mean values within a column with different superscripts were significantly different (P<0.05)
wxyz Mean value within row with different superscripts were significantly different (P<0.05)

**Table 4.10**: Summary of cleavage rate (mean ± SEM) of ICSI derived embryos for different sperm capacitation chemicals treatments in goats

| Sperm capacitation treatment | *No. of injected oocytes | Cleavage rate (%) | | | |
|---|---|---|---|---|---|
| | | 2-cells | 4-cells | 8-cells | Morula |
| Heparin | 184 | $63.35\pm4.20^{az}$ (n=119) | $43.84\pm4.38^{ay}$ (n=91) | $27.01\pm3.91^{ax}$ (n=61) | $6.04\pm1.98^{aw}$ (n=15) |
| Theophylline | 140 | $66.99\pm4.49^{az}$ (n=92) | $54.12\pm4.91^{ay}$ (n=73) | $36.19\pm4.97^{ax}$ (n=48) | $4.90\pm2.15^{aw}$ (n=8) |
| Average | 162 (n=324) | $65.08\pm3.06^{z}$ (n=211) | $48.73\pm3.30^{y}$ (n=164) | $31.38\pm3.14^{x}$ (n=109) | $5.50\pm1.45^{w}$ (n=23) |

\* Mean percentage of embryo development was based on oocytes used for ICSI
[a] Mean value within a column with same superscripts were not significantly different (P>0.05)
[wxyz] Mean value within row with different superscripts were significantly different (P<0.05)

**Table 4.11**: Summary of cleavage rate (mean ± SEM) of ICSI derived embryos for different sperm movements in goats

| Sperm capacitation treatment | *No. of injected oocytes | Cleavage rate (%) | | | |
|---|---|---|---|---|---|
| | | 2-cells | 4-cells | 8-cells | Morula |
| Rapid | 159 | $71.00\pm4.17^{az}$ (n=109) | $56.62\pm4.69^{by}$ (n=89) | $39.51\pm4.70^{bx}$ (n=61) | $9.21\pm2.85^{bw}$ (n=16) |
| Slow | 165 | $59.88\pm4.36^{az}$ (n=102) | $41.80\pm4.49^{ay}$ (n=75) | $24.24\pm4.05^{ax}$ (n=48) | $2.24\pm0.94^{aw}$ (n=7) |
| Average | 162 (n=324) | $65.08\pm3.06^{z}$ (n=211) | $48.73\pm3.30^{y}$ (n=164) | $31.38\pm3.14^{x}$ (n=109) | $5.50\pm1.45^{w}$ (n=23) |

* Number of embryo development was based on oocytes used for ICSI
[ab] Mean value within a column with different superscripts were significantly different (P<0.05)
[wxyz] Mean value within row with different superscripts were significantly different (P<0.05)

**4.4     EFFECTS OF OOCYTE FACTORS ON ICSI PERFORMANCE IN**

**GOAT (EXPERIMENT 4)**

This experiment was divided into two parts: (i) to investigate the effects of LOPU cycle on yield of oocytes and (ii) to evaluate the effects of IVM duration on maturation rate and ICSI- derived embryonic development.

**4.4.1 The Effect of LOPU Cycle on Yield of Oocytes**

There were 3 repeated cycles of LOPU procedure performed using 16 donor goats. The oocytes obtained were classified based on COC layer criterion and was divided into 5 grades, which were Grades A, B, C, D and E, respectively (Figure 4.4).

Table 4.12 shows the number of oocytes obtained from Grades A, B, C, D and E for different oocyte recovery (OR) cycles. Generally, the number of oocytes recovered decreased when the cycle increased (OR1, OR2 and OR3) with the values of $5.47\pm0.67$, $3.94\pm0.44$ and $3.09\pm0.50$ respectively. The OR1 retrieved number was significantly higher ($P<0.05$) than OR2 and OR3. When analysed the each grades of oocytes, Grades B and D gave no significance difference ($P>0.05$) between the OR cycle. In Grade A, the number of oocytes obtained in OR1 and OR2 was significantly higher than OR3, which were $1.50\pm0.34$ and $1.25\pm5.27$ versus $0.56\pm0.13$. In Grades C, the number of oocytes obtained in OR1 was significantly higher ($P<0.05$) than OR2 or OR 3, which were $2.41\pm0.36$ versus $1.25\pm0.23$ and $1.06\pm0.31$, respectively. The Grade E gave higher significantly difference ($P<0.05$) in OR2 and OR3 compared to OR1 ($0.22\pm0.07$ and $0.34\pm0.13$ versus $0.03\pm0.03$, respectively).

Table 4.13 shows the oocyte recovery rate for different grades of oocytes. According to the results obtained, the recovery rates of oocytes for Grades A, B, C and

D were not significantly different (P>0.05) between the OR cycles (OR1, OR2 and OR3). Except in Grade E, the OR3 shows significantly higher oocytes recovery rate compared to OR1 (10.35±4.37% versus 0.26±0.26%, respectively). However, OR2 recovery rate was not significantly different (P>0.05) from OR1 or OR3.

When analysed the percent oocyte recovery for each grade per OR cycle, generally Grade C oocytes was higher in LOPU procedure. In OR1, the recovery rates of Grades D and E oocytes were not significantly different (P>0.05) (4.16±1.90% and 0.26±0.26%, respectively). Grades A and B (24.78±4.73% and 21.39±4.31, respectively) were not significantly different to each other (P>0.05), but they were significantly higher (P<0.05) than Grades D and E. The C oocytes recovered were significantly higher compared to other grades, whereby the value of Grade C was 40.04±5.22%. In OR2 cycle, the oocyte recovered in Grades A, B and C gave significantly higher results than Grades D and E, 29.36±5.27%, 21.95±5.01% and 26.50±4.37% versus 7.69±2.40% and 8.30±3.64%, respectively. In OR3 cycle, there were no significant difference (P>0.05) for all grades of oocytes recovered.

**Table 4.12**: Number (mean ± SEM) of oocytes per ovary obtained after LOPU according grades for different OR cycle in goats

| OR cycle | No. of ovaries | No. of oocytes recovery per ovary | No. of oocytes recovered per ovary | | | | |
|---|---|---|---|---|---|---|---|
| | | | Grade A | Grade B | Grade C | Grade D | Grade E |
| OR 1 | 32 | $5.47\pm0.67^{b}$ (n=175) | $1.50\pm0.34^{by}$ (n=48) | $1.25\pm0.27^{ay}$ (n=40) | $2.41\pm0.36^{bz}$ (n=77) | $0.28\pm0.13^{ax}$ (n=9) | $0.03\pm0.03^{ax}$ (n=1) |
| OR 2 | 32 | $3.94\pm0.44^{a}$ (n=126) | $1.25\pm5.27^{bz}$ (n=40) | $0.94\pm0.22^{az}$ (n=30) | $1.25\pm0.23^{az}$ (n=40) | $0.28\pm0.08^{ay}$ (n=9) | $0.22\pm0.07^{aby}$ (n=7) |
| OR 3 | 32 | $3.09\pm0.50^{a}$ (n=99) | $0.56\pm0.13^{ayz}$ (n=18) | $0.66\pm0.20^{ayz}$ (n=21) | $1.06\pm0.31^{az}$ (n=36) | $0.41\pm0.15^{ay}$ (n=13) | $0.34\pm0.13^{by}$ (n=11) |
| Average (Total) | 32 (n=96) | $4.17\pm0.33$ (n=400) | $1.40\pm0.14^{yz}$ (n=106) | $0.95\pm0.13^{y}$ (n=91) | $1.57\pm0.19^{z}$ (n=153) | $0.32\pm0.07^{x}$ (n=31) | $0.2\pm0.05^{x}$ (n=19) |

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

**Table 4.13**: Percentage (mean ± SEM) of oocytes obtained after LOPU according grades for different OR cycle in goats

| OR cycle | No. of ovaries | No. of oocytes recovery | Percent oocytes recovery per ovary | | | | |
|---|---|---|---|---|---|---|---|
| | | | Grade A | Grade B | Grade C | Grade D | Grade E |
| OR 1 | 32 | $5.47 \pm 0.67^{b}$ (n=175) | $24.78 \pm 4.73^{ay}$ (n=48) | $21.39 \pm 4.31^{ay}$ (n=40) | $40.04 \pm 5.22^{az}$ (n=77) | $4.16 \pm 1.90^{ax}$ (n=9) | $0.26 \pm 0.26^{ax}$ (n=1) |
| OR 2 | 32 | $3.94 \pm 0.44^{a}$ (n=126) | $29.36 \pm 5.27^{az}$ (n=40) | $21.95 \pm 5.01^{az}$ (n=30) | $26.50 \pm 4.37^{az}$ (n=40) | $7.69 \pm 2.40^{ay}$ (n=9) | $8.30 \pm 3.64^{aby}$ (n=7) |
| OR 3 | 32 | $3.09 \pm 0.50^{a}$ (n=99) | $23.38 \pm 6.38^{az}$ (n=18) | $18.95 \pm 6.40^{az}$ (n=21) | $24.01 \pm 5.56^{az}$ (n=36) | $13.37 \pm 5.37^{az}$ (n=13) | $10.35 \pm 4.37^{bz}$ (n=11) |
| Average (Total) | 32 (n=96) | $4.17 \pm 0.33$ (n=400) | $25.84 \pm 3.15^{yz}$ (n=106) | $20.76 \pm 3.04^{y}$ (n=91) | $30.18 \pm 2.99^{z}$ (n=153) | $8.40 \pm 2.07^{x}$ (n=31) | $6.10 \pm 1.70^{x}$ (n=19) |

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

### 4.4.2 The Effects of IVM Duration on Maturation Rate and ICSI- Derived Embryonic Development

According to results obtained in Table 4.8, Grades D and E were shown to have low number of oocytes matured per ovary. Therefore, only oocytes from Grades A, B and C were selected for subsequent ICSI experiments. There were two different IVM durations (18-21 hours and 22- 25 hours) were assigned for the ICSI procedure. The cleavage rates for different grades of oocytes with respective IVM duration are depicted in Table 4.14. A total of 327 matured oocytes this experiment were used for this experiment, in which 157 and 170 oocytes were used for 18-21 hours and 22-25 hours IVM duration, respectively. At the 18-21 hours IVM duration, Grade A oocytes showed significantly the highest ($P<0.05$) cleavage rates for all stages of development, followed by Grades B and C. At the 22-25 hours IVM duration, the cleavage rates for all grades of oocytes were not significantly different ($P>0.05$) among the grades.

Regardless of oocyte grades, no significant differences in maturation rates and cleavage rates for all stages embryonic development between 18-21 and 22-25 hours IVM durations. In addition, it was found that the first polar body formation for 18-21 hours IVM duration was not clear and not fully visible as protrusion as those of 22-25 hours IVM duration (Figure 4.5).

**Table 4.14**: Cleavage rate (mean ± SEM) of ICSI derived embryos at different IVM duration for different grades of goat oocytes

| IVM duration | Grade of oocyte | *No. of injected oocyte | Cleavage rate (%) | | | |
|---|---|---|---|---|---|---|
| | | | 2-cell | 4-cell | 8-cell | Morula |
| 18-21 hours | A | 62 | $59.20\pm7.23^{bz}$ (n=42) | $47.41\pm7.15^{bxz}$ (n=33) | $33.05\pm7.18^{bx}$ (n=23) | $11.93\pm5.22^{bx}$ (n=6) |
| | B | 32 | $31.38\pm7.92^{az}$ (n=20) | $20.46\pm6.17^{ayz}$ (n=13) | $10.92\pm5.12^{axy}$ (n=6) | $0.00\pm0.00^{ax}$ (n=0) |
| | C | 63 | $35.95\pm7.34^{az}$ (n=34) | $24.45\pm5.89^{az}$ (n=22) | $9.28\pm3.21^{ay}$ (n=12) | $1.01\pm0.71^{ay}$ (n=2) |
| | Average (Total) | 78.5 (n=157) | $42.17\pm4.48^{z}$ (n=96) | $30.78\pm3.88^{y}$ (n=68) | $17.75\pm3.30^{x}$ (n=41) | $4.31\pm1.83^{w}$ (n=8) |
| 22-25 hours | A | 54 | $57.52\pm8.02^{az}$ (n=43) | $43.71\pm8.00^{ayz}$ (n=37) | $31.24\pm7.18^{ay}$ (n=26) | $3.98\pm1.78^{ax}$ (n=6) |
| | B | 49 | $41.11\pm6.99^{az}$ (n=29) | $31.39\pm6.79^{az}$ (n=24) | $26.94\pm6.25^{az}$ (n=20) | $1.94\pm1.37^{ay}$ (n=2) |
| | C | 67 | $46.41\pm7.03^{az}$ (n=44) | $37.06\pm6.44^{az}$ (n=37) | $13.71\pm3.26^{ay}$ (n=22) | $4.32\pm2.05^{ay}$ (n=7) |
| | Average (Total) | 85 (n=170) | $48.34\pm4.26^{z}$ (n=116) | $37.39\pm4.09^{y}$ (n=98) | $23.96\pm3.89^{x}$ (n=68) | $3.41\pm1.01^{w}$ (n=15) |

\* Number of injected oocyte was based on MII stage oocyte that used for ICSI, in respectively grade
[ab] Mean values within a column with different superscripts were significantly different (P<0.05)
[wxyz] Mean value within row with different superscripts were significantly different (P<0.05)

**Table 4.15**: Cleavage rate (mean ± SEM) of ICSI derived embryos at different IVM duration regardless grade of goat oocytes

| IVM duration | *No. of injected oocytes | *Maturation rate | Cleavage rate (%) | | | |
|---|---|---|---|---|---|---|
| | | | 2-cells | 4-cells | 8-cells | Morula |
| 18-21 hours | 157 | $97.34 \pm 7.86^{a}$ (n=157/169) | $42.17 \pm 4.48^{az}$ (n=96) | $30.78 \pm 3.88^{ay}$ (n=68) | $17.75 \pm 3.30^{ax}$ (n=41) | $4.31 \pm 1.83^{aw}$ (n=8) |
| 22-25 hours | 170 | $89.01 \pm 2.41^{a}$ (n=170/195) | $48.34 \pm 4.26^{az}$ (n=116) | $37.39 \pm 4.09^{ay}$ (n=98) | $23.96 \pm 3.89^{ax}$ (n=68) | $3.41 \pm 1.01^{aw}$ (n=15) |
| Average (Total) | 163.5 (n=327) | $93.10 \pm 4.06$ (n=326/364) | $45.31 \pm 3.09^{z}$ (n=212) | $34.14 \pm 2.83^{y}$ (n=166) | $20.91 \pm 2.40^{x}$ (n=109) | $3.85 \pm 1.03^{w}$ (n=23) |

\* Number of injected oocytes was based on oocytes used for ICSI
\*\* (n=157/169): 156= Number of matured oocytes; 169= Number of oocytes recovered
$^{ab}$ Mean value within a column with different superscripts were significantly different (P<0.05)
$^{wxyz}$ Mean value within row with different superscripts were significantly different (P<0.05)
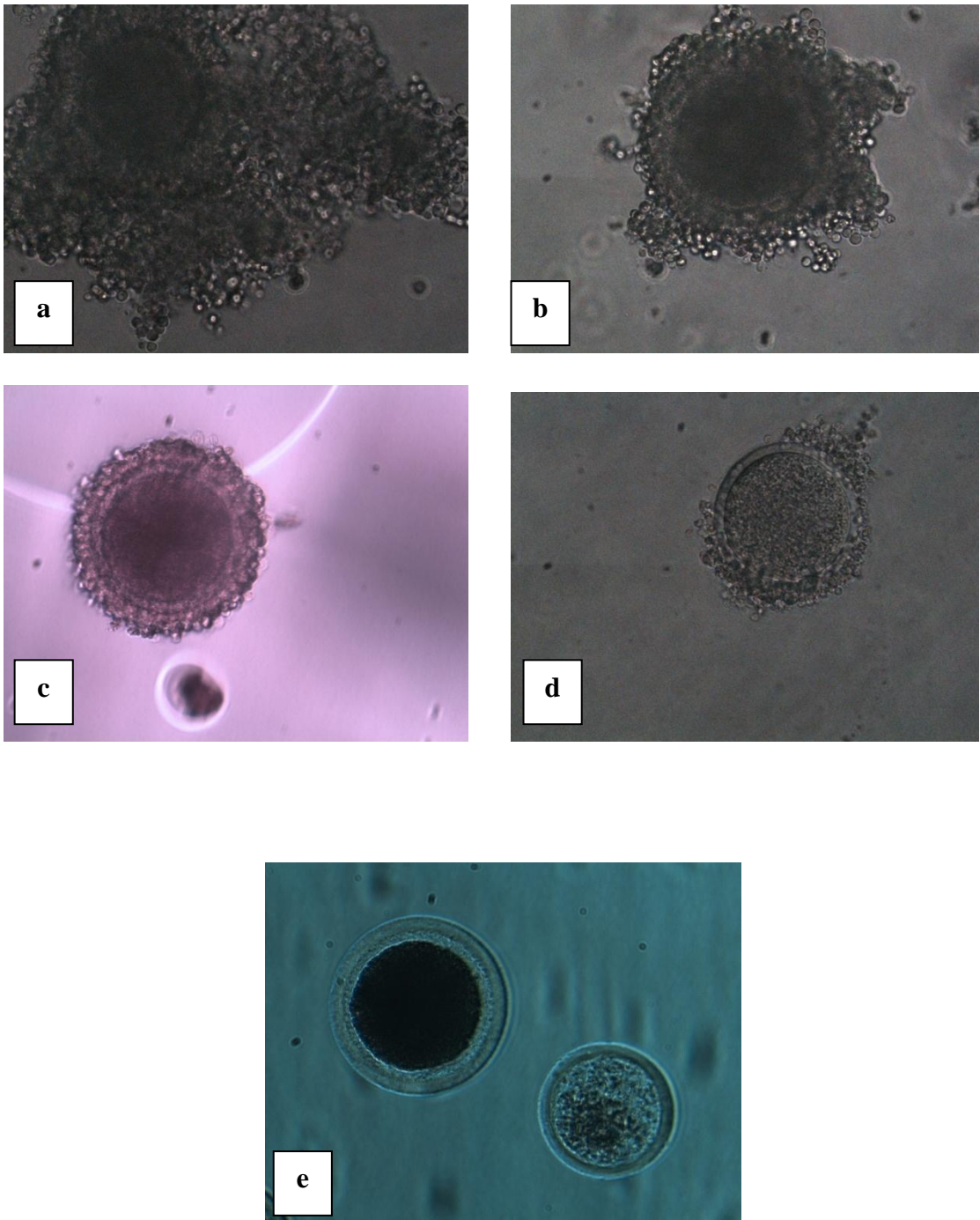
**Figure 4.4**: Morphology of different grade of goat oocytes. (a) Grade A, (b) Grade B, (c) Grade C, (d) Grade D and (e) Grade E.
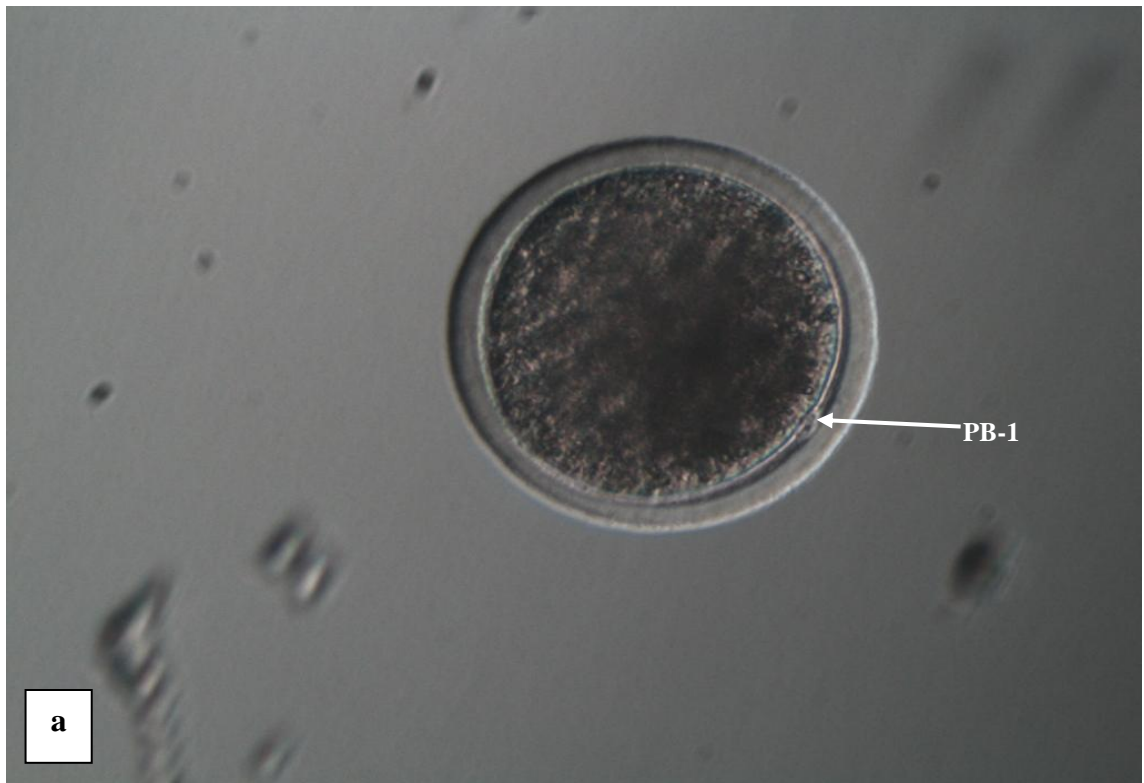
**Figure 4.5**: Morphological of matured goat oocyte with presents of first polar body. (a) at IVM duration of 18-21 hours and (b) at IVM duration of 22-25 hours.
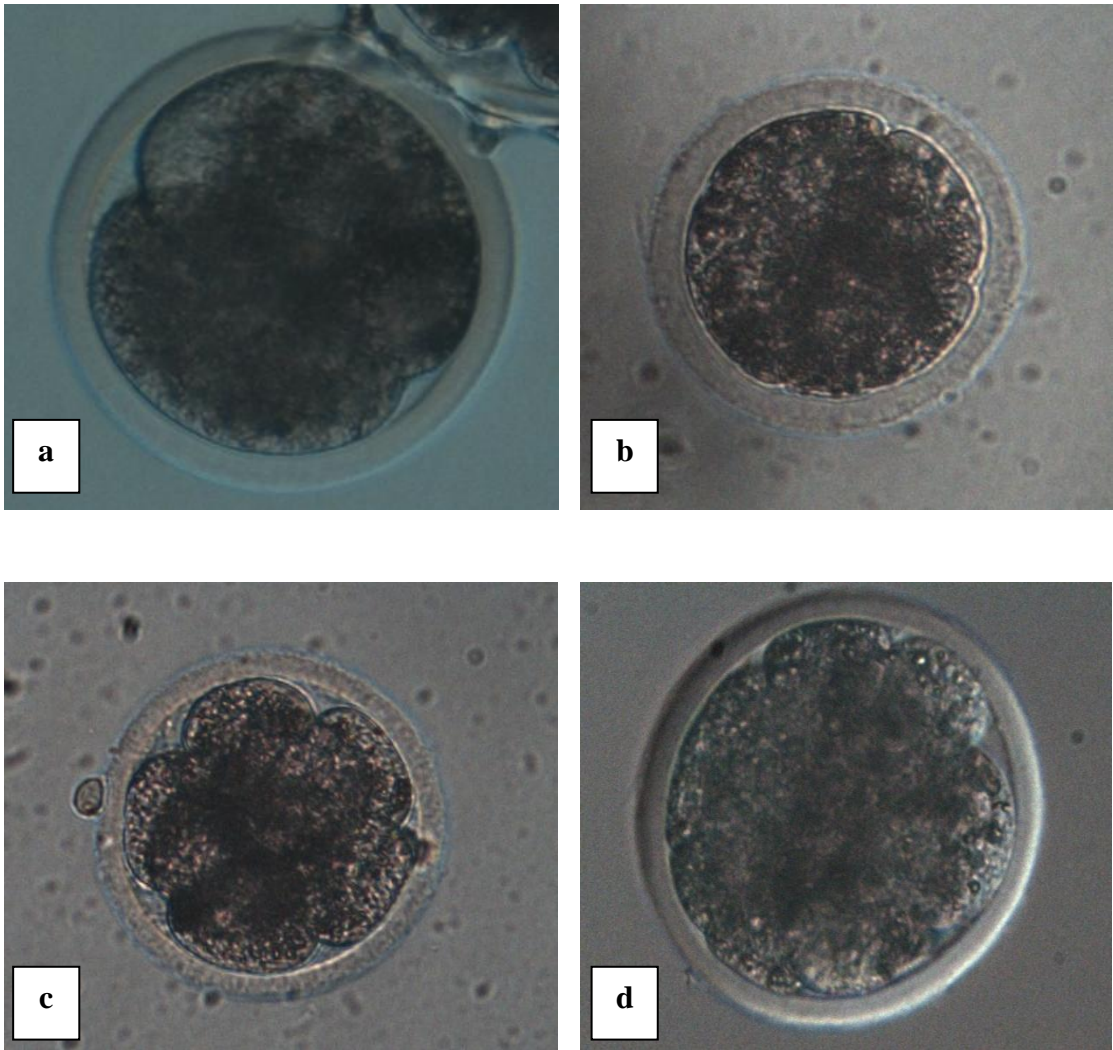
**Figure 4.6**: Morphological of goat embryo development. (a) 2-cell embryo, (b) 4-cell embryo, (c) 8 -ell embryo and (d) morula.
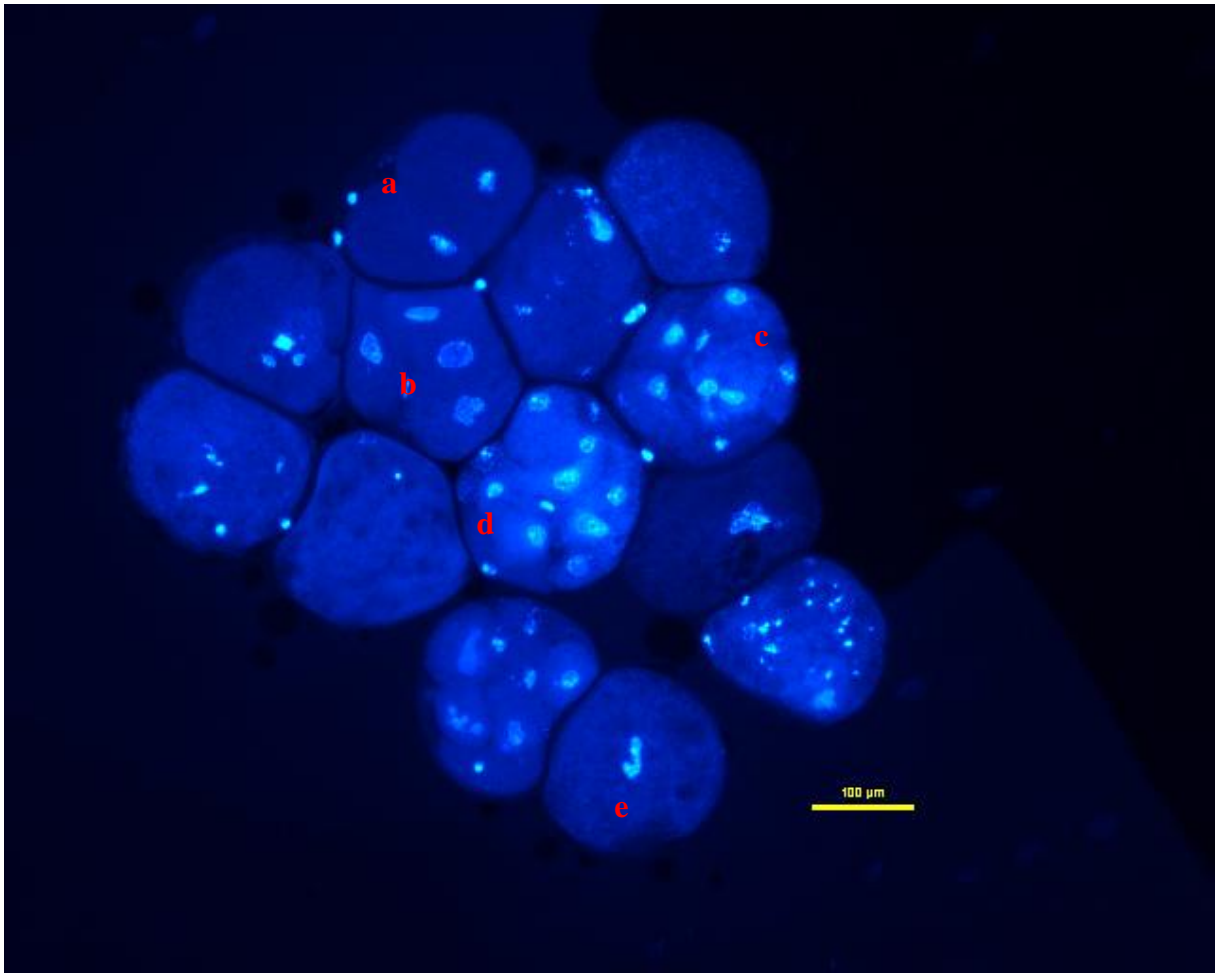
**Figure 4.7**: Hoechst staining. (a) 2-cell embryo, (b) 4-cell embryo, (c) 8-cell embryo, (d) morula and (e) unfertilised oocyte.

**Chapter 5**

**5.0 DISCUSSION**

# Chapter 5

## 5.0 DISCUSSION

## 5.1 EFFECT OF SPERM FACTORS ON ICSI PERFORMANCE IN MICE (EXPERIMENT 1)

Before beginning the ICSI experiment, the baseline data of oocytes showed that 73.16% of oocytes were in MII stage (i.e. presence of polar body), 16.67% absence of polar body and 10.14% dysmorphic form (i.e. fragmented, disintegrated or degenerated). Only the MII stages of oocytes were used in ICSI experiment.

ICSI technique was mainly used to overcome motility and morphological deficiencies that may occur during traditional fertilisation (Goosens *et al.,* 2003). The fertilisation processes such as capacitation, acrosome reaction and membrane fusion may bypass through ICSI technique. Chromatin remodeling will happen once the sperm nucleus undergoes several structural changes in ooplasmn. During this moment, the perinuclear material, acrosome and cell membrane were also together entering the ooplasmn (Ajduk *et al.,* 2006). However, the chromatin remodeling might be interfered by perinuclear material, acrosome and cell membrane, even though these components can be disintegrated inside the ooplasmn (Ramalho-Santos *et al.,* 2000; Katayama *et al.,* 2002; Sutovsky *et al.,* 2003). Therefore, it has been suggested to have a full capacitation state before injecting the sperm for the occurrence of acrosome reactions in the ooplasmn (Sathananthan *et al.,* 1997).

Heparin is a highly sulfated glycoaminoglycans that become natural inducer of sperm capacitation in cattles (Parish *et al*., 1989; Leclerc *et al.,* 1992) and goats (Cox *et al.,* 1994; Cognie *et al.,* 1995). Heparin can induce the capacitation by reducing the intracellular calcium-mediator calmodulin (CaM) concentration from sperm cells. This

may reduce the $Ca^{2+}$ pump activity and resulting increased back the intracellular $Ca^{2+}$ and activate the $Ca^{2+}$ dependent enzymes and subsequently bring the event of sperm capacitation. Theophylline is derived from methylxanthine has been used in human (Loughlin *et al.,* 1992), mouse (Fisher *et al.,* 1975), hamster (Cornwall *et al.,* 1986), pig (Yoshioko *et al.,* 2003) and cattle (Takahashi *et al.,* 1993) for sperm capacitation inducer. Theophylline can enhance the sperm motility by raising cyclic AMP levels and produce abundant energy in sperm (Cornwall *et al.,* 1986; Kajihara *et al.,* 1990).

Both sperm capacitation chemicals (Heparin and Theophylline) were used as treatments for mouse sperm capacitation studies. The results have shown that Heparin gave significantly higher (P<0.05) cleavage rates than Theophylline group at 2- and 4-cell stages. This shows that Heparin gave better results as sperm capacitation chemicals at early stage of embryonic development compared to Theophylline. The later stages of embryonic development (8-cell and morula stages) were found to be not significantly different (P>0.05) among the two chemicals. It has been suggested that Heparin has the ability to speed up the cleavage rate compared to Theophylline at early stage of embryonic development in mice. Perhaps, it was reported that the developmental arrest at 5- to 8- cell stage on Day 3 and with a decrease in blastocyst formation on Day 5 (Miller and Smith, 2001). They reported that there were associations between chemical activation with aberrant zygotic gene activation (in late paternal effect factor). This was suggested as to why the developmental rate of Heparin treated sperm gave good performance during early stage of development and not later stages of development.

In a study by Kawakami *et al*. (1999), they showed that the percentage of motile sperm and hyperactivated sperm were increased by addition of Heparin and Theophylline. However, Theophylline gave a higher percentage of motile and hyperactivated sperm compared to Heparin. Both chemicals did not give effect on the percentage of acrosome reaction.

Based on results obtained by Kawakami *et al.* (1999), the acrosome reaction rates were not necessarily correlated with the percent of motile and hyperactivated sperm. The motile and hyperactivated sperm are indicators of sperm capacitation. Capacitation is generally defined as a modification of the sperm surface that involves that mobilisation and/or removal of certain surface components from plasma membrane such as glycoproteins, decapacitation factor, acrosome-stabilising factor and acrosin inhibitor (Fraser, 1984 and 2008; Morales *et al.,* 2003). The functional changes in sperm are preparing them to undergo acrosome reaction, promote the motility patterns and subsequently fertilised with the matured oocytes. During normal fertilisation, sperm nucleus decondensation and oocytes activation is occurred within ooplasmn and sperm sub-membrane components (Perry *et al*., 1999). In ICSI procedure, the sperm membrane breakdown by sperm penetration into oolemma was different with IVF and normal fertilisation. The sperm capacitation before ICSI is not sufficient compare to IVF and normal fertilisation because the sperm also capacitate when naturally passing through several natural barriers such as cumulus cells and zona pellucida. Thus, the insufficient of sperm capacitation process could contribute the persistence of sperm acrosome or its substructures and these might bring the insufficient sperm decondensation and prevent the importance of maternal nuclear proteins (Hewitson *et al.,* 2000).

In order to increase sperm membrane permeabilisation, acrosome reaction and sperm head decondensation following ICSI, there were several treatments have been used including freezing and thawing (Perreault *et al.,* 1988), crushing the sperm tail with a micropipette (Keskintepe *et al.,* 1997) or laser shot (Montag *et al.,* 2000), removing the acrosome and tail by sonification (Keefer, 1989; Goto, 1993) and treated by various chemicals such as heparin (Izquierdo *et al.,* 1998; Wei and Fukui, 1999, Kawakami, *et al.,* 1999; Katska-Ksiazkiewicz *et al.,* 2004), theophylline (Kawakami *et*

*al.,* 1999; Ebner *et al.,* 2011), calcium ionophore (Chen and Seidel, 1997; Wei and Fukui, 1999).

These studies also involved comparison between sperm capacitation chemical treatment (Heparin and Theophylline) and sperm movement (Rapid and Slow). Rapid sperm was score by rapid (motile) movement and the Slow sperm was score by locally motile movement (Giwercman *et al.,* 2003). In this study, a normal morphology of sperm was selected to perform ICSI. This is to reduce any risk of defects effect from sperm abnormalities. Even though there has been reported that sperm morphological abnormality might not necessarily affecting the fertilisation and pregnancy rates after ICSI as long as the sperm is matured (from ejaculated sperm) (Mansour *et al.,* 1995; Nagy *et al.,* 1995), the precaution step through selecting normal morphology sperm has been taken.

According to the results, even though insignificance (P>0.05), generally the Heparin group gave higher cleavage rates than Theophylline group for all the developmental stages regardless of the sperm movement. Similarly, the Rapid group gave higher cleavage rates than Slow group regardless of the sperm capacitation chemical treatment (P>0.05). As for sperm movement, when disregard sperm capacitation chemical treatment, there was no significant difference (P>0.05) in the embryonic development of cleavage rate at all stages of development (2-cell to morula stage). It can be inferred that the sperm movement with the normal morphology has same ability to perform ICSI-derived embryo. In addition, the sperm already capacitated with the sperm capacitation chemicals, either Heparin or Theophylline. It has been reported that the immotile sperm can show some motility after 2 to 3 hours of incubation (Nijs *et al.,* 1996) which is in contrast to the results of presence study whereby sperm were incubated in the sperm capacitation chemicals treatment medium for 1 hour, similar to that of Kato and Nagao (2009). It was found that acrosome

reaction needs only 1 hour of incubation with Heparin or Theophylline. These also might lead both of sperm movement (Rapid and Slow) criteria obtained same opportunity to fertilise the matured oocyte. The slow sperm movement is not affecting the results of embryonic development which may be due to only the structure of the cell was defective but the nuclei were still alive for nuclear remodeling or decondensation to activate the oocytes for fertilisation and further embryonic development (Goto, 1997).

## 5.2    EFFECT OF OOCYTE FACTORS ON ICSI PERFORMANCE   IN MICE (EXPERIMENT 2)

It was reported that the quality of oocytes produced by superovulation was affected by several factors such as dose and timing of gonadotrophin (Edgar *et al.,* 1987; Vergara *et al.,* 1997), weight (Hogan *et al.,* 1986), age (Hogan *et al.,* 1986; Ozgunen *et al.,* 2001) and strain of mice (Goh, 2008;   Ainul Bahiyah, 2010). In this study, the timing of gonadotrophin becomes factor of interest. Most of previous studies, researchers chose the range of 13-15 hours of interval duration of oocytes collection with post-hCG administration such as at 13 hours (Hillier *et al.,* 1985), 14 hours (Martin-Coello *et al.,* 2008), 14.5 hours (Vergara *et al.,* 1997).  This duration were mainly chosen because generally, the mouse ovulation starts after 11 to 12 hours post-hCG administration (Hogan *et al.,* 1986).

In this experiment, two groups of post-hCG administration durations (13-15 hours  and 16-18 hours) were evaluated to determine their effects on the total numbers of oocytes per ovary which were $13.91\pm0.61$   and $13.57\pm0.54$, respectively ($P>0.05$). There was no difference in the number of oocytes retrieved between the two post-hCG administration durations. However, percentage of polar body present was higher in 13-15 hours group ($75.61\pm1.64\%$) compared with 16-18 hours ($67.37\pm1.50\%$). Conversely, the percent for polar body absent was significantly higher at 16-18 hours post-hCG administration duration compared with 13-15 hours duration with the values of $12.50\pm1.55\%$ versus $21.82\pm1.49\%$, respectively. This might be due to the aging of oocytes (post-maturity) at 16-18 hours. Matured oocytes (MII stage) should be fertilised within the window of optimal fertilisation, which may be different for  different species such as in mice (8-12 hours), rats (12-14 hours), rhesus monkeys and humans (<24 hours) (Austin, 1974). The event of ovulation (first polar body or MII stage) and

fertilisation is optimal at 11-14 hours, while, the extrusion of second polar body is occured at 17-23 hours of post-hCG administration (Hogan *et al.,* 1986). In addition, if the oocytes are not fertilised within the fertilisation window, it will undergo a time-dependent deterioration in quality and this process is known as oocyte aging (Miao *et al.,* 2009). The polar body absence was found in 16-18 hours post-hCG administration duration might be due to the oocytes started having physical changes, for examples, perivetelline space larger and the first polar body either started to degenerate or deviated from the MII spindle which was undergoing the process of aging (Miao *et al.,* 2004).

There was a few percentage of dysmorphic oocytes at both duration groups. Both of duration groups (13-15 hours and 16-18 hours) show no significant difference in percentage of dysmorphic oocytes. The presence of dysmorphic oocytes may be due to the exposure with hyaluronidase during cumulus cells removal. Hyaluronidase can activate the mouse oocytes (Kaufman, 1983) if inappropiate or higher concentration. However, in ICSI, the hyaluronidase concentration was reduced to 80 IU/ ml to avoid activation but for this case, the exposure time of oocytes to this chemical might initiate the oocytes to activate and becomes parthenotes or fragmented. Other than that, the DNA fragmentation also could occur in the first polar body and oocyte cytoplasmn, indicating they were undergoing apoptotic changes (Fujino *et al.,* 1996). Apoptosis is a morphological process that cause by 'cellular suicide' programme. Degenerating oocytes with cytoplasmic fragmentation were frequently found at oocyte retrieval (Wyllie, 1981). However, the reason why the cytoplasmic fragmentation occurs is still unclear (Fujino *et al.* 1996)

In this study, only the oocytes with presence of polar body were used for ICSI experiments. the absence of polar body and dysmorphismn oocytes were discarded in order to reduce the risk of low quality embryonic development. This was because good quality oocytes would affect subsequent embryonic development after fertilisation

(Wang and Sun, 2007). The embryonic development cleavage rates at 2-, 4-, 8-cell and morula stages were significant higher (P<0.05) at 13-15 hours compared to 16-18 hours duration (67.95±1.06% versus 57.02±1.33%, 53.51±1.13% versus 41.90±1.98%, 39.40±1.01% versus 22.21±1.62%, and 14.83±1.09% versus 9.77±1.02%, respectively). These results clearly showed that the post-hCG administration duration at 13-15 hours was the better duration for oocytes retrieval and embryonic development through ICSI procedure. Oocytes aging could have occurred at 16-18 hours duration. The oocyte aging before fertilisation may cause low embryonic development rates and also early pregnancy failure in several mammalian species (Wilcox *et al.,* 1998). Thus, the use of aged oocyte should be minimised or avoided in order to obtain good embryonic development and pregnancy.

## 5.3    EFFECTS OF SPERM FACTORS ON ICSI PERFORMANCE IN GOAT (EXPERIMENT 3)

This experiment was carried out to study the effect of two sperm factors (sperm capacitation chemicals treatment and sperm movement) on ICSI derived embryonic developmental competency. In order to optimise the embryonic developmental rate for these experiments, the quality of oocytes obtained from LOPU procedure were identified and only good quality of oocytes grades were selected for this study. Therefore, before start the ICSI studies, the baseline data of oocytes were graded based on COCs layer criterion known as Grades A, B, C, D, and E, similarly done by Rahman (2008) and Kong (2010). Only Grades A, B and C oocytes were used compared to Grades D and E for ICSI experiment. The selection was done based on the maturation rate performance, where the maturation rates of Grades A, B, C were 96.35±2.11%, 87.59±4.69% and 84.40±2.78%, while for the Grades D and E were 51.09±9.59% and 4.17±4.17%, respectively.

Sperm maturation process was occurred in male reproductive tract and become motile while transported in the epididymis (Yanagimachi, 1994). However, this maturation is not sufficient for fertilisation and they need to undergo the additional 'maturation' process, termed 'capacitation' to acquire the ability to penetrate oocyte and fertilised in normal fertilsation process. This sperm capacitation process is requirements for triggering sperm acrosome reaction. Sperm acrosome reaction is a process of releasing the proteolytic enzyme to enable the sperm membrane permeabilisation and penetration through the oocyte zona pellucida. A full sperm capacitation state and acrosome reaction mechanism (Sathananthan *et al.,* 1997) is not only required in normal fertilisation or IVF but also in ICSI procedure. This is because the insufficient of sperm capacitation process could contribute the persistence of sperm acrosome or its

substructures and these might bring the insufficient sperm decondensation and prevent the important of maternal nuclear proteins (Hewitson *et al.,* 2000).

In order to increase sperm capacitation, acrosome reaction and sperm head decondensation following ICSI, there were several treatments have been used including freezing and thawing (Perreault *et al.,* 1988), crushing the sperm tail with a micropipette (Keskintepe *et al.,* 1997) or laser shot (Montag *et al.,* 2000), removing the acrosome and tail by sonification (Keefer, 1989; Goto, 1993) and treated by various chemicals such as heparin (Izquierdo *et al.,* 1998; Wei and Fukui, 1999, Kawakami, *et al.,* 1999; Katska-Ksiazkiewicz *et al.,* 2004), theophylline (Kawakami *et al.,* 1999; Ebner *et al.,* 2011), calcium ionophore (Chen and Seidel, 1997; Wei and Fukui, 1999).

Heparin and Theophylline were used for sperm capcitation chemicals treatment in this experiment (this treatment also similarly conducted in Experiment 2 in mice experiment). Heparin is a highly sulfated glycoaminoglycans that become natural inducer of sperm capacitation in cattles (Parish *et al.*, 1989; Leclerc *et al.,* 1992) and goats (Cox *et al.,* 1994; Cognie *et al.,* 1995). Heparin can induce the capacitation by reducing the intracellular calcium-mediator calmodulin (CaM) concentration from sperm cells. This may reduce the $Ca^{2+}$ pump activity and resulting increased back the intracellular $Ca^{2+}$ and activate the $Ca^{2+}$ depend enzymes and subsequently bring the event of sperm capacitation. Theophylline is derived from methylxanthine have been used in human (Loughlin *et al.,* 1992), mouse (Fisher *et al.,* 1975), hamster (Cornwall *et al.,* 1986), Pig (Yoshioko *et al.,* 2003) and cattle (Takahashi *et al.,* 1993) for sperm capacitation inducer. Theophylline can enhance the sperm motility by raising cyclic AMP levels and produce abundant energy in sperm (Cornwall *et al.,* 1986; Kajihara *et al.,* 1990).

In the studies of both effect of sperm capacitation and sperm movement, generally, there were no significance different ($P > 0.05$) in Heparin group. However, in

each grades of oocytes for sperm movement (Rapid and Slow) showed different pattern of embryonic development. The slow sperm in Grade A oocytes group at 2-, 4- and 8-cell stages gave higher embryonic development cleavage rates than Rapid group. In morula, the Rapid group was higher than Slow group. Grade B group, showed higher embryonic development rate at 2-cell stage from Slow group. Meanwhile, the Rapid group showed higher embryonic development cleavage rate at 4- and 8-cell stage. The Rapid group of Grade C oocytes showed higher cleavage rates for all stages of embryonic development rates (2-, 4-, 8-cell and morula stages). When combined the grades (Grades A, B and C), only in 4-cell stage embryonic development showed higher cleavage rate for Rapid group. The rest of embryonic development cleavage rates (2-, 8-cell and morula stages) were highly showed in Slow group. In overall discussion for Heparin group, most of the early stage of embryo development (2-cell stage) was higher when using Slow sperm movement for all grades of oocytes, except for Grade C. However, there were no significantly different analyses were found when comparing this parameter (Rapid and Slow). Therefore, it still can assume that, the Slow sperm has the ability to undergo same fertilisation capability. Goto (1997) has reported that slow sperm movement is not affecting the results of embryonic development may be due to only the structure of the cell is defect but the nuclei were still alive for nuclear remodeling or decondensation to activate the oocytes for fertilisation and further embryonic development (Goto, 1997). However, there are still need more studies to support this statement because some of sperm chromatin remodeling and abnormalities should be identified to confirm the ability and effective of using Slow sperm for the successful of embryonic development and pregnancy.

In Theophylline group, generally the results showed higher rates of embryonic development in all grades using Rapid sperm movement. The Grade A oocytes group gave significantly higher when using Rapid compared to the Slow sperm movement at

2-, 4-, and 8-cell stage (with the values of 95.83±4.17% versus 63.27±9.10%, 83.33±12.60% versus 47.96±9.92% and 77.08±12.96% versus 20.15±8.52%, respectively). In Group B oocytes group, only at 2-cell stage showed significantly different when Rapid group was compared with Slow sperm movement group (84.38±5.98% versus 50.00±11.89%). The Grade C oocyte group gave no significant difference (P>0.05) for all embryonic developmental rates. The combined grades oocytes group (Grades A, B and C) also gave significant higher using Rapid than Slow sperm movement group at all developmental stage (2-, 4-, 8-cell and morula stages) (85.42±4.17% versus 54.35±6.20%, 71.39±6.49% versus 42.28±6.31%, 57.43±7.63% versus 21.63±5.34% and 10.76±5.03% versus 0.88±0.62%, respectively). According to the results, it may be concluded that in Theophylline group, the Rapid sperm movement gave better embryonic development cleavage rates at all stages.

When disregard the sperm movement, Theophylline group gave higher cleavage rates than Heparin group. However, both sperm capacitation chemicals showed no significant difference (P>0.05) in cleavage rate at all developmental stages. In agreement with Kawakami *et al.,* (1999), Theophylline gave a higher percentage of motile and hyperactivated sperm compared to Heparin. However, it was not found any significance different of acrosome reaction rate (Kawakami *et. al.* 1999). This might be one of the reason why no significance different found of embryonic developmental rates in using of both sperm capacitation chemicals treatment.

As for sperm movement, when disregard sperm capacitation chemical treatment, there was no significant difference (P>0.05) at 2-cell stage cleavage rate. However, at other stages of development (4-, 8-cell and morula stages), the Rapid group gave significantly higher (P<0.05) than Slow group, where the respective values were 56.62±4.69% versus 41.80±4.49%, 39.51±4.70% versus 24.24±4.05% and 9.21±2.85% versus 2.24±0.94%. The embryonic developmental might be delay of cleavage speed at

early stage of embryo. However, the significance findings were dominantly showed in using Rapid sperm for later stage of development. It was reported that, early paternal effect may cause delay of cleavage speed and contribute of poor quality of embryo development (Menezo, 2006). However, in other report, the sperm defective is not directly associated with early paternal effects (Lee *et al.,* 2009). The time of fertilisation is controlled in ICSI indicates that the early cleavage of embryos mainly influenced by some intrinsic factors within the embryo (Sakkas *et al.,* 1998). Sperm become second important factors compared to oocyte for a successful of embryonic developmental. It can be assumed that later stage of development was significantly different may due to has any intrinsic factor of oocytes were fully trigger for further development.

According to results obtained, the ability of both sperm capacitation chemicals treatment has same ability to fertilise and develop embryos. The Rapid sperm movement was clearly gave better choice for ICSI procedure, even the slow sperm also has the ability to fertilise and develop embryo.

## 5.4    EFFECTS OF OOCYTE FACTORS ON ICSI PERFORMANCE IN

## GOAT (EXPERIMENT 4)

### 5.4.1    The Effect of LOPU Cycle on Yield of Oocytes

Laparoscopic ovum pick-up (LOPU) is an alternative procedure in order to provide consistent quality of oocytes for IVP programme. LOPU is minimally invasive and faster post-operative recovery compared to the standard laparotomy (Koeman *et al.,* 2003; Tibary *et al.,* 2005).  LOPU is reported to be an efficient method for oocytes provider (Baldassarre *et al*., 2002; Pierson *et al*., 2004, Abdullah *et al.,* 2007, Rahman *et al.,* 2008a and 2008b; Kong, 2010). LOPU allows repetition of the laparoscopic procedure more frequently and more times during the reproductive life of a valuable female (Baldassarre *et al*., 2007), ability of producing embryos and offspring from animal which not capable to reproduce by multiple ovulation-embryo transfer (MOET) and artificial insemination (AI), including prepubertal (Baldassarre and Karatzas, 2004) and aged goats (Baldassarre *et al*., 2007). The LOPU technique avoids several causes of the poor results related with superovulation, such as poor ovulation rate, early regression of corpus luteum (CL) and poor fertilisation (Baldassarre and Karatzas, 2004).

LOPU procedure can be conducted for hormonal stimulated or unstimulated animals. In the present experiment, the 16 goats were used 3 times and stimulated with 60 hours post-PMSG + hCG. Our previous study had shown that the 60 or 72 hours of post-FSH + hCG gave optimised yield of good quality of goats oocytes for IVM and embryo production (Abdullah *et al.,* 2008).  The repeated oocyte recovery (OR) surgeries was conducted at 3 months interval.

Generally, the number of oocytes recovered decreased when the cycle increased (OR1, OR2 and OR3) with the values of 5.47±0.67, 3.94±0.44 and 3.09±0.50 respectively. The OR1 retrieved number was significantly higher (P<0.05) than OR2 and OR3. The total number of oocytes retrieved were decreased may be due to the repeated follicular punctured during the procedure might alter endocrine profiles slightly and subsequently cause minor morphological changes in ovaries (Petyim *et al.,* 2001). When analysed the each grades of oocytes, Grades B and D gave no significance difference (P>0.05) between the OR cycle. In Grade A, the number of oocytes obtained in OR1 and OR2 was significantly higher than OR3, which were 1.50±0.34 and 1.25±5.27 versus 0.56±0.13. In Grades C, the number of oocytes obtained in OR1 was significantly higher (P<0.05) than OR2 or OR 3, which were 2.41±0.36 versus 1.25±0.23 and 1.06±0.31, respectively. The Grade E gave higher significantly difference (P<0.05) in OR2 and OR3 compared to OR1 (0.22±0.07 and 0.34±0.13 versus 0.03±0.03, respectively). When analysed by oocytes recovery rate, Grades A, B, C and D were not significantly different (P>0.05) between the OR cycles (OR1, OR2 and OR3). Except in Grade E, the OR3 shows significantly higher oocytes recovery rate compared to OR1 (10.35±4.37% versus 0.26±0.26%, respectively). However, OR2 recovery rate was not significantly different (P>0.05) from OR1 or OR3. This is in agreement with Pierson *et al*., (2004); Rahman *et al*., (2007). According to their findings, LOPU can be repeated up to 5 times in the goats at different intervals and in different seasons with little or no important change in overall response. Therefore, LOPU was capable to be repeated more than 3 times.

When analysed the percent oocyte recovery for each grade per OR cycle, generally Grade C oocytes was higher in LOPU procedure. In OR1, the recovery rates of Grades D and E oocytes were not significantly different (P>0.05) (4.16±1.90% and 0.26±0.26%, respectively). Grades A and B (24.78±4.73% and 21.39±4.31,

respectively) were not significantly different to each other (P>0.05), but they were significantly higher (P<0.05) than Grades D and E. The C oocyte recovered was significantly higher compared to other grades, whereby the value of Grade C was 40.04±5.22%. In OR2 cycle, the oocyte recovered in Grades A, B and C gave significantly higher results than Grades D and E, 29.36±5.27%, 21.95±5.01% and 26.50±4.37% versus 7.69±2.40% and 8.30±3.64%, respectively. In OR3 cycle, there were no significant difference (P>0.05) for all grades of oocytes recovered. The Grade C oocytes were highly obtained followed by Grade A, B, D and finally, lowest yield was Grade E oocyte. This may be due to the oocytes were half 'cook' or matured during the time of ovum pick-up (OPU). It has been known that the cumulus cells could be easily loosened when going matured. In addition, the needle size and vacuum pressure during LOPU procedure could lead the loosened of cumulus cells. Therefore, the preservation of cumulus vestment in recovered oocytes was good (Baldassarre *et al.,* 1994 and 2007). The Grade E oocyte was increased by the increasing of OR cycle. This probably due to the effects of hormonal treatments repetition used for ovulation induction that generally followed by decreasing fertility in goats. It also has linked to the presence of anti-hormone antibodies (i.e. eCG antibodies or PMSG + hCG antibodies) that might be showed immune response to previous treatments (Roy *et al.,* 1999; Drion *et al.,* 2001). It can be concluded that Grades A, B and C oocytes could be highly significantly retrieved in LOPU that could be used for ICSI experiments.

**5.4.2   The Effects of IVM Duration on Maturation Rate and ICSI- Derived**

**Embryonic Development**

Similar with Experiment 3, the Grades D and E have low number of oocytes matured per ovary. Therefore, only oocytes from Grades A, B and C were selected for subsequent ICSI experiments. Again, it believed that oocyte quality used will influence the *in vitro* embryo production in goats (Katska-Ksiazkiewicz *et al.,* 2007)

In previous studies in the same laboratory, Rahman (2008a) was conducting goats ICSI experiments using 27 hours of IVM duration with and without considering the oocyte quality. This was similarly studied with previous researcher (Wang *et al.,* 2003; Jimenez-Macedo *et al.*, 2005, 2006, and 2007). While, Kong (2010) reported that the IVM duration at 22-25 hours gave better maturation rate compared at 26-29 hours (71.6% versus 38.7%, respectively). In other report showed maturation rate at 16-24 hours gave 21.0% to 72.0% after the initiation of maturation process (Cognie *et al.,* 2003). In the present experiment, two different IVM durations (18-21 hours and 22- 25 hours) were assigned for the ICSI procedure. It was similarly carried out by Kong (2010). The range of IVM duration at 18-21 hours was chosen because we hypothesised that the hormonal treated of donor goats (60 hours of post-PMSG + hCG) from LOPU procedure could give good quality of oocytes and subsequently enable to fertilised and developed. At this range of IVM duration it may has probability the oocytes was matured.  According to the results, even insignificance (P>0.05) of maturation rates in both IVM duration, generally at 18-21 hours IVM durations was higher than 22-25 hours (97.34±7.86% versus 89.01±2.41%, respectively). This was inferred that the IVM duration at 18-21 hours has same ability to matured oocytes with at 22-25 hours. Moreover, in presence study it found that the maturation rates were improved than previous study by Kong (2010) at the 22-25 hours, where the maturation rates were 89.0

% versus 71.6%, respectively. In the IVM duration at 18-21 hours, Grade A oocytes showed significantly the highest (P<0.05) cleavage rates for all stages of development, followed by Grades B and C. This has shown that the oocyte quality via the role cumulus cells can promote necessary maturation for embryonic development. Previous studies obtained better maturation rate when culture better quality of oocytes with at least 4 complete layers (Jimenez-Macedo *et al.,* 2005, 2006 and 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 cells.

At the 22-25 hours IVM duration, the cleavage rates for all grades of oocytes were not significantly different (P>0.05) among the grades. The cleavage rates at 2-cell stage for Grades A and B was generally found to be lowed compared than Kong (2010) (57.5% and 41.1% versus 64.5% and 64.5%, respectively). However, for Grade C, the cleavage rate at 2-cell stage was slightly higher in presence study compared than in Kong (2010) (46.4% versus 44.6%). These differences might be due to the factors of number of samples used. When comparing with studied by Rahman (2008a) who used FSH + hCG treated donor goats, the cleavage rates of Grades A, B and C (84.0%, 66.7% and 57.8%, respectively) were higher compared to presence studies. The difference between these experiments may be due to the types of hormone used that bring more effective by using FSH compared to PMSG (Armstrong *et al.,* 1983, Nuti *et al.,* 1987). In addition, the PMSG was found to influence to disrupt normal fertilisation (Moor *et al.,* 1985) and also decrease the fertilisation, embryonic development and pre-implantation stage of development (Miller and Armstrong, 1981; Evans and Armstrong, 1983).

Regardless of oocyte grades, no significant differences in maturation rates and cleavage rates for all stages embryonic development between 18-21 and 22-25 hours IVM durations. These indicating that the IVM duration at 18-21 hours have same

fertilisation and embryonic developmental ability with 22-25 hours of IVM duration. Even though, there was insignificance in both IVM duration embryonic developmental, the 22-25 hours IVM duration were higher than at 18-21 hours in all embryonic development stages (2-cell to morula stages). It well known that the oocytes maturation were involved of nuclear and cytoplasmic maturity in order to obtain the success rate of ICSI (Kahraman *et al.,* 2000).During conducting the experiments, the first polar body formation morphology for 18-21 hours IVM duration was not clear and not fully visible as protrusion as those of 22-25 hours IVM duration. This probably because the matured oocytes undergo 18-21 hours of IVM duration were 'young' or just early matured. However, this suggestion was still unclear and need more studies. In addition, the extrusion of first polar body was occurred before the oocyte able to become fertilised, that happened before the cytoplasm was completely maturated, then the resumption if meiosis might fail or be incomplete (Kubiak, 1989). It has been suggested that the formation of first polar body may reflect an asynchrony between nuclear and cytoplasmic maturation (Eichcnlaub-Riuer *et al.,* 1995) that would effects the ability of cells to support pronuclear formation after ICSI. The maturation of nuclear is easier and faster than cytoplasmn (Kubiak, 1989; Krisher, 2004). The degree of cytoplasmn maturation will determines the developmental competence of IVM oocyte to undergo further embryonic development (Combelles *et al.,* 2002; Inoue *et al.,* 2008). If the oocytes were inadequate duration of IVM culture, the oocytes development can be impaired. The kinetics of immature and IVM oocytes were demonstrated during meiotic maturation and has a close relationship with spindle assembly. In human, the spindle configuration anomalies were related with reproduction failure (Szczygriet and Kurprisz, 2001). However, the spindle assembly and maturation duration has yet to be performed.

However, if the oocytes were fast *in vitro* matured, extruded the first polar body early and has been completing the nuclear and cytoplasm maturation during IVM, the possibility to developing blastocyst stage is more clear (Dominko and First, 1992 and 1997). The prolongation of metaphase II arrest before ICSI was lead to reduce the successful fertilisation and embryo development (First *et al.,* 1998).

Therefore, according to these studies, it can be inferred that, the IVM duration for goats oocytes at 18-21 hours has same ability of embryonic developmental with at 22-25 hours. The Grade A oocyte was obviously gave higher rate of maturation and better embryonic development compared. However, the Grades B and C also could be used for IVP programme (IVM and ICSI), in order to obtain good embryonic development.

## 5.5    GENERAL DISCUSSION

The sperm and oocyte factors related with conventional ICSI technique are being focused in the present study. From the findings of this study, it is feasible that ICSI can be used routinely as an alternative IVP procedure to produce embryonic development in mouse and goat, provided that optimisation of this procedure will be studied in detail in the future. Several factors that influence the outcomes of this study have been discussed in the previous section. It is well-known that ICSI technique was developed and applied as one of the ART to overcome the male infertility problems such as motility and abnormalities in human (Palermo *et al.,* 1992). Similar attempts were made for mice (Kimura and Yanagimachi, 1995) and goats (Wang *et al*., 2003). Even though the sperm factors were not a major problem in ICSI, it can influence the embryonic development rate. It has been suggested that sperm need to be capacitated before carrying out the ICSI procedure in order to produce viable embryos (Sathananthan *et al.,* 1997). In

addition, oocyte quality can influence the success of embryonic development (Wang and Sun, 2007). The aging factor by pre- and post-maturation of oocytes was studied in this research. From the findings of this study, embryonic development can be achieved from Heparin-sperm capacitation treatment, using any sperm movement (Rapid or Slow) and 13 to 15 hours of post-hCG administration oocytes. For goat study, LOPU is good procedure to provide consistent good oocytes quality (Grades A, B and C) and can be repeated using the same donor up to 3 times. Rapid sperm movement is a better choice for ICSI-embryonic development. Both sperm capacitation chemical treatments (Heparin and Theophyline) have the same potential to produce ICSI-embryonic development.

### 5.5.1   Influence of Sperm and Oocytes Factors on *In Vitro* Production

The requirement of sperm and oocyte is different for different types of *in vitro* production (IVP). According to present study, most of the experiments were carried out using intracytoplasmic sperm injection (ICSI) as a device of *in vitro* production. The success of embryo development in IVP is influenced by sperm and oocyte factors (Xia, 1997; Dumoulin *et al.,* 2000; Ebner *et al.,* 2000; Tesarik *et al.,* 2002). Previous reports claimed that sperm factors was not become a major detrimental effect on ICSI-derived embryo development as long the sperm nucleus has intact genetic integrity (Yanagimachi, 2005). It has been suggested that sperm factors is responsible for initiating the oocyte activation (William, 2002). For this case, an appropriate condition and complete sperm capacitation before ICSI is important in order to increase sperm permeabilisation, acrosome reaction and sperm head decondensation for fertilisation process. Various treatments could be used but the most practically sperm capacitation treatment is by chemical treatment. In this research, a single treatment of Heparin and

Theophylline had been compared and studied for this purpose. There was no previous studies did the same comparable in mouse and goat sperm, only reported in dog (Kawakami *et al.,* 1999). It has been suggested that the sperm nuclei stability were different in species-species, probably due to the different disulfide bonding in sperm nuclei (Perreault *et al.,* 1988). Therefore the pattern of results for mouse and goat study gave different finding in sperm capacitation chemicals treatments. The sperm movement (motility) reflects sperm vitality. Normally, the locally motile and immotile sperm were considered poor sperm and not being selected in normal fertilisation or IVF. However, in ICSI, this two sperm criteria have the potential to fertilise the oocytes might be due to sperm nuclei is still alive and just die as a cells (Goto *et al.,* 1997). However, in other study, the normal morphology and motility may carry hidden defects that can interfere with the embryo development. Thus, especially in human, only the good sperm will be used for ICSI. Unfortunately, in this experiment, there was no study conducted on mechanism at molecular and ultrastructural level in order to identify and know the hidden sperm defect factor in using of particular sperm capacitation chemical and sperm movement criteria.

The oocyte factors with special reference to oocyte quality and aging have been studied in this research. The interesting part of this study is the mouse and goat experiments showed that the duration of post-hCG (mouse study) and IVM (goat study) oocyte aging by post-maturity could affect the yield of embryo and the oocyte should undergo equal nuclear and cytoplasmic maturation before performing ICSI. In mouse experiment, the same age of mouse oocytes retrieved at 13-15 hours of post-hCG administration duration gave good embryonic development. In addition this range of duration is within the optimal fertilisation window for mouse. In goat experiment, the good quality oocytes (Grades A, B and C) with full cumulus cells vestment obviously obtained from LOPU-hormonal treated donor (PMSG + hCG). The lower Grades of

oocytes (Grades D and E) were less obtained but will increase with the repeated number of LOPU cycle. The first LOPU cycle (OR1) gave higher yield of oocytes and then it will decrease for the next following LOPU cycle (OR2 and OR3). Based on the findings, the first LOPU cycle was significantly higher ($P<0.05$), while the second and third LOPU cycle were not significantly different from each other. However, OR2 and OR3 are still recommended to be used for the oocytes sources. Therefore, it is practical assume that LOPU procedure can be repeated up to 3 times in the same donor. According to these results there might be some adverse effect of hormone used for the next LOPU cycle. Moreover, the interval between the next cycles, the good animal physiological preparation and post-LOPU treatment effects should be optimised for obtaining good and high yield of oocytes. However, some of the problems have to be solved such as the tissue adhesion cause by surgery can be reduced by the application of warm saline (~38.5⁰C), the animal were sedated and anaesthetised with particular reagent, time and dose, off-feed and water to prevent the donor vomiting during LOPU, good feeding and post treatment for donor after LOPU (refer Section 3.3.4). In IVM duration study, the findings show equal potential to produce embryo development at (18-21 hours and 22-25 hours), even though maturation rates found to be higher at 18-21 hours. The Grades A, B and C were already considered as a good quality of oocytes. Therefore, no obvious differences of embryonic development at 22-25 hours of IVM duration. However, in group 18-21 hours of IVM duration, the Grade A oocyte showed higher embryonic development compared to others (Grades B and C). Probably, due to the Grade A with full cumulus cells vestment initiating earlier nuclear and cytoplasmic maturation and subsequently able to develop embryo. However, the ultrastructural and molecular study was not performed to observe the maturation mechanism and chromosome remodeling using the different quality of oocytes and IVM duration.

### 5.5.2   Intracytoplasmic Sperm Injection (ICSI)

The preparation of ICSI microtools and procedural were generally based on previous in-house laboratory research (Rahman, 2008a, b; Kong, 2010 and Ainul Bahiya 2010) unless otherwise stated. The procedural of ICSI in different species is the same except in term of medium used and microneedles size. Generally, 3 types of based medium used i) for sperm with sperm capacitation medium ii) hepes contained medium iii) 10% of PVP (Uehara and Yanagimachi, 1976). The arrangements of mediums on the dish were not specific. The media were placed in small microdroplet covered by mineral oil for the smooth microneedle handling. It has been found that the sperm were difficult to catch for immobilised because of the rapid movement of the sperm in the microdroplet. Therefore, in order to solve this problem, the microdroplets were prepared in longer or flatten shape. Through this, the sperm movement was limited in the microdroplet and subsequently, they were queuing at the edge of droplet. Later, the process of catch sperm or immobilised could be directly done in the droplet without placing in PVP. In hepes contained medium (for placing oocytes), the microdroplets were good in round shape to facility during handling oocyte manipulation (Keskinetepe *et al.,* 1997; Wang *et al.,* 2003; Jimenez-Macedo *et al.,* 2005). Usually 3-5 oocytes were placed in one microdroplet (according to the oocyte grades). The injected oocyte will be placed down the microdroplet to separate the non-injected oocytes. This is practical to speed up the procedure. Extra microdroplets were made to spare the clean droplet and also for microneedle cleaning place.

Generally the mouse sperm size is smaller than goat sperm. However, because of the morphology of the sperm have 'hook'and the goat sperm shape is round shape, the mouse inner diameter of ICSI microneedle were prepared slightly bigger than the real size (Ahmadi *et al.,* 1995)  (4-8 µm: mice and 8-10 µm: goat). The outer diameter of holding microneedle was outer diameter (120-150 µm: mice and 150-180 µm: goat).

However, the inner diameter was 25-30 μm. During the making of microneedles, there were many problems encountered, for examples to obtain a good size of ICSI and holding microinjection, making spike for ICSI microinjection, bending the microneedle without close the hole of bending site in desire angle, the risk of broken microneedle and contamination. The process of making ICSI microneedle is much difficult (Yanagida *et al.,* 2001) compared to holding microneedle. The ICSI microneedle needs to make a straight cut at the tips of needle with the desire size. The straight cut will do by assist of heat-shock of touched glass bead. In this process, the experienced and patience were needed followed by manipulating the heat score of the glass bead. Next, the microneedle was grinded to make a bevel (45°) (Van Sterghem *et al.,* 1993) for 2-3 minutes. Then, the microneedle was allowed to be rinsed by hydrofluoric acid (10%, 5 seconds), distilled water (10 seconds) and 70% alcohol (3-5 seconds). This was done to avoid contamination of glass debris from grinding process. Then, a short spike was making with heat touch with glass bead at the tips of bevel (Nagy *et al.,* 1995; Palermo *et al.,* 1996). The spike should not be too long because it will cause the inner diameter of the ICSI microneedle become small and long shape cause of heat effect. The inner size should be in round form. If not the sperm could not enter the microneedle. Last parts is bending process by placing the microneedle in horizontal position and apply the heat until they were bent at 25-30 degrees with microforge to allow a horizontal displacement on the microscope stage. Same procedure to bend the holding microneedle but the heat level is slightly higher than ICSI microneedle because of the diameter is bigger in holding microneedle. In order to prevent the contamination the microneedle were rinsed with alcohol (70%) and placed in individual holder. Then it will dried oven (60°C) and subsequently it will UV light sterilisation for 30 minutes before used.

During handling the ICSI procedure, the holding microneedle were set-up at micromanipulator first at correct position and followed by ICSI microneedle. Then both

of microneedles were immerged in mineral oil and the experimenter will align both microneedles and allowed the mineral oil equilibrating the inner pressure. Once the mineral oil stop entering the inner, the hepes contained medium (for allocating oocytes) were let to enter the ICSI microneedle. This was done to avoid high pressure during aspirating in and out the sperm during injection into the cytoplasmn.

The procedure of ICSI will start with catch the sperm to place the sperm into PVP (10%) for immobilization (Uehara and Yanagimachi, 1976). The sperm tail was score with rapid and gently keen by spike (Fishel *et al.,* 1995; Van de Bergh *et al.,* 1995; Vanderzwalmen *et al.,* 1998) or bevel of ICSI microneedle on the tail. Once the sperm were stop moving, the sperm was aspirated in the tail first in the ICSI microneedle with the minimal amount of PVP. For the information, some sperm was able to be immobilised in sperm microdroplet with the same immobilisation procedure. This step would help to reduced the time and avoid the PVP enter the ooplasmn. However, there were no comparison study was done for identify of this effect of this factor. After that, the immobilised sperm were injected into ooplasmn by gently rapid force of the injecting microneedle to the zona pellucida and subsequently the ooplasmn membrane (Esfandiari *et al.,* 2005). Not to forget, the oocytes polar body position should be at 12 or 6 o'clock (Nagy *et al.,* 1995; Joris *et al.,* 1998). During injection the image of oocyte should be sharped and focused especially the membrane and polar body image. This will help the injection process occur at the correct placed and subsequently the sperm image inside the microneedle could be adjust and see, then the process of aspirating in ooplasmn and aspirating out the sperm inside the ooplasmn become smoothly (Vanderzwalmen *et al.,* 1996; Calillo *et al.,* 1998). Finally, gently the ICSI microneedle was withdrawn slowly from the oocyte without bring out the sperm out or damage the oocyte. In this part, the role of ICSI microneedle spike is important (Nagy *et al.,* 1995; Palermo *et al.,* 1996; Yanagida *et al.,* 2001). The spike must be sharp and the

inner pressure of the microneedle is in well condition. In addition, the suction pressure of holding microneedle to hold the oocyte should not too high just enough to hold the oocyte in static placed and less movement.

In summary, the ICSI procedure itself could be a main influenced in order to obtain good ICSI procedure. These experiments were conducted using the conventional ICSI technique (Zhou *et al.,* 2004; Jimenez-Macedo *et al.,* 2005, 2006, 2007; Rahman 2008a, b; Kong 2010; Ainul Bahiya, 2010). Even though, the application of piezo-driven was widely used in mouse experiment (Kimura and Yanagimachi, 1995) or some in goat (wang *et al.,* 2003). The application of conventional ICSI is still practical and just need more technical experienced, microneedles preparation improvement and preserved the good quality of sperm and oocytes for ICSI. The duration of the whole process should be minimised and try to handle it not more than 20 minutes. The longer time taken will make the samples become risk of death or degenerated (Keskinetepe *et al.,* 1997). The ICSI of manipulation process was doing on the stage warmer to avoid the samples death (Keskinetepe *et al,* 1997; Wang *et al.,* 2003; Jimenez-Macedo *et al.,* 2005).

### 5.5.3 Embryonic Development via ICSI

ICSI procedure itself may lead the nuclear and cytoplasmn component (physical disruption) that necessary for oocyte continued development (Miller and Smith, 2001). In other issue,the post-ICSI oocytes were placed 30 minutes in equilibrated TCM 199+ Foetal bovine serum (FBS) (10%) before oocyte activation. This is the requirement duration to let the oocyte cytoskeleton and spindle to relax and recover back to normal form for further activation. In addition, the activation would begin 30 minutes after sperm injection (Dozortsev *et al.,* 1995a and b).

The actions of sperm factor on embryonic development are referred to paternal effect. The paternal effect is divided in to early and late paternal effect. The early paternal effect may delay the cleavage speed and increase the fragmentation in embryo development (Menezo, 2006). While, the late paternal effect may involve sperm aneuploidy, DNA damage, abnormal chromatin packaging that resulted from defective sperm (Borini *et al.,* 2006). The sperm factor is not related in fertilising ability but it is associated with high rate of developmental arrest at 5- to 8-cell stages on Day 3 and decreased blastocyst formation on Day 5 in this cycle (Miller and Smith, 2001). Therefore, the good quality of sperm selection from earlier ICSI procedure influenced the results of ICSI-derived embryos. The hidden parameter that could not seen by naked eye should be investigated through ultrastructural or molecular experiment to confirm the quality of sperm used.

Previous studies have achieved better embryo development when they were cultured in groups (well-in-drop, WID) compare than cultured individually (microdrop individual culture, MIC). (Keefer *et al.* 1994; Moessner and Dodson, 1995). However, this study used MIC because the small number of oocytes available and need to be identification after culture, especially in goat experiment. The mouse experiment culture was used WID because the large number of oocytes. Due to this aspect, in the findings

the mouse embryo developmental rates are much better than goat embryo developmental rates.

Glucose in media was used as an energy substrate at the earlier stage of embryonic development. However, the cleavage stage embryos have a limited capacity to utilise glucose (Reiger *et al.,* 1992; Thompson *et al.,* 1996). Glucose in the culture medium has an adverse effect on embryo development and it is not required until Day 3 or 4 of development (Kim *et al.,* 1993). However, the granulose cell monolayers can utilise the glucose from culture medium and depleting the levels and give pyruvate in return (Teotia *et al.,* 2001). It has been reported that glucose free medium (Lim *et al.,* 1993) were gave higher morula rate development (40% versus 22%) compared cultured glucose contained medium (Quinn, 1995). Nevertheless, a high glucose concentration is also detrimental to embryo development (Thompson *et al.,* 1996). The embryos culture medium should be changed or replaced on Day 3 using glucose free medium. In other hand, on the Day 2 or 3 development, the capable embryos, which attaining blastocyst stage embryos were recommended to be transferred to recipient (Palermo *et al.,* 1998).

### 5.5.4 Future Direction

This preliminary study covering the selected sperm and oocyte factors that related with LOPU, IVM and ICSI experiment. This research had been carried out in two different species, which are mice and goat. The pattern of embryonic development in both laboratory scale (mouse) and farm scale (goat) could be seen through this experiment. The author was experienced with some problems and most of the problems encountered were able to overcome. Thus, the presented results might be influenced by several factors, such as author's learning curve; in terms of preparing the ICSI microtools, sperm and oocytes handling; mouse breeding management to obtain consistent healthy, weight, and age mouse for experiment; farm management (goat management) for pre- and post-LOPU experiment; and all regarding embryo culture media and system. Even though the findings in present study gave basis physiological aspect, this information could be beneficial in order to understand the process and mechanism of sperm and oocyte factors that affected ICSI-derived embryo development. The further study should be conducted at molecular or ultrastructural levels at small (laboratory) and also at large scale (farm).

The information obtained through this finding could be contributed and applied for the farm sectors. With the good knowledge and understanding of sperm and oocyte factors, it is not possible that ICSI technique could be widely applied together with other technique such as LOPU and ET, in order to make an efficient IVP and subsequently obtain good breed of offspring. Therefore, a detailed or extended research related with ICSI is good for being study in the future includes: the effects of sperm capacitation chemicals treatment in term of types chemicals used, concentration, toxicity, mechanism and adverse effect on ICSI experiment; the detailed effects and mechanismn of sperm motility and morphology on early and latest ICSI-embryo development; the effect of IVM duration related with LOPU-derived oocytes and the

effects of different oocytes quality and selection at different IVM duration; study the details of low quality of LOPU-derived oocytes (i.e dysmorphism, less or no COCs) at ultrastructural and molecular level in goat; study the repeated goat LOPU adverse effects in order to obtained the good quality supplies, optimal interval repeated of LOPU cycle, ovarian physiology status of donor; the technical aspect of convensional ICSI microtools preparation and procedure could be improved by medium used and the optimal timing of ICSI experiment; the important of oocyte activation in term of types and the optimal timing of activation. Last but not least, the effect of manipulation or improvement of media for embryo developing and culture system that suitable for ICSI-embryo development.

**Chapter 6**

**6.0 CONCLUSIONS**

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This study presents the *in vitro* development embryos via intracytoplasmic sperm injection (ICSI) with special reference to sperm and oocytes factors correlating with embryonic developmental performance in mouse and goat species. According to the findings, it can be inferred that:

a) Mouse and goat embryos were produced from ICSI technique under the local setting of ABEL, University of Malaya, Malaysia.

b) Mouse ICSI-embryonic development was improved by using Heparin as sperm capacitation chemical treatment.

c) The sperm movement factor (Rapid and Slow) has same potential to produce mouse ICSI-derived embryos.

d) The post-hCG administration duration at 13 to 15 hours gave good quality of matured oocytes and embryonic developmental rate compared with at 16-18 hours duration.

e) Sperm capacitation chemicals (Heparin and Theophylline) gave same potential of goat ICSI-embryonic development.

f) Rapid sperm movement gave better goat ICSI-embryonic development compared to slow sperm movement.

g) LOPU can be repeated up to 3 times in the same hormonal treated goat donor (60 hours of post-PMSG+hCG) without detrimental effect.

h) Higher Grade C oocytes were obtained from LOPU compared to Grades A and B.

i) Lower Grades D and E oocytes were obtained compared to Grades A, B and C.

j) OR1 gave better quantity and quality oocytes, followed by OR2 and OR3.

k) Maturation rate of goat oocytes at 18 to 21 hours was higher than 22 to 25 hours of IVM duration. However, both IVM durations had the same potential ICSI-derived embryo development.

l) Grade A oocytes gave higher cleavage rates at 18 to 21 hours compared to Grades B and C.

m) The earlier selection of good oocyte quality factor (based on cumulus cells expansion) influenced the subsequent success of embryonic development.

n) It can be concluded that:

    i) For mouse study, embryonic development can be achieved from Heparin-sperm capacitation treatment, using any sperm movement (Rapid or Slow) using the 13 to 15 hours of post-hCG administration oocytes.

    ii) For goat study, LOPU is good procedure to provide consistent good oocytes (Grades A, B and C) and can be repeated using the same donor up to 3 times. Rapid sperm movement is a better choice for ICSI-embryonic development. Both sperm capacitation chemical treatments (Heparin and Theophyline) have the same potential to produce ICSI-embryonic development.

**REFERENCES**

# REFERENCES

Abdullah, R.B., S.L. Liow, A.N.M.A. Rahman, W.K. Chan, W.E. Wan Khadijah and S.C. Ng. 2008. Prolonging the interval from ovarian hyperstimulation to laparoscopic ovum pick-up improved oocyte yield, quality, and developmental competence in goats. Theriogenology: 70: 765-771.

Abe, H. And H. Hoshi. 2003. Evaluation of bovine embryos produced in high performance serum-free media. Journal of Reproduction Development. 49: 193-202.

Abecia, J.A., S.M. Rhind, T.A. Bramleyand S.M. McMillen. 1995. Steroid production and LH receptor concentrations of ovarian follicles and corpora lutea and associated rates of ova wastage in ewes given high and low levels of food intake before and after mating. Animal Science. 61: 57-62.

Abeydeera, L.R. 2002. *In vitro* production of embryos in swine. Theriogenology. 57: 257-273.

Adam, G.P. Comparative patterns of follicle development and selection in ruminants. Journal of Reproduction Fertility. 1999: 17-32.

Adamiak, S.J., k. Mackie, R.G. Watt, R. Webb and K.D. Sinclair. 2005. Impact of nutrition on oocyte quality: cumulative effects of body composition and diet leading to hyperinsulinemia in cattle. Biology of Reproduction. 73: 918-926.

Agrawal, K.P., T. Sharma, C. Sexena and N. Sharma. 1995. Chronology of first meiotic events of caprine oocytes matured *in vitro*. Indian Journal of Animal Sciences. 65: 285-288.

Ahmadi, A.A., S.C. Ng, S.L. Liow, J. Ali, A, Bongso and S.S. Ratnam. 1995. Intracytoplasmic sperm injection of mouse oocytes with 5 mM $Ca^{2+}$ at different intervals. Human Reproduction. 75: 442-451.

Ainul Bahiyah, A.B. 2010. *In vitro* and *in vivo* developmental competence of intact, *in vitro*-derived and cryopreserved murine embryos. PhD Thesis. University of Malaya, Kuala Lumpur.

Ajduk, A., Y. Yamauchi and M.A. Ward. 2006. Sperm chromatin remodeling after intracytoplasmic sperm injection differs from that of in vitro fertilization. Biology of Reproduction. 75: 442-451.

Alberio, R., J. Olivera, A. Roche, J. Alabart and J. Folch. 2002. Performance of a modified ovum pick-up system using three different FSH stimulation protocols in ewes. Small Ruminant Research. 46: 81-87.

Ammoun, I., T. Encinas, A. Veiga-Lopez, J.M. Ros. I. Contreras, P. Gonzalez-Anover, M.J. Cocero, A.S. McNeilly and A. Gonzalez-Bulnes.2006. Effect of breed on kinetics of ovine FSH and ovarian response in superovulated sheep. Theriogenology, 66: 896-905.

Amoah, E.A. and S. Gelaye. 1990. Superovulation, synchronisation and breeding of does. Small Ruminant Reseacrh. 3: 63-72.

Anguita B., M.T. Paramio, R. Morato, R. Romaguera, A.R. Jimenez-Macedo, T. Mogas and D. Izquierdo. 2009. Effect of the appopyosis rate observed in oocytes and cumulus cells on embryo development in prepubertal goats. Animal Reproduction in Science. 116: 95-106.

Arlotto, T., J.L. Schwartz, N.L. First and M.L. Leibfried-Rutledge. 1995. Aspect if follicle and oocyte stage that affect *in vitro* maturation and development of bovine oocytes. Theriogenology. 45: 943-956.

Armstrong, D.T., A.P. Pfitzner, G.M. Warners, M.M. Ralph and R.F. Seamark. 1983. Endocrine responses of goats to superovulation with PMSG and FSH. Journal of Reproduction and Fertility. 67: 395-401.

Armstrong, D.T. 2001. Effect of maternal age on oocyte developmental competence. Theriogenology. 55: 1303-1322.

Austin. C.R. In: Whitter J.R. (ed). 1974. Concept of development . Sineur Associates Inc., Publisher.

Avery, B., A. Hay-Schmidt, P. Hyttel and T. Greve. 1998. Embryo development. Oocyte morphology and kinetics of meiotic maturation in bovine oocyte exposed to 6-dimethykaminopurine prior to *in vitro* maturation. Molecular Reproduction Development. 50: 334-344.

Balaban, B., B. Urman, A. Sertak, C. Alatas, S. Aksoy and R. Mercan. 1998. Oocyte morphology does not affect fertilization rate, embryo quality and implantation rte after intracytoplasmic sperm injection.Human reproduction. 13: 3431-3433.

Baldassarre, H., C.C. Funus, T.E. Castro and E.I. Cabrera Fischer. 1994. Technique for efficient recovery of sheep oocytes by laparoscopic folliculocentesis. Animal Reproduction Science. 35: 145-150.

Baldassarre, H., C.C. Funus, D.G. De Matos and H. Pessi. 1996. *In vitro* production of sheep embryos using laparoscopic folliculogenesis: Alternative gonadotrophin treatments for stimulation of oocyte donors. Theriogenology. 45: 707-717.

Baldassarre, H., B. Wang, N. Kafidi, C. Keefer and A. Lazaris and C.N. Karatzas. 2002. Advances in the production and propagation of transgenic goats using laparoscopic ovum pick-up and *in vitro* embryo production technologies. Theriogenology. 57: 275-284.

Baldassarre, H., B. WangN. Kadifi, M. Gauthier, N. Neveu, J. Lapointe, L. Sneek, M. Leduc, F. Duguay, J.F. Zhou,  A. Lazaris and C.N. Karatzas. 2003. Production of transgenic goats by pronuclear microinjection of in vitro produced zygotes derived from oocytes recovered by lapascopy. Theriogenology. 59: 831-839.

Baldassarre, H. and C.N. Karatzas. 2004. Advanced assisted reproduction technologies (ART) in goats. Animal Reproduction Science. 82-83: 255-266.

Baldassarre, H., K.M. Rao, N. Neveu, E. Brochu, I. Begin. E. Behboodi and D.K. Hockley. 2007. Laparoscopic ovum pick-up followed by *in vitro* embryo production for the reproductive rescue of aged goats of high genetic value. Reproduction, Fertility and Development. 19: 612-616.

Ball, G.D., M.L. Leibfried, R.W. Lenz, R.L. Az, B.D. Bavister and N.L. First. 1983. Factors affecting successful *in vitro* fertilisation of bovine follicular oocytes. Biology of Reproduction. 28: 717-725.

Baril, G., P. Brebion and P. Chesne. 1993. Handbook for embryo transfer in sheep and goats. 115: 1014-1099.

Bates, G.W., A. Saunders and A.E. Sowers. 1987. Electrofusion. *In*: Cell Fusion. *Editors*: A.E. Sowers. Plenum Press, New York. pp. 367-395.

Bavister, B.D. 1995. Culture of preimplantation embryos: facts and artifacts. Human Reproduction Update. 1: 91-148.

Bavister, B.D., T.A. rose-Hellekant and T. Pinyopummiter. 1992. Development of *in vitro* matured/ *in vitro* fertilized bovine embryos into morulae and blastocyst in defined culture medium. Theriogenology. 37: 127-146.

Ben-Yosef, D. and R. Shalgi. 2001. Oocyte activation: lessons from human infertility. Trends in Molecular Medicine. 7: 163-169.

Bhatia, B., B. Wang, H. Baldassarre and C.L. Keefer. 2002. *In vitro* fertilisation of goat oocytes using fresh and frozen-thawed spermatozoa. Theriogenology. 57:568 (abstract).

Bindon, B.M., L.P. Piper, L.P. Cahill, M.A. Draincourt and T. O'Shea. 1986. Genetic and hormonal factors affecting superovulation. Theriogenology. 25: 51-70.

Blondin, P. and M.A. Sirard. 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. Molecular Reproduction and Development. 41: 54-62.

Booth, P.J., P. Holm and H. Callesen. 2005. The effect of oxygen tension on porcine embryonic development is dependent on embryo type. Theriogenology. 63: 2040-2052.

Borini, A., N. Tarozzi, D. Bizzaro, M.A Bonu, L. Fava and C. Flamigni. 2006. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. Human Reproduction. 21: 2876-2881.

Bormann, C.L., E.M. Ongeri and R.L. Krisher. 2003. The effect of vitamins during maturation of caprine oocytes on subsequent developmental potential *in vitro*. Theriogenology. 59: 1373-1380.

Brackett, B.G. and G. Oliphant. 1975. Capacitation of rabbit spermatozoa in vitro. Biology of Reproduction. 12: 260-274.

Brackett, B.G. and K.A. Zuelke. 1993. Analysis of factors involved in the *in vitro* production of bovine embryos. Theriogenology. 39: 43-64.

Bravini-Gandolfi, T.A.L. and F. Gandolfi. 2001. The maternal legacy to the embryo: cytoplasmic components and their effects in early development. Theriogenology. 55: 1255-1276.

Bretzlaff, K.N. and N. Madrid. 1985. Synchronisation of oestrus and fertility in goats with norgestomet ear implants. Theriogenology. 16: 587-591.

Bretzlaff, K.N. and N. Madrid. 1989. Clinical use if norgestomet ear implants or intravaginal pessaries for synchronization of oestrus in anoestrus dairy goats. Theriogenology. 31: 419-423.

Buccione, R., A.C. Schroeder and J.J. Eppig. 1990. Interactions between somatic cells and germ cells thrpughout mammalian oogenesis. Biology of Reproduction. 43: 543-547.

Burruel, V., H. Yanagimachi and W.K. Whitten. 1996. Normal mice develop from oocytes injected with spermatozoa with grossly mishappen heads. Biology of Reproduction. 55: 709-714.

Byers S.L., S.J. Payson and R.A. Taft. 2006. Performance of ten inbred mouse strains following assisted reproduction technologies (ARTs). Theriogenology. 65: 1716-1726.

Byers, S.L., Wiles, M.V. and R.A. Taft. 2008. Surgical oocyte retrieval (SOR): a method for collecting mature mouse oocyte without euthanasia. Journal of the American Association. Laboratory Animal Science. 48: 44-51.

Calvin, H.I., K. Grasshans and E.J. Blake. 1986. Estimation and manipulation of gluthione levels in prepubertal mouse ovaries and ova, relevance to sperm nucleus transformation in the fertilizad egg. Gamete Research. 6: 107-114.

Carnevale, E.M., D.R. Bergfelt and O.J. Ginther. 1993. Aging effects on follicular activity and concentrations of FSH, LH and progesterone in mares. Animal Reproduction Science. 31: 287-299.

Carnevale, E.M., M.J. Hermenet and O.J. Ginther. 1997. Age and pasture effects on vernal transition in mares. Theriogenology. 47: 1009-1018.

Carnevale, E.M., M. Uson, J.J. Bozzola, S.S. King, S.J. Scmitt and H.D. Gates. 1999. Comparison of oocytes from young and old mares with light microscopy. Theriogenology. 51: 229.

Catt, J. and S.L. Rhodes. 1995. Comparative intracytoplasmic sperm injection (ICSI) in human and domestic. Reproduction, Fertility and Development. 7: 161-167.

Catt, S.L., J. W. Catt, M.C. Gomez, W.M. Maxwell and G. Evans. 1996. Birth of a male lamb derived from an in vitro matured oocyte fertilised by intracytoplasmic injection of a single presumptive male sperm. The Veterinary Record. 139: 494-495.

Ceconi, S., R. D'Aurisio and R. Colonna. 1996. Role of antral follicle development and cumulus cells on *in vitro* fertilisation of mouse oocytes. Journal of Reproduction and Fertility. 107: 207-214.

Chang, M.C. 1951. Fertilising capacity of spermatozoa deposited into the fallopian tubes. Nature. 168: 697-698.

Chatot, C.L., C.A. Ziomek, B.D. Bavister, J.L. Lewis and I. Torres. 1989. An improved culture medium supports development of random bred 1 cell mouse embryos *in vitro*. Journal of Reproduction and Ferility. 86: 679-688.

Chen, L., P.T. Russell and W.J. Larsen. 1993. Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumuls mass. Molecular Reproduction and Development. 34: 87-93.

Chen, S.H. and G.E. Seidel. 1997. Effect of oocyte activation and treatment of spermatozoa on embryonic development following intracytoplasmic sperm injection in cattle. Theriogenology. 48: 1265-1273.

Chen, N.Q., S.L. Liow, R.B. Abdullah, W.K. Embong, W.Y. Yip, L.G. tan, G.Q. Tong and S.C. Ng. 2006. Developmental competence of transported *in vitro* matured macaque oocytes. Reproductive Biomedicine Online. 12: 50-59.

Choi, T., F. Aoki, M. Mori, M. Yamashita, Y, Nagahama and K. Kohmoto. 1991. Activation of p34cdc2 protein kinase activity in meiotic and mitotic cell cycles in mouse oocytes and embryos. Development. 113: 789-795.

Choi, Y.H., C.C. Love, L.B. Love, D.D. Varner, S. Brinsko and K. Hinrichs. 2002. Developmental competence *in vivo* and *in vitro* of *in vitro* matured equine oocytes fertilized by intracytoplasmic sperm injection with fresh or frozen-thawed spermatozoa. Reproduction. 123: 455-465.

Christmann, L., T. Jung and R.M. Moor. 1994. MPF components and meiotic competence in growing pig oocytes. Molecular Reproduction and Development. 38: 85-90.

Chung, J.T., C.L. Keefer and B.R. Downey. 2000. Activation of bovine oocytes following intracytoplasmic sperm injection (ICSI). Theriogenology. 53: 1273-1284.

Cochran, R., M. Meintjes, B. Reggio, D. Hylan, J. Carter, C. Pinto, D. Paccamonti and R.A. Godke. 1998. Live foals produced from sperm-injected oocytes derived from pregnant mares. Journal of Equine Veterinary Science. 18: 736-740.

Cognie, Y. 1999. State of the art in sheep-goat embryo transfer. Theriogenology. 51: 105-116.

Cognie, Y., G. Baril, N. Poulin and P. Mermillod. 2003. Current status of embryo technologies in sheep and goat. Theriogenology. 59: 171-188.

Cognie, Y., N. Poulin, Y. Locatelli and P. Mermillod. 2004. State-of-the-art production, conservation and transfer of *in vitro* produced embryos in small ruminants. Reproduction, Fertility and Development. 16: 437-445.

Cohen, J., M. Alikani, S. Munne and G. D. Palermo. 1994. Micromanipulation in clinical management of fertility disorders. Seminars in Reproductive Endocrinology. 12: 151-168.

Combelles, C.M., N.A. Cekleniak, C. Racowsky, and D.F. Albertini. 2002. Assesement of nuclear and cytoplasmic maturation in in vitro matured human oocytes. Human Reproduction. 17: 1006-1016.

Corcoran, D., T. Fair, S. Park, D. Rizos, O.V. Patel, G.W. Smith, P.M. Coussens, J.J. Ireland, M.P. Boland, A.C.O. Evans and P. Lonergan. 2006. Suppressed expression of genes involved in transcription and translation in *in vitro* compared with *in vivo* cultured bovine embryos. Reproduction. 131: 651-660.

Cornwall, G.A., T.B. Vindivich, D., Harter, C. Robinson, J., Chang. 1986. Induction and enhancement of progressive motility in hamster caput epididymal spermatozoa. Biology of Reproduction. 35: 1065-1074.

Creed, J., T.G. McEvoy, J.J. Robinson, R.P. Aitken, R.M. Palmer and I.S Robertson. 1994. The effect of pre-ovulatory nutrition on the subsequent development of superovulated sheep in ova in an *in vitro* culture system. Animal Production. 58: 447 (abstract).

Crister, J.K., F.C. Gunsett and R.P. Winch. 1979. Factors affecting ova transfer in LImousin, Maine-anjou and Simmental cattle. Theriogenology. 11: 95.

Crister, F.S., M.L. Liebfried Rutledge, W.H. Eyestone, D.L. Northey and N.L. First. 1986. Acquisition of developmental competence during maturation *in vitro*. Theriogenology. 25: 150.

Crosier, A.E., P.W. Farin, M.J. Dykstra, J.E. Alexander and C.E. Farin. 2000. Ultrastructural morphometry of bovine compact morulae produced *in vivo* and *in vitro*. Biology of Reproduction. 62: 1459-1465.

Crosier, A.E., P.W., Farin, M.J. Dykstra, J.E. Alexander and C.E. Farin. 2001. Ultrastructural morphometry of bovine blastocysts produced *in vivo* and *in vitro*. Biology of Reproduction. 64: 1375-1385.

Crozet, N., J. Motlik and D. Szollosi.1981. Nucleolar fine structure and RNA synthesis in porcine oocytes during early stages of antrum formation. Journal of Cell Biology. 41: 35-42.

Crozet, N., M. Ahmed-Ali and M. P. Dubos. 1995. Developmental competence of goat oocytes from follicles of different size categories following maturation, fertilization and culture *in vitro*. Journal of Reproduction and Fertility. 103: 293-298.

Crozet, N., M. Dahirel and L. Gall. 2000. Meiotic competence of *in vitro* grown goat oocytes. Journal of Reproduction and Fertility. 118: 367-373.

Damiani, P., R.A. Fissore, J.B. Cibelli, C.R. Long, J.J. Balise and J.M. Robl. 1996. Evaluation of developmental competence, nuclear and ooplasmic maturation of calf oocytes. Molecular Reproduction and Development. 45: 521-534.

Damiani, P., R.A. Fissore and R.T. Duby. 1998. Biochemical and physiological maturation of cow and calf oocytes. Theriogenology. 49: 310 (abstract).

De Castro, T., E. Rubianes, A. Menchaca and A. Rivero. 1999. Ovarian dynamics, serum estradiol and progesterone concentrations during the interovulatory interval in goats. Theriogenology. 52: 399-411.

De Matos, D.G. and C.C. Furnus. 2000. The importance of having high glutathione (GSH) level after bovine *in vitro* maturation in embryo development effect of β-mercaptoethanol, cysteine and cystine. Theriogenology. 53: 761-771.

De Matos, D.G., C.C. Funus, D.F. Moses and H. Baldassarre. 1995. Effect of cysteamine on glutathione level and development capacity of bovine oocyte matured *in vitro*. Molecular Reproduction and Development. 42: 432-436.

de Paz, P., A.J. Sanchez, J. De la Fuente, C.A. Chamorro, M. Alvarez, E. Anel and L. Anel. 2001. Ultrastructural and cytochemical comparison between calf and cow oocytes. Theriogenology. 55: 1107-1116.

De Smedt, V., N. Crozet, M. Ahmed-Ali, A. Martino and Y. Cognie. 1992. *In vitro* maturation and fertilization of goat oocytes. Theriogenology. 37: 1049-1060.

De Sutter, P., D. Dozortsev, C. Quian and M. Donth. 1996. Oocyte morphology does not with fertilization rate and embryo quality after intracytoplasmic sperm injection. Human Reproduction. 11: 595-597.

Deb, S.M. and S.L. Goswami. 1990. Cytogenic analysis of goat oocytes cultured *in vitro*. Italian Journal of Animal Science. 5: 137-143.

Dedieu, T., L. Gall, N. Crozet, C. Sevellec and S. Ruffini. 1996. Mitogen-activated protein kinase activity during goat ocyte maturation and the acquisition of meiotic competence. Molecular Reproduction and Development. 45: 351-358.

Dekel, N. 1996. Protein phosphorylation/dephosphorylation in the meiotic cell cycle of mammalian oocyte. Review of Reproduction. 1: 82-88.

Demoustier, M.M., J.F. Beckers, P.Van der Zwalmen, J. Closset, J.L. Gillared and F.R. Ectors. 1988. Determination of porcine plasma folltropin levels during superovulation treatment in cow. Theriogenology. 30: 379-386.

Dhruba, M, and A.L. Majumdar. 2002. Comparative study on *in vitro* development of goat embryos cultured in medium containing L-glutamine alone or with essential and nonessential amino acids. Indian Journal of Dairy Science. 55: 145-149.

Dielman, S.J. P.J. Hendriksen, D. Viuff, P.D. Thomsen, P. Hyttel, H.M. Knijn, C. Wrenzycki, T.A. Kruip, H. Niemann, B.M. gadella, M.M. Bevers and P.L. Vos. 2002. Effects of *in vivo* prematuration and *in vivo* final maturation on developmental capacity and quality of pre-impalantation embryos. Theriogenology. 57: 5-20.

Diskin, M.G., D.R. Mackey, J.F. Roche and J.M. Sreenan. 2003. Effects of nutrition and metabolix status on circulating hormones and ovarian follicle development in cattle. Animal Reproduction Science. 78: 345-370.

Dobbs, K.E., D.A. Dumesic, J.A. Dumesic and S.S. Shapiro. 1994. Differences in serum follicle stimulating hormone uptake after intramuscular and subcutaneous human menopausal gonadotrophin injection. Fertility and Sterility. 62: 978-983.

Dominko T. and N.L. First. 1992. Kinetics of bovine oocyte maturation maturation allow selection for developmental competence and is affected by gonadotrophins. Theriogenology. 37: 203 (abstract).

Dominko, T. and N.L. First. 1997. Timing of meiotic progression in bovine oocytes and its effect on early embryo development. Molecular Reproduction and Development. 47: 456-467.

Donaldson, L.E. 1984. The effect of the age of the donor cow on embryo production. Theriogenology. 21: 963-967.

Donnay, I., R.J. Partridge and H.J. Leese. 1999. Can embryo metabolism be used for selecting bovine embryos before transfer? Reproduction Nutrition Development. 39: 523-533.

Downing, J.A., J. Joss, P. Connell and R.J. Scaramuzzi. 1995. Ovulation rate and the concentration of gonadotrophic and metabolic hormones in ewes fed lupin grain. Journal of Reproduction and fertility. 103: 137-145.

Dozortsev, D., C. Qian, A. Ermilov, A. Rybouchinkin, P. De Sutter, and M. Dhont. 1995a. Sperm-associated oocyte-activating factor is released from the spermatozoon within 30 minutes after injection as a result of sperm-oocyte interaction. Human Reproduction. 12: 2792-2796.

Dozortsev, D., P. De Sutter, A. Rybouchinkin, and M. Dhont. 1995b. timing of sperm and oocyte nuclear progression after intracytoplasmic sperm injection. Human Reproduction. 10: 3012-3017.

Dozortsev, D., C. Qian, A. Ermilow, A. Rybouchkin, P.D. Sutter and M. Dhont. 1997. Sperm-associated oocyte-activating factor is released from the spermatozoon within 30 minutes after injection as a result of the sperm-oocyte interaction. Human Reproduction. 12: 2792-2796.

Driancourt, M.A. 2001. Regulation of ovarian follicular dynamics in farm animals: impilication for manipulation of reproduction. Theriogenology. 55: 1211-1239.

Drion, P.V., R.D. Roover, J. Houtain, E.M. McNamara, B. Remy, J. Sulon ans J. Beckers. 2001. Increase of plasma eCG binding rate after administration of repeated high dose of eCG to cows. Reproduction Nutrition Development. 41: 207-215.

Dumoulin, J.C., E. Coonan, M. Bras, L.C. van Wissen, R. Ignoul-Vancvuchelen, and J.M Bergers-Jansen. 2000. Comparison of *in-vitro* development of embryo originating from either conventional *in-vitro* fertilization or intracytoplasmic sperm injection. Human Reproduction. 15: 402-409.

East, N.E. and J.D. Rowe. 1989. Subcutaneous progestin implants versus intravaginal sponges for dairy goat oestrus synchronisation during the transitional period. Theriogenology. 32: 921-928.

Ebner, T., C. Yaman, M. Moser, M. Sommergruber, O. Feichtinger, and G. Tews. 2000. Prognostic value of first polar body morphology on fertilization rate and embryo quality in intracytoplasmic sperm injection. Fertility and Sterility. 15: 427-430.

Ebner, T., G. Tews, R.B. Mayer, S. Ziehr, W. Arzt, W. Costamoling and O. Shebl. 2011. Pharmacological stimulation of sperm motility in frozen and thawed testicular sperm using the dimethylzanthine theophylline. Fertility and Sterility. 96: 1331-1336.

Edgar, D.H., K.M. Walley and J.A. Mills. 1987. Effects of high-dose and multiple dose gonadotrophin stimulation on mouse oocyte quality as assessed by preimplantation development following *in vitro* fertilization. Journal of *In Vitro* fertilization and Embryo Transfer. 4: 273-276.

Edwards, R.G. 1965. Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. Nature. 208: 349-351.

Eichenlaub-Riuer, U. H. Schmiady, H. Kentenich, and D. Soewarto. 1995. Recurrent failure in polar body formation and premature chromosome condensation in oocytes from a human patient : indicators of asynchrony in nuclear and cytoplasmic maturation. Human Reproduction.10: 2343-2349.

Ellington, J.E., E.W. Carney, P.B. Farell, M.E. Simkin and RH. Foote. 1990. Bovine 1 to 2-cell embryo development using a simple medium in three oviduct epithelial cell coculture systems. Biology of Reproduction. 41: 268-276.

Elmazar, M.M., R. Vogel and H. Spielmann. 1989. Maternal factors influencing development of embryos from mice superovulated with gonadotrophin. Journal of Reproductive and Toxicology. 3: 135-138.

Eppig, J.J. 1996. Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. Reproduction, fertility and Development. 8: 485-489.

Epping J.J. and Schroeder A.C. 1989. Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization in vitro. Biology Reproduction. 41(2): 268–276.

Erbach, G.T., J.A. Lawitts, V.E. Papaioannou and J.D. Biggers. 1994. Differential growth of the mouse preimplantation embryo in chemically defined media. Biology of Reproduction. 345: 1027-1033.

Esfandiari, N., M.H. Javed, L. Gotlieb and R.F. Casper. 2005. Complete failed fertilisation after intracytoplasmic sperm injection-analysis of 10 years data. International Journal of Fertility and Women's Medicine. 50: 187-192.

Evans, G. and D.T. Armstrong. 1983. Reproduction in fertilisation rate in vitro of oocytes from immature rats induced to superovulate. Journal of Reproduction and Fertility. 70: 131-135.

Evans, G. and W.M.C. Maxwell. 1987. Salamon's Artificial Insemination of sheep and goats. Butterworths, Sydney, Australia.

Fabian, D., J. Koppel and P. Maddox-Hyttel. 2005. Apoptotic process during mammalian preimplantation development. Theriogenology. 64: 221-231.

Fair, T., P. Hyttel and T. Greve. 1995. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. Molecular Reproduction and Developement. 42: 437-442.

Ferguson, E.M., C.J. Ashworth, S.A. Edwards, N. Hawkins, N. Hepburn and M.G. Hunter. 2003. Effect of different nutritional regimes before ovulation on plasma concentrations of metabolic and hormones and oocyte maturation in gilts. Reproduction. 126: 61-71.

First, N.L., M.L. Leibbfried-Rutledge and M.A. Sirard. 1988. Cytoplasmic control of ocytes maturation and species differences in the development of maturational competence. In: Meiotic Inhibition: Molecular Control of Meiosis. Editors: F.P. Haseltine and N.L. First. National Institutes Health. New York. p.1.

Fishel, S., F. Lisi, L. Rinaldi, S. Green and K. Dowell and S. Thornton. 1995. Systematic examination of immobilizing spermatozoa before intracytoplasmic sperm injection in the human. Hum. Reprod., 10: 497-500.

Fisher D.L. and K.P., Gunaga. 1975. Theophylline induced variations in cyclic AMP content in superovulated preimplantation mouse embryo. Biology of Reproduction. 12: 471-476.

Fisher-Brown, A.E., A. Crooks, S. Leonard, R. Monson, D. Northey and J.J. Rutledge. 2005. Partituition following transfer of embryos produced in two media under two oxygen concentrations. Animal Reproduction Science. 87: 215-228.

Flores-Foxworth, G. 1997. Reproductive biotechnologies in the goat. In: Current Theraphy in Large Animal. Theriogenology. Editors: R.S. Youngquist. W..B. Saunders. Philadelphia. pp 560-568.

Fraser L.R. 1984. Mouse sperm capacitation in vitro involves loss of a surface-associated inhibitory component. Journal of Reproduction Fertility. 72: 373-384.

Fraser L.R. 2008. The role of small molecules in sperm capcitation. Theriogenology. 70: 1356-1359.

Freitas, V.J., G. ba and J. Saumande. 1997. Oestrus synchronisation in dairy goats: use of fluorogestone acetate vaginal sponges or norgestomet ear implants. Animal Reproduction Science. 46: 237-244.

Fry, R., L. Cahill, J. Cummins, B.M. Bindon, L.R. Piper and I. Clarke. 1987. The half-life of follicle stimulating hormone in ovary intact and ovariectomized Booroola and control Merino ewes. Journal of Reproduction and Fertility. 81: 611-615.

Fujinami, N, Y. Hosoi, H. Kato, K. Matsumoto, K. Saeki, and A. Iritani. 2004. Activation with ethanol improves embryo development of ICSI-derived oocytes by regulation of kinetics of MPF activity. Journal of Reproduction and Development. 50: 171-178.

Fujino, Y., K. Ozaki, S. Yamamasu, F. Ito, I. Matsuoka, E. Hayashi, H. Nakamura , S. Ogita, E. Sato and M. Inoue. 1996. DNA fragmentation of oocytes in aged mice. Human Reproduction. 11: 1480-1483.

Fukui, Y., A.M. Glew, F. Gandolfi and R.M. Moor. 1988. *In vitro* culture of sheep oocytes matured and fertilised *in vitro*. Theriogenology. 29: 883-891.

Funahashi, H. And B.N. Day. 1993. Effects of the duration of exposure to hormone supplements on cytoplasmic maturation of pig oocytes *in vitro*. Journal of Reproduction and Fertility. 98: 179-185.

Funahashi, H., T.C. Cantley and B.N. Day. 1997. Synchronisation of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following *in vitro* fertilisation. Biology of Reproduction. 57: 49-53.

Gall, L., V. De Smedt, N. crozet, D. Ruffini and C. Sevellec. 1996. Meiotically incompetent and competent goat oocytes: Timing of nuclear events and protein phosphorylation. Theriogenology. 46. 825-835.

Garcia-Rosello, E., P. Coy, F.A. Garcia Vazquez, S. Ruiz and C. Matas. 2006. Analysis of different factors influencing the intracytoplasmic sperm injection (ICSI) yield in pigs. Theriogenology. 66: 1857-1865.

Gardener, D.K. 1994. Mammalian embryo culture in the absence of serum or somatic cell support. Cell Biology International. 18: 1163-1179.

Gardener, D.K. 2004. The road to single embryo transfer. The Journal of Clinical Embryology. 7: 16-26.

Gates, A.H. 1971. Maximizing yield and developmental uniformity of eggs. *In*: Methods in Mamalian Embryology. *Editor*: J.C. Daniel. Freeman, San Francisco. pp. 86-116.

Gates, A.H. and J.L. Bozarth. 1978. Ovulation in the PMSG-treated immature mouse: Effect of dose, age, weight, puberty, season and strain (BALB/c, 129 and $C129F_1$ hybrid). Biology of Reproduction. 18: 407-505.

Geshi, M., C.R. Youngs and T. Nagai. 1999. Addition of cystiamine to a serum-free maturation medium enhances *in vitro* development of IVM-IVF bovine oocytes. Journal of Mammalian Ova Research. 16: 135-140.

Gilchrist, R.B. and J.G. Thompson. 2007. Oocyte maturation: emerging concepts and technologies to improve developmental potential *in vitro*. Theriogenology. 67: 6-15.

Giwercman, A. J. Richthoff, H. Hjoullund, J.P. Bonde, K. Jepson, B. Frohm and M. Spano. 2003. Fertility and Sterility. 80: 1404-1412.

Gjorret, J.O., H.M. Knijn, S.J. Dieleman, b. Avery, L.I. Larsson and P. Maddox-Hyttel. 2003. Chronology of apoptosis in bovine embryos produced *in vivo* and *in vitro*. Biology of Reproduction. 69: 1193-1200.

Goh, S.Y. 2008. Effect of strains of mice and their crossbreds on IVF performance. Thesis of Bachelor Science. University of Malaya, Kuala Lumpur.

Gomez, M.C., J.W. catt, G. Evans and W.M. Maxwell. 1998. Sheep oocyte activation after intracytoplasmic sperm injection (ICSI). Reproduction, Fertility and Development. 10: 197-205.

Gong, J,G., D.G. Armstrong, G. Bazter, C.O. Hogg. P.C. Garnsworthy and R. Webb. 2002. The effect of increased dietary intake on superovulatory response to FSH in heifers. Theriogenology. 57: 1591-1602.

Gonzalez-Bulnes, A., R.M. Garcia-Garcia, J. Santiago-Moreno, A. Lopez-Sebastian and M.J. Cocero. 2002. Effect of follicular status on superovulatory response in ewes is influenced by presence of corpus luteum at first FSH doe. Theriogenology. 58: 1607-1614.

Gonzalez-Bulnes, A., J.A. Carrizosa, C. Diaz-Delfa, R.M. Garcia-Garcia, B. Urrutia, J. Santiago-Moreno, M.J. Cocero and A. Lopez-Sebastian. 2003. Effects of ovarian follicular status on superovulatory response of dairy goats to FSH treatment. Small Ruminant Research. 48: 9-14.

Gonzalez-Bulnes, A., J. Santiago-Mareno, R.M. Gracia-Garcia, C.J. Souza, A. Lopez-Sebastian and A.S. McNeilly. 2004. Effects of GnRH antagonist treatment on gonadotrophin secreation, follicular development and inhibin A secretion in goats. Theriogenology. 61: 977-985.

Gonzalez-Bulnes, A., F. Berlinguer, F. M.J. Cocero, R.M. Garcia-Garcia, G. Leoni, S. Naitana, I. Rosati, S. Succu and A. Veiga-Lopez. 2005. Induction of the presence of corpus luteum during superovulatory treatments enhances *in vivo* and *in vitro* blastocyst output in sheep. Theriogenology. 64: 1392-1403.

Goosens, E., V. Fredickx, G. De Block, A.C. Van Steirteghem and H. Tournaye. 2003. Reproductive capacity of sperm obtained after germ cell transplantation in a mouse model. Human Reproduction. 18: 1874-1880.

Goto, K., A. Kinoshita, Y. Takuma and K. Ogawa. 1990. Fertilisation of bovine oocytes by the injection of immobilised, killed apermatozoa. Veterinary Research. 139: 494-495.

Goto, K.1993. Bovine microfertilisation and embryo transfer. Molecular Reproduction and Development. 36: 288-290.

Goto, K. 1997. Current status and future of micromanipulation-assisted fertilisation in animals and human. Journal of Reproduction and Development. 43: 107-119.

Graff, K.J., M. Meintjes, J.B. Paul, V.W. Dryer, R.S. Denniston, C.A. Ziomek and R.A. Godke. 1995. Ultrasound-guided oocyte recovery from FSH-treated goats for IVF. Theriogenology. 43: 223 (abstract).

Graff, K.J., M. Meintjes, V.W. Dyer, R.S. Denniston, C.A. Ziomek and R.A. Godke. 1999. Transvaginal ultrasound-quided oocyte retrieval following FSH stimulation of domestic goats. Theriogenology. 51: 1099-1119.

Greve, T., H. Callesen, P. Hyttel, R. Hoier and R. Assey. 1995. The effects of exogenous gonadotrophins on oocyte and embryo quality in cattle. Theriogenology. 43: 41-50.

Grupen, C.G., H. Nagashima and N.B. Nottle. 1997. Asynchronous meiotic progression in porcine oocytes matured *in vitro*: a cause of polyspermic fertilisation? Reproduction, Fertility and Development. 9: 187-191.

Gutierrez, C.G., J. Oldham, T.A. Bramley, J.G. Gong, B.K. Campbell and R. Webb. 1997. The recruitment of ovarian follicles is enhanced by increased dietary intake in heifer. Journal of Animal Science. 75: 1876-1884.

Gyu-Jin, R., S. Balasubramanian, K. Dong-Sik, S. Woo-Jin, C. Sang-Rae, K. Jung-Gon, B.K. Mohana and C. Sang-Young. 2007. Influence of *in vitro* oxygen concentrations on preimplatation embryo development, gene expression and production of hanwoo calves following embryo transfer. Molecular Reproduction and Development. 74: 486-496.

Hagemann, L.J., S.E. Beaumont, M.Berg, M.J. Donnison, A. Ledgard, A.J. Peterson, A. Schurmann and H.R. Tervit. 1999. Development during single IVP of bovine oocytes from dissected follicles: interactive effects of estrous cycle stage, follicle size and atresia. Molecular Reproduction and Development. 53: 451-458.

Hamano, K., X. Li, X. Qian, K. Funauchi, M. Furudate and Y. Minato. 1999. Gender preselectionin cattle with intracytoplasmic sperm injected, flow cytometrically sorted sperm heads. Biology of Reproduction. 60: 1994-1997.

Hamberger, L., A. Sjogren, K. Lundin, B. Soderlund and L. Nilsson, C. Bergh, W.U.B.M. Wikland, P. Svalander, A. H. Jakobsson and A.S. Forsberg. 1995. Microfertilization techniques- The Swedish experience. Reprod. Fertil. Dev., 7: 263-268.

Hammond, J. Jr. 1949. Recovery and culture of tubal mouse ova. Nature. 163:28-29.

Han, Z.B., G.C. Lan, Y.G. Wu, D. Han, W.G. Feng, J.Z. Wang and J.H. Tan. 2006. Interactive effects of granulose cell apoptosis, follicle size, cumulus-oocyte complex morphology, and cumulus expansion on the developmental competence of goat oocytes: a study using the well-in-drop culture system. Reproduction. 132: 749-758.

Hansen, P.J. 2007. Releasing the promise of IVF in cattle- an overview. Theriogenology. 65: 119-125.

Harada, M., T. Miyano, K. Matsumura, S. Osaki, M. Miyake and S. Kato. 1997. Bovine oocytes from early antral follicle grow to meiotic competence in vitro: effect of FSH and hypoxanthine. Theriogenology. 48: 743-755.

Harvey, A.J., K.L. Kind, M. Pantaleon, D.T. Armstrong and J.G. Thompson. 2004. Oxygen-regulated gene expression in bovine blastocysts. Biology of Reproduction. 71: 1108-1119.

Hashimoto, N. and T. Kishimoto. 1988. Regulation of meiotic metaphase by a cytoplasmic maturation-promoting factor during mouse oocyte maturation. Developmental Biology. 126: 242-252.

Hashlamoun, L.A. and G.J. Killian. 1985. Effect of timing of ovum recovery, cumulus cells, sperm preincubation time and pH on *in vitro* fertilization in C57BL/6mice. Archives of Andrology. 15: 159-171.

Hasler, J.F. 1992. Current status and potential of embryo transfer and reproductive technology in dairy cattle. Journal of Dairy Science. 75: 2857-2879.

Hatakeyama, M., M. Sawa and K. Oishi. 1994. fertilization by microinjection of cryopreserved sperm in the sawfly, *Athalia rosae* (Hymenoptera). Journal of Insect Physiology. 40: 909-912.

Hewitson, L., C. Simerly, T. Dominko and G. Schatten. 2000. Cellular and molecular events after *in vitro* fertilization and intracytoplasmic sperm injection. Theriogenology. 53: 79-85.

Hewitson, L., T. Dominko, D. Takashi, C. Martinovich, J. Ramalho-Santos, P. Sutovsky, J, Fanton, D. Jacob, D. Monteith, M. Neuringer, D. Battaglia, S.C. and G. Schatten. 1999. Unique checkpoints during first cell cycle of fertilization after intracytoplasmic sperm injection in rhesus monkey. Nature Medicine. 5: 431-433.

Hiller, S.G., A.K.S. Siddiquey and R.M.L. Winston. 1985. Fertilization *in vitro* of cumulus-enclosed mouse oocytes: effect of timing of the ovulatory hCG injection. International Journal of Fertility. 30: 34-38.

Hirabayash, M., M. Kato, T. Aoto, A. Sekimoto, M. Ueda, I. Miyoshi, N. Kasai and S. Hochi. 2002. Offspring delivered from intracytoplasmic injection of transgenic rat sperm. Transgenic Research. 11: 221-228.

Hiramoto, Y. 1962. Microinjection of live spermatozoa in sea urchin eggs. Journal of Experimental Cell Reseacrh. 27: 416 (abstract).

Hishinuma, M. and J. Sekine. 2004. Influence of theophylline supplementation of canine epididymal spermatozoa. Journal of Veterinary Medical Science. 66: 1417-1419.

Hogan, B., F. Constantini and E. Lacy. 1986. Manipulating the mouse embryos. A Laboratory Manual. 1st edition. Cold Spring Harbor Laboratory. USA.

Hogan, B., R. Beddington, F. Constantini and E. Lacy. 1994. Manipulating the mouse embryos. A laboratory manual. 2nd edition, Cold Spring Harbor Laboratory. USA.

Holness, D.H,m D.H. Hale and C.T. McCable. 1980. Ovarian response to pregnant mare serum gonadotrophin and prostaglandin $F_2$ in Africander and Meshona Cows.Theriogenology. 14: 375-381.

Holtz, W. and B. Sohnrey. 1992. Oestrus induction during the anoestrus season in goats by means of intravaginal pessaries or subcutaneous implants. *In*: Recent Advances in goat Production. *Editor*: R.R. Lokeshwar. Nutan rinters. New Delhi. pp. 1284-1289.

Hooper, K., M. Lane and D.K. Gardner. 2001. Reduced oxygen concentration increases mouse embryo development and oxidative metabolism. Theriogenology. 55: 334 (abstract).

Horiuchi, T., C. Emuta, Y. Yamauchi, T. Oikawa, T. Numabe and R. Yanagimachi. 2002. Birth of normal calves after intracytoplasmic sperm injection of bovine oocytes: A methodological approach. Theriogenology. 57: 1013-1024.

Hoshi, K., K. Yanagida, H. Yazawa, H. Katayose and A. Sato, 1994. Pregnancy and delivery after intracytoplasmic sperm injection of an immobilized, killed spermatozoon into an oocyte. Journal of Assisted Reproduction and Genetics. 11: 325-326.

Hosoi, Y. M. Miyake, K. Utsumi and A. Iritani. 1988. Development of rabbit oocytes after microinjection of spermatozoon. Proceedings of 11th Congerss on Animal Reproduction and Artificial Insemination, Dublin, Ireland. pp. 331-333.

Houghton, F.D. and H.J. Leese. 2004. Metabolism and developmental competence of the preimplantation embryo. European Journal of Obsterics and Gynecology and Reproductive Biology. 115: 92-96.

Huang, T., Y. Kimura and H. Yanagimachi. 1996. The use of piezo micromanipulation for intracytoplasmic sperm injection of human oocytes. Journal of Assisted Reproduction and Genetics. 13: 320-328.

Hunter, R.H.F. 1989. Aging of the unfertilized cow egg *in vivo*: how soon is fertility comprised. The Veterinary Record. 124: 489-490.

Hunter, R.H.F. and T. Greve. 1997. Could artificial insemination of cattle be more fruitful? Penalties associated with aging eggs. Reproduction in Domestic Animals. 32: 137-142.

Hwang, S., E. Lee, J. Yoon, B. Yoon, J. Lee, D. Choi. 2000. Effects of electric stimulation on bovine oocyte activation and embryo development in intracytoplasmic sperm injection procedure. Journal of Assisted Reproductive and Genetics. 17: 310–314.

Hyttel, P., T. Fair, H. Callesen and T. Greve. 1997. Oocyte growth, capacitation and final maturation in cattle. Theriogenology. 47: 23-32.

Inoue, A. R. Nakajiman, M. Nagata, and F. Aoki. 2008. Contribution of the oocytes nucleus and cytoplasm to the determination of meiotic and developmental competence in mice. Human Reproduction. 23: 1377-1384.

Iritani, A. 1991. Micromanipulation of gametes for *in vitro* assisted fertilisation. Molecular Reproduction and Development. 28: 199-207.

Iwasaki, S. and X. Li.1994. Experimental production of triploid bovine embryos by microinjection of two sperm and their development. Journal of Reproduction and development. 40: 317-322.

Izquierdo, D., P. Villamediana, M.J. Palomo, T. Mogas and M.T. Paramio. 1998. Effect of sperm capacitation and fertilization media on IVF and early embryo development of prepubertal goat oocytes. Theriogenology. 49: 1501-1513.

Izquierdo, D., P. Villamediana, M. Palomo, T. Mogas and M.T. Paramio. 1999. Effect of sperm capacitation and fertilization media on IVF and early embryo development of prepubertal goat oocytes. Theriogenology. 49: 1501-1513.

Izquierdo, D., P. Villamediana, M. Lopez-Bejar and M.T. Paramio. 2002. Effect of *in vitro* and *in vivo* culture on embryo development from prepubertal goat IVM-IVF oocytes. Theriogenology. 57: 1431-1441.

De Smedt, V., N. Crozet, M. Ahmed-Ali, A. Martino and Y. Cognie. 1992. *In vitro* maturation and fertilization of goat oocytes. Theriogenology. 37: 1049-1060.

Jainudeen, M.R., H. Wahid and E.S.E. Hafez. 2000. Sheep and goats. *In*: Reproduction in farm animals. *Editors*: B.Hafez and E.S.E. Hafez. Lippincott Eilliams and Wilkins, Philadelphia, USA. pp. 172-181.

Jimenez-Macedo, A.R., D. Izquierdo, B. Anguita and M.T. Paramio. 2005. Comparison between intracytoplasmic sperm injection and *in vitro* fertilisation employing oocytes derived from prepubertal goats. Theriogenology. 64: 1249-1262.

Jimenez-Macedo, A.R., B. Anguita, D. Izquierdo, T. Mogas and M.T. Paramio. 2006. Embryo development of prepubertal goat oocytes fertilised by intracytoplasmic sperm injection (ICSI) according to oocyte diameter. Theriogenology. 66: 1065-1072.

Jimenez-Macedo, A.R., M.T. Paramio, B. Anguita, R. Morato, R. Romaguera, T. Mogas and D. Izquierdo. 2007. Effect of ICSI and embryo biopsy on embryo development and apoptosis according to oocyte diameter in prepubertal goats. Theriogenology. 67: 1399-1408.

Joris, H.C., Z. Nagy, H. Van de Velde, A. De Vos and A.C. Van Steirteghem. 1998. Intracytoplasmic sperm injection: laboratory set-up and injection procedure. Human Reproduction. 3: 76-86.

Kahraman, S., K. Yakin, E. Donmez, H. Samli, M. Bahce, G. cengiz, S. Sertyel, M. Samli, and N. Imirzalioglu. 2000. Relationship between granular cytoplasm of oocytes and pregnancy outcome following intracytoplasmic sperm injection. Human Reproduction. 15: 2390-2393.

Kajihara, Y., N. Kometani, S. Kobayashi, Y. Koshiba, K. Hishiyama, K. Shiraiwai and K. Goto. 1990. Pregnancy rates and births after co-culture of cumulus cells with bovine embryos derived from *in vitro* fertilization of *in vitro* matured follicular oocytes. Theriogenology. 33: 264.

Kane. M.T. 1979. Fatty acids as energy sources for culture of one-cell rabbit ova to viable morulae. Biology of Reproduction. 20: 323-332.

Kane, M.T. 1985. A low molecular weight extract of bovine serum albumin stimulates rabbit blastocyst cell division and expansion *in vitro*. Journal of Reproduction and Fertility. 73: 147-150.

Karja, N.W., P. Wongsrikeao, M. Murakami, B. Agung, M. Fahrudin, T. Nagai and T. Otoi. 2004. Effects of oxygen tension on the development and quality of porcine *in vitro* fertilised embryos. Theriogenology. 62: 1585-1595.

Katayama, M., M. Koshida and M. Miyake. 2002. Fate of acrosome in ooplasmn in pigs after IVF and ICSI. Human Reproduction. 17: 2657-2664.

Katayose, H, K. Yanagida, T. Shinoki, T. Kawahara, T. Horiuchi and A. Sato. 1999. Efficient injection of bull spermatozoa into oocytes using a piezo-driven pipette. Theriogenology. 52: 1215-1224.

Kato, Y. and Y. Nagao. 2009. Effect of PVP on sperm capacitation status and embryonic development in cattle. Theriogenology. 72: 624-635.

Katska, L. And Z. Smorg. 1984. Number and quality of oocytes in relation to age of cattle. Animal Reproduction Science. 72: 624-635.

Katska-Ksiazkiewicz, L. B. Rynska, B. gajda and Z. Smorag. 2004. Effect of donor stimulation, frozen semen and heparin treatment in the efficiency of in vitro embryo production in goats. Theriogenology. 62: 576-586.

Katska-Ksiazkiewicz, L., J. Opiela and B. Rynska. 2007. Effect of oocyte quality, semen donor ans embryo co-culture system in the efficiency of blastocyst production in goats. Theriogenology. 62: 576-586.

Kartz-Jaffe, M.G., D.W. Linck, W.B. Schoolcraft and D.K. Gardner. 2005. A proteomic analysis of mammalian preimplantation embryonic development. Reproduction. 130: 899-905.

Kaufman, M. 1983. Early mammalian development: parthenogenetic studies. Cambridge University Press, Cambridge.

Kawakami, E., T. Arai and U. Nakamura. 1999. Effect of medium containing heparin andtheophylline on capacitation and metabolite enzyme activities of ejaculated spermatozoa from dogs with asthenozoospermia. Animal Reproduction Science. 54: 251-259.

Keefer, C.L. 1989. Fertilisation by sperm injection in the rabbit. Gamete Research. 22: 59-69.

Keefer, C.L., S.L. Stice, A.M. Paprocki and P. Golueke. 1994. *In vitro* culture of bovine IVM-IVF embryos: cooperative interaction among embryos and the role of growth factors. Theriogenology. 41: 1323-1331.

Kelly, R.W., K.E. Thompson, H. Hawker, S.F. Crosbie and J.C. McEwan. 1983. Live weight ovulation rate and wool growth responses of light and heavy ewes to differential feeding. New Zealand Journal of Experimental Agriculture. 11: 219-224.

Keskintepe, L., G.M. Darwish, A.I. Younis and B.G. Brackett. 1994. *In vitro* development of morulae from immature caprine oocytes. Zygote. 2: 97-102.

Keskintepe, L., G.C. Luvoni, S.J. Rzucidlo and B.G. Brackett. 1996. Procedural improvements for *in vitro* production of viable uterine stage caprine embryos. Small Ruminant Research. 20: 247-254.

Keskintepe, L., P. Morton, S.E. Smith, M.J. Tucker, A.A. Simplico and B.G. Bracket. 1997. Caprine blastocyst formation following intracytoplasmic sperm injection and defined medium. Zygote. 5: 261-265.

Kharche, S.D., A.K. Goel, S.K. Jindal and N.K. Sinha. 2006. In vitro maturation of caprine oocytes in different concentrations of oestrus goat serum. Small Ruminant research. 64: 186-189.

Kharche, S.D., A.K. Goel, S.K. Jindal, E.N. yadav, R. Sinha and N.K. Sinha. 2009. Effect of serum albumin supplementation on *in vitro* capacitation and fertilisation of caprine oocytes. Small Ruminant Research. 81: 85-89.

Khurana, N.K. and H. Niemann. 2000. Energy metabolism in preimplantation bovine embryos derived *in vitro* and *in vivo*. Biology of Reproduction. 62: 847-856.

Kim, J.H., K. Niwa, J.M. Lim and K. Okuda. 1993. Glucose requirements at different developmental stages of *in vitro* fertilised bovine embryos cultured in semi-defined medium. Theriogenology. 39: 875-886.

Kimura, Y. and R. Yanagimachi. 1995. Intracytoplasmic sperm injection in the mouse. Biology of Reproduction. 52: 709-720.

Kimura, Y., R. Yanagimachi, S.Kuratake, H. Bortkiewicz, A.C.F. Perry and H. Yanagimachi. 1998. Analysis of mouse oocyte activation suggest the involvement of sperm perinuclear material. Biology of Reproduction. 58: 1407-1415.

Kitagawa, Y., K. Suzuki, A. Yoneda and T. Watanabe. 2004. Effects of oxygen concentration and antioxidants on the *in vitro* development ability, production of reactive oxygen species (ROS) and DNA fragmentation on porcine embryos. Theriogenology. 62: 1186-1197.

Knijn, H.M., C. Wrenzycki, P.J. hendriksen, P.L. Vos, D. Herrmann, G.C. van der Weijden, H. Niemann and S.J. Dieleman. 2002. Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocyst derived *in vitro* and *in vivo*. Reproduction. 124: 365-375.

Koeman, J., C.L. Keefer, H. Baldassarre and B.R. Downey. 2003. Developmental competence of prepubertal and adult goat oocytes cultured in semi-defined media following following laparoscopic recovery. Theriogenology. 60: 870-879.

Kojima, N., T.T. Stumpf. A.S. Cupp, L.A. Werth, M.S. Roberson, M.W. Wolfe, R.J. Kittok and J.E. Kinder. 1992. Progestins and exogenous progesterone do not mimic the corpus luteum in regulation in LH and 17β-estradiol in circulation of cows. Biology Reproduction. 47: 1009.

Kojima, F.N., E.G.M. Bergfeld, M.E. Wehrman, A.S. Cupp, K.E. Fike, D.V. Mariscal-Aguayo, T. Sanchez-Torres, M. Garcia Winder, D.T. Clopton, A.J. Roberts and J.E Kinder. 2003. Frequency of luteinizing hormone pulses in cattle influences duration of persistence of dominant ovarian follicles, follicular fluid concentrations of streroids, and activity of insulin-like growth factor binding proteins. Animal Reproduction Science. 77: 187-211.

Kong, S.C. 2010. *In vitro* production of caprine embryos through intracytoplasmic sperm injection (ICSI) technique. MSc. Dissertation. University of Malaya, Kuala Lumpur.

Kono, T., K. Jones, A. Bos-Mikich. D.G. Whittingham, and Carroll, J. 1996. A cell cycle-associated change in Ca2/tranembryonic viability. sients in mouse embryos during the first mitotic division. Journal Cell of Biology. 132: 915–923.

Krisher, R.L., M. Lane and B.D. Bavister.. 1999. Developmental competence and metabolism of bovine embryos cultured in semi-defined and defined culture media. Biology of Reproduction. 60: 1345-1352.

Krisher, R.L. 2004. The effect of oocyte quality on development. Journal of Animal Science. 82: 14-23.

Kubiak, J.Z. 1989. Mouse oocytes gradually develop the capacity for activation during the metaphase II arrest. Development of Biology. 136: 537-545.

Kuhholzer, B., S. Muller, A. Treuer, J. Seregi, U. Besenfelder and G. Brem. 1997. Repeated endoscopic ovum pick-up in hormonally untreated ewes: a new technique. Theriogenology. 48: 545-550.

Kuretake, S., Y. Kimura, K. Hoshi and R. yanagimachi. 1996. Fertilization and development of mouse oocytes injected with isolated sperm heads. Biology of reproduction. 55: 789-795.

Lacham-Kaplan, O. and A. Trounson. 1995. Intracytoplasmic sperm injection in mice: increased fertilization and development to term after induction of the acrosome reaction. Human Reproduction. 10: 2642-2649.

Lacham-Kaplan, O. and A. Trounson. 2003. Oocyte activation after intracytoplasmic sperm injection with frozen sperm without cryoprotectants results in live offspring from inbred and hybrid mouse strains. Biology of Reproduction. 69: 1683-1689.

Lambert, R.U., M.A. Sirard, C. Bernard, R. Beland, J.E. Rioux, P. Leclere, D.P. Menard and M. Bedoya. 1986. *In vitro* fertilisation of bovine oocytes matured *in vivo* and collected at laparoscopy. Theriogenology. 25: 117-133.

Landau, S., R. Braw-Tal, M. Kaim, A. Bor and I. Bruckental. 2000. Preovulatory follicular status and diet affect the insulin and glucose content of follicles in high-yielding dairy cows. Animal Reproduction Science. 64: 181-197.

Lane, M. and D.K. Gardner. 1992. Effect of incubation volume and embryo density on the development and viability of mouse embryos *in vitro*. Human Reproduction. 7: 558-562.

Lane, M. 2001. Mechanisms for managing cellular and homeostatic stress *in vitro*. Theriogenology. 55: 225-236.

Lane, M. and D.K. Gardener. 2005. Understanding cellular distruptions during early embryo development that perturb viability and fetal development. Reproduction, fertility and Development. 17: 371-378.

Lazzari, G., C. Galli and R.M. Moor. 1994. Functional changes in teh somatic and germinal compartments during follicle growth in pigs. Animal Reproduction Science. 35: 119-130.

Le Gal, F., L. Gall and V. De Smedt. 1992. Changes in protein sysnthesis pattern during *in vitro* maturation of goat oocytes. Molecular Reproduction and Development. 32: 1-8.

Lechniak, D., D. Kaczmarek, D. Stanisawski and T. Adamowicz. 2002. The ploidy of *in vitro* matured bovine oocyte. Theriogenology. 57: 1303-1308.

Leclerc, P., M.A. Sirard, J.G. Chafouleas and R.D. Lambert. 1992. Decrease in calmodulin concentaryions during heparin-induced capacitation in bovine spermatozoa. Journals of Reproduction and Fertility Ltd. 94: 23-32.

Lee, S.H, H. Song, Y.S. Park, M.K. Koong, I.O. Song and J.H. Jun. 2009. Poor sperm quality affects clinical outcomes of intracytoplasmic sperm injection in fresh and subsequent frozen-thawed cycles: potential paternal effects on pregnancy outcomes. Fertility and Sterility. 91: 778-804.

Legge, M and M.H. Sellens. 1991. Free radicals scavengers ameliorate the two-cell block in mouse embryo. Human Reproduction. 6: 867-871.

Lehloenya, K.C. and J.P.C. Greyling. 2009. Effect of route of superovulatory gonadotrophin administration on embryo recovery rate of Boer goat does. Small ruminant Research. 87: 39-44.

Lehtonen, E. and R. Kankondi. 1987. Rate of gonadotrophin-induced abnormalities in mouse ova is related to the site of hormone administration. Journal of Reproduction and Fertility. 80: 613-617.

Lerner, S.P., W.V. Thayne, B.D. Baker, T. Henschen, S. Meredith, E.K. Inskeep, R.A. Daily, P.A. Lewis and R.L. Butcher. 1986. Age, dose of FSH and other factors affecting superovulation in Holstein cows. Jpurnal of Animal Science. 63: 176-183.

Leyva, V., Buckrell, B.C. and J.S. Walton. 1998. Regulation of follicular activity and ovulation in ewes by exogenous progestagen. Theriogenology. 50: 395-416.

Lim, J.M., J.H. Kim, K. Okuda and K. Niwa. 1993. Effect of the presence of glucose during fertilisation and/or culture in a chemically semi-defined medium on the development of bovine oocytes matured and fertilised *in vitro*. Journal of Reproduction and Development. 39: 237-242.

Lim, J.M., B.C. Reggio, R.A. Godke and W. Hansel. 1999. Development of *in vitro* derived bovine embryos cultured in 5% $CO_2$ in air or in 5% $O_2$, 5% $CO_2$ and 90% $N_2$. Human Reproduction. 14: 458-464.

Lin, T.P. 1966. Microinjection of mouse oocytes. Science. 151: 333 (abstract).

Liu, L. And X. Yang. 1996. Interplay of maturation-promoting factor and mitogen-activated protein kinase inactivation during metaphase- to -interphase transition of activated bovine oocytes. Biology of Reproduction. 61: 1-7.

Liu, R., A. Rybouchkin, J. Van der Elst and M. Dhont. 2002. Fertilization of mouse oocytes from *in vitro* matured preantral follicles using classical *in vitro* fertilization or intracytoplasmic sperm injection. Biology of Reproduction. 67: 575-579.

Lonergan, P., P. Monagham, D. Rizos, M.P. Boland and L. Gordon. 1994. Effect of follicle size if bovine oocyte quality and developmental competence following maturation, fertilisation and culture *in vitro*. Molecular Reproduction and Development. 37: 48-53.

Lonergan, P., H. Khatir, C. Carolan and P.Mermillod. 1997. Bovine blastocyst production *in vitro* after inhibition of oocyte meiotic resumption for 24 h. Journal if Reproduction and Fertility. 109: 355-365.

Lopes, A.S., C. Wrenzycki, N.B. Ramsing, D. Herrmann, H. Niemann, P. Lovendahl, T. Greve and H. Callesen. 2007. Respiration rates correlate with mRNA expression of G6PD and GLUT1 genes in individual bovine *in vitro* produced blastocyst. Theriogenology. 68: 223-236.

Loughlin K.R. and A. Agarwal. 1992. Use of theophylline to enhance sperm function. Archives of Andrology. 28: 99-103.

Luvoni, G.C., L. Keskintepe and B.G. Brackett. 1996. Improvement in bovine embryo production *in vitro* by gluthathione-containing culture media. Molecular Reproduction and Development. 43: 437-443.

Lv, L.H., W.B. Yue, W.Z. Liu, Y.S. Ren, F.Z. Li, K.B. Lee and G.W. Smith. 2010. Effect of oocyte selection, oestradiol and antioxidant treatment on in vitro maturation of oocytes collected from prepubertal Boer goats. Italian Journal of Animal Science. 9: 50-54.

Ma, V., N. Crozet and L. Gall. 1994. Morphological and functional changes accompanying the acquisition of meiotic competence of ovarian goat oocytes. Journal of Experimental Zoology. 269: 128-139.

Macas, E., B. Imthurn, M. Roselli, and P.J. Keller. 1996. The chromosomal complements of multipronuclear human zygotes resulting from intracytoplasmic sperm injection. Human Reproduction. 11: 2496-2501.

Magarey, G.M. and K.E. Mate. 2003. Fertilization following intracytoplasmic sperm injection of *in vivo* and *in vitro* matured oocytes from an Australian marsupial, the tammar wallaby (*Macropus eugenii*). Zygote. 11: 339-346.

Mahmood, S., G.L. Koul and J.C. Biswas. 1991. Comparative efficacy of FSH-P and PMSG on superovulation in Pashmina goats. Theriogenology. 35: 1191-1196.

Malhi, P.S., G.P. Adams, R.J. Mapleatoft and J. Singh. 2008. Superovulatory response in a bovine model of reproductive aging. Animal Reproduction Science. 66: 1257-1266.

Malik, R.K., I.S. Lohan, O.P. Dhanda, O.K. Hooda and S. Singh. 1999. Peritoneal fluid from rabbits or goats as media for *in vitro* maturation, fertilisation and initial culture of caprine oocytes. Animal Reproduction Science. 54: 195-201.

Mansour, R.T., M.A. Aboulghar and G.I. Serour. 1994. Study of the optimum time for human chorionic gonadotrophin-ovum pick-up interval in *in vitro* fertilization. Journal of Assisted Reproduction and Genetics. 11: 478-481.

Mansour, R.T., Aboulghar M.A., G.I. Serour, Y.M. Amin and A.M Ramzi. 1995. The effect of sperm parameters on the outcome of intracytoplasmic sperm injection. Fertility and Sterility. 64: 982-986.

Mantovani, R., W.J. Enright, M.G. Keane, J.F. Roche and M.P. Bolan. 1993. Effect of nutrition and dose of follicle stimulating hormone (FSH) on superovulatory response in beef heifers. Proceedings of the 9th AETE Meeting. Lyon, France. p. 234 (abstract).

Markert, C.L. 1983. Fertilisation of mammalian eggs by sperm injection. Journal of Experimental Zoology. 228: 195-201.

Marston, J.H. and M.C. Chang. 1964. The fertilisable life of ova and their morphology following delayed insemination in mature and immature mice. Journal of Experimental Zoology. 15: 237-251.

Martino, A., T. Mogas, M.J. Palomo and M.T. Paramio. 1994. Meiotic competence of prepubertal goat oocytes. Theriogenology. 41: 473-485.

Martino, A., T. Mogas, M.J. Palomo and M.T. Paramio. 1995. *In vitro* maturation and fertilisation of prepubertal goat oocytes. Theriogenology. 43: 473-485.

Martin, M.J. 2000. Development of in vivo-matured porcine oocytes following intracytoplasmic sperm injection. Biology of Reproduction. 63: 109-112.

Martin-Coello, J., R. Gonzalez, C. Crespo, M. Gomendio and E.R.S. Roldan. 2008. Superovulation and *in vitro* oocyte maturation in three species of mice (*Mus musculus, Mus spretus* and *Mus spicilegus*). Theriogenology. 70: 1004-1013.

Masui, Y. and C. Markert. 1971. Cytoplasmic control of nuclear behaviour during meiotic maturation of frog oocytes. Journal of Experimental Zoology. 177: 129-146.

Mayor, P., M. Lopez-Bejar, E. Rodriguez-Gonzalez and M.T. Paramio. 2001. Effects of the addition of glutathione during maturation on in vitro fertilisation of prepubertal goat oocytes. Zygote. 9: 323-330.

McEvoy, T.G., J.J. Robinson, R.P. Aitken, P.A. Findlay, R.M. Palmer and I.S. Robertson. 1995. Dietary-induced suppression of pre-ovulatory progesterone concentrations in superovulated ewes impairs the subsequent *in vivo* and *in vitro* development of their ova. Animal Reproduction Science. 39: 89-107.

McGaughey, R.W. and C. Polge. 1972. Cytogenetic analysis of pig oocytes matured *in vitro*. Journal of Experimental Zoology. 176: 155-162.

McGaughey, R.W. 1977. The maturation of oocytes in minimal defined culture media with varied macromolecular supplements and varied osmolarity. Experimental Cell Research. 109: 25-30.

McNeilly, A.S. 1985. Effect of charges in FSH induced by bovine follicular fluid and FSH infusion in the preovulatory phase on subsequent ovulation rate and corpus luteum function in the ewe. Journal of Reproduction and fertility. 74: 661-668.

Medan, M., A.H. Shalaby, S. Sharawy, G. Watanabe and K. Taya. 2002. Induction of oestrus during the non-breeding season in Egyptian Baladi goats. Journal of Veterinary Medical Science. 64: 83-85.

Melican, D. and W.G. Garvin. 2008. Repeated superovulation, non-surgical embryo recovery, and surgical embryo transfer in transgenic dairy goats. Theriogenology. 69: 197-203.

Mellado, M., R. Olivas and F. Ruiz. 2000. Effect of buck stimulus on mature and prepubertal norgestomet-treated goats. Small Ruminant Research. 36: 269-274.

Menchaca, A. and Rubianes. 2001 . Effect of high progesterone concentration during the early luteal phase on the length of the ovulatory cycle of goats. Animal Reproduction Science. 68: 69-76.

Menezo, Y.J. 2006. Paternal and maternal factors in preimplantation embryogenesis: interaction with the biochemical environment. Reproductive Biomedical Online. 12: 616-621.

Mermillod, P., B. Oussaid and Y. Cognie. 1999. Aspects of follicular and oocyte maturation that effect the developmental potential of embryos. Journal of Reproduction and Fertility. 54: 449-460.

Miao, Y., S. Ma, X. Liu, D. Miao, Z. Chang, M.J. Luo and J. Tan. 2004. Fate of the first polar bodies in mouse oocytes. Molecular Reproductive Development. 69: 66-76.

Miao, Y.L., K. Kikuchi, Q.Y. Sun and H. Schatten. 2009. Oocyte aging: cellular and molecular changes, developmental potential and reversal possibility. Human Reproduction. 15 (5): 573-585.

Mihm, M. And E.C. Bleach. 2003. Endocrine regulation of ovarian antral follicle development in cattle. Animal Reproduction Science. 78: 217-237.

Miller J.E. and T.T. Smith. 2001. The effect of intracytoplasmic sperm injection and semen parameters on blastocyst development *in vitro*. Human Reproduction. 16: 918-924.

Miller, B.G., and D.T. Armstrong. 1981. Effects of a superovulatory dose of pregnant mare serum gonadotrophin on ovarian function, serum oestradiol and progesterone levels and early embryo development in immature rats. Biology of Reproduction. 25: 261-271.

Ming-Wen, L. And K.C.K. Lloyd. 2006. Intracytoplasmic Sperm Injection in Mouse. *In*: Principle and Practice, Mamamalian and Avian Transgenesis-New Approaches. *Editors*: S. Pease and C.Lois. Springer-Verlag, Berlin Heidelberg. pp. 23-40.

Mizoguchi, H. And W.R. Dukelow. 1980. Effect of timing of hCG injection on fertilization in superovulated hamsters. Biology of Reproduction. 23: 237-241.

Moessner, J. and W.C. Dodson. 1995. The quality of human embryo growth is improved when embryos are cultured in groups rather than separately. Fertility and Sterility. 64: 1034-1035.

Mogas, T., A. Martino, M.J. Palomo and M.T. Paramio. 1997. Effect of method of recovery on the number and type of oocytes obtained for IVM. Journal of Reproduction and Fertility. 9:52 (abstract).

Mohd Noor Hisham, M.N. 2006. Effect of oestrus synchronisation and superovulation on progesterone and oestradiol levels in relation to oocytes recovery in goats. Institute of Biological Sciences. MSc Dissertation. The University of Malaya, Kuala Lumpur.

Monniaux, D., D. Chupin and J. Saumande. 1983. Superovulatory response in cattle. Theriogenology. 19: 55-81.

Montag, M., V. Tok, S.L. Liow, A. Bongso and S.C. Ng. 1992. *In vitro* decondensation of mammalian sperm and subsequent formation of pronuclei-like structures for manipulation. Molecular Reproduction and Development. 33: 338-346.

Montag, M., K. Rink, G. Delacretaz and H. van der Ven. 2000. Laser-induced immobilization and plasma membrane permeabilization in human spermatozoa. Human Reproduction. 15: 846-852.

Moor, R.M., J.C. Osborn and I.M. Crosby. 1985. Gonadotrophin-induced abnormalities in sheep oocytes adter superovulation. Journal of Reproduction and Fertility. 74: 167-172.

Morel, M.C.D., J.R. Newcombe and J.C. Swindlehurst. 2005. The effect of age on multiple ovulation rates, multiple pregnancy rates and embryonic vesicle diameter in the mare. Theriogenology. 63: 2482-2493.

Morris, L.H.A. and W.R. Allen. 2002. Reproductive efficiency of intensively managed through bred mares in mares in Newmarket. Equine Veterinary Journal. 34: 51-60.

Motlik, J. and J. Fulka. 1976. Breakdown of the germinal vesicle in pig oocytes in vivo and in vitro. Journal of Experimental Zoology. 198: 155-162.

Nagai, T., M. Ebihara, A. Onishi and M. Kubo. 1997. Germinal vesicle stages in pig follicular oocytes collected by different methods. Journal of Reproduction and Development. 43: 339-343.

Nagy, Z.P., J. Liu, H. Joris, G. Verheyen, H. Tournaye and M. Camus. 1995. The results of intracytoplasmic sperm injection is not related to any of three basic sperm parameters. Human Reproduction. 10: 1123-1129.

Nagy, A., M. Gertsenstein, K. Vintersten and R. Behringer. 2003. Manipulating the Mouse Embryo: A Laboratory Manual. Third edition. Cold Spring Harbour Laboratory Press, New York, USA.

Nakagawa, K., S. Yamano, N. Moride, M. Yamashita, M. Yoshizawa and T. Aono. 2001. Effect of activation with Ca ionophore A23187 and puromycin on the development of human oocytes that failed to fertilize after intracytoplasmic sperm injection. Fertility and Sterility. 76: 148-152.

Nasr-Esfahani, M.H. and M.H. Johnson. 1992. Effects of glucose, glutamine, ethylenediaminetetraacetic acid and oxygen tension on the concentration of the reactive oxygen species and on development of the mouse preimplantation embryo *in vitro*. Journal of Reproduction and Fertility. 96: 219-231.

Negrao, S., M. Nibart and P. Humblot. 1997. Negative effects of overfeeding on superovulatory response and embryo production in dairy heifers. Proceeding of the 13th AETE Meeting. Lyon, France. p. 186 (abstract).

Ng, S.C., P. Martelli, S.L. Liow, S. Herbert and S.H. Oh. 2002. Intracytoplasmic injection of frozen-thawed epididymal spermatozoa in a nonhuman primate model, the cynomolgus monkey (*Macaca fascicularis*). Theriogenology. 58: 1385-1397.

Nijs, M., P. Vanderzwalmen, B. Vandamme, G. Segal-Bertin, B. Lejeune, L. Segal, E. van Roosendaal and R. Schoysman. 1996. Fertilizing ability of immotile spermatozoa after intracytoplasmic sperm injection. Human Reproduction. 11: 2180-22185.

Noel, B., J.L. Bister, B. Pierquin and R. Paquay. 1994. Effects of FGA and PMSG on follicular growth and LH secretion in Suffolk ewes. Theriogenology. 41: 719-727.

Nonogaki, T., Y. Noda, K. Narimoto, Y. Umaoka and T. Mori. 1991. Protection from oxidative stress by thioredoxin and superoxide dismutase of mouse embryos fertilized *in vitro.* Human Reproduction. 6: 1305-1310.

Nowshari, M.A., E. Yuswiati, M. Puls-Kleingeld and W. Holtz. 1992. Superovulation in prepubertal and adult goats treated with PMSG or pFSH. *In:* Recent Advances in Goat Production. *Editor:* R.R. Lokeshwar. Nutan Printers. New Delhi. pp. 1358-1363.

Nuti, L.C., B.S. Minhas, W.C. Baker, J.S. Capehart and P. Marrack. 1987. Superovulation and recovery of zygotes from Nubian and Alphine dairy goats. Theriogenology. 28: 481-488.

O'Callaghan, D., H. Yaakub, P. Hyttel, L.J. Spicer and M.P. Boland. 2000. Effect of nutrition and superovulation on oocyte morphology, follicular fluid composition and systemic hormone concentrations in ewes. Journal of Reproduction and Fertility. 118: 303-313.

Ocampo, M.B., L.C. Ocampo, I.S. Ryu, T. Mori, J. Ueda and H, Kanagawa. 1993. Effects of culture time, ovarian activity and sera in the nuclear and cytoplasmic maturation of pig oocytes in vitro. Animal Reproduction Science. 34: 135-146.

Ock, S.A., J.S. Bhak, S.Balasubramanian, H.J. Lee, S.Y. Choe and G.J. Rho. 2003. Different activation treatments for successful development of bovine oocytes following intracytoplasmic sperm injection. Zygote. 11:69-76.

Ogura , A., K. Inoue and J.Matsuda. 1999. Mouse spermatid nuclei can support full term development after premature chromosome condensation within mature oocytes. Human Reproduction. 14: 1294-1298.

Ohta, H., Y. Sakaide and T. Wakayama. 2009. Age- and substrain-dependent sperm abnormalities in BALB/c mice and functional assessment of abnormal sperm by ICSI. Human Reproduction. 24: 775-781.

Oikawa, T., N. Takada, T. Kikuchi, T. Numabe, M. Takenaka and T. Horiuchi. 2005. Evaluation of activation treatments for blastocyst production and birth of viable calves following bovine intracytoplasmic sperm injection. Animal Reproduction Science. 86: 187-194.

Oliveira, M.A.L., A.I. Guido and P.F. Lima. 2001. Comparison of different protocols used to induce and synchronise oestrus cycle of Saanen goats. Small Ruminant Research. 40: 149-153.

Ongeri, E.M., C.L. Bormann, R.E. Butler, D. Melican, W.G. Gavin, Y. Echelard, R.L. Krisher and E. Bohboodi. 2001. Development of goat embryos after *in vitro* fertilisation and parthenogenetic activation by different methods. Theriogenology. 55: 1933-1945.

Orsi, N.M. and H.J. Leese. 2001. Protection against reactive oxygen species during mouse preimplantation embryo development: role of EDTA, oxygen tension, catalase superoxide dismutase and pyruvate. Molecular Reproduction and Development. 59:44-53.

Ozgunen, K.T., S. Erdogan, N. Mazmanoglu, I. Pamuk, G. Logoglu and T. Ozgunen. 2001. Effect of gonadothrophin dose on oocyte retrieval in superovulated BALB/c mice. Theriogenology. 56: 435-445.

Palermo, G., H. Joris, M.P. Derde and A.C. van Steirteghem. 1992. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet. 340: 17-18.

Palermo, G., J. Cohen, M. Alikani, A. Adler and Z. Rosenwaks. 1995. Development and implementation of intracytoplasmic sperm injection (ICSI). Reproduction, Fertility and Development. 7: 211-218.

Palermo, G.D., M. Alikani, M. Bertoli, L.T. Colombero, F. Moy, J. Cohen and Z. Rosenwaks. 1996. Oolema characteristics in relation to survival and fertilisation patterns of oocytes treated by intracytoplasmic sperm injection. Human Reproduction. 11: 172-176.

Palermo, G., G. Ergun, T. Takeuchi. 1998. ICSI outcome by the number of embryos replaced. *In*: Treatment of infertility: the new frontiers. *Editors*: M. Filcori and C. Flamigni. Communications Media for Education, Princeton Junction. USA. pp. 283-290.

Palma, G.A. and F. Sinowatz. 2004. Male and female effects on the *in vitro* production of bovine embryos. Anatomia, Hstologia, Embryologia. 33: 257-262.

Palomo, G.D., M. D. Izquierdo, T. Mogas and M.T. Paramio. 1999. Effect of semen preparation on IVF of prepubertal goat oocytes. Theriogenology. 51: 927-940.

Papadopoulus, S., P. Lonergan, V. Gath, K.M. Quinn, A.C. Evans, D. O'Callaghan and M.P. Bolan. 2001. Effect of diet quantity and urea supplementation on oocyte and embryo quality in sheep. Theriogenology. 55: 1059-1069.

Parish, J.J., J.L. Susko-Parrish, R.R. Handrow, M.M. Sims and N.L. First. 1989. Capacitation of bovine spermatozoa by oviduct fluid. Biology of Reproductive. 40: 1020-1025.

Parr, R.A., I.F. Davis, R.J. Fairclough and M.A. Miles. 1987. Overfeeding during early pregnancy reduced peripheral progesterone concentration and pregnancy rate in sheep. Journal of Reproduction and Fertility. 80: 317-320.

Pavlok, A., A. Lucas-Hahn and H. Niemann. 1992. Fertilisation and developmental competence of bovine oocytes derived from different categories of antral follicles. Molecular Reproduction and development. 31: 63-67.

Pawshe, C.H., S.M. Totey, and S.K. Jain. 1994. A comparison of three methods of recovery of goat oocytes for *in vitro* maturation. Theriogenology. 42: 307-320.

Pawshe, C.H., A. Palanisamy, M. Taneja, S.K. Jain and S.M. Totey. 1996. Comparison of various maturation treatments on *in vitro* maturation of goat oocytes and their early embryonic development and cell numbers. Theriogenology. 42: 117-125.

Pawshe, C.H. and S.M. Totey. 2003. *In vitro* maturation, fertilization and embryo of goat oocytes: A review. Indian Journal of Animal Science. 73: 615-619.

Payne, D. 1995. Micro-asissted fertilization. Reproduction, Fertility and Development. 7: 831-839.

Perrault, S.D., R.R. Barbee, K.H. Elstein, R.M. Zucker and C.L. Keefer. 1988. Interspecies differences in the stability of mammalian sperm nuclei assessed *in vivo* by microinjection and *in vitro* by flow cytometry. Biology of Reproduction. 39: 157-167.

Perreault, S.D., R.R. Barbee, K.H. Elstein, R.M. Zucker and C.L. Keefer, 1988. Interspecies differences in the stability of mammalian sperm nuclei assessed *in vivo* by sperm microinjection and *in vitro* by flow cytometry. Biology Reproduction., 39: 157-167.

Perry, A.C., A. Rothman, J.I. de las Heras, P. Freinstein, P. Mombearts, H.J. Cooke and T. Wakayama. 2001. Efficient metaphase II transgenesis with different transgene archetypes. Nature Biotechnology. 19: 1071-1073.

Perry, A.C., T. Wakayama, H. Kishikawa, T. Kasai, M. Okabe, Y. Toyoda and R. Yanagimachi. 1999. Mammalian transgenesis by intracytoplasmic sperm injection. Science. 14: 180-183.

Petyim, S., R. bage, M. Forsberg, H. Rodriguez-Martinez and B. Larsson. 2001. Effects of repeated follicular puncture on ovarian morphology and endocrine parameters in dairy heifers. Jpurnal of Veterinary Medicine. A, Physiology, Phatology, Clinical Medicine. 48: 449-463.

Phua, A.C.Y. 2006. Developmental of a PCR-based method for sex determination of caprine embryos produced from *in vitro* maturation, fertilisation and culture techniques. MSc. Dissertation. University of Malaya, Kuala Lumpur.

Picazo, R.A., M.J. Cacero, M.L. Barragan and A. Lopez-Sebastian. 1996. Effects of LH administration at the end of an FSH superovulatory regimen on ovulation rate and embryo production in the three breeds of sheep. Theriogenology. 45: 1065-1073.

Pierson, J., B. Wang, N. Neveu, L. Sneek, F, Cote, C.N. Karatzas and H. Baladassarre. 2004. Effect of repetition, interval between treatments and season on the results from laparoscopic ovum pick-upin goats. Reproduction Fertility and Development. 16: 795-799.

Pincus, G. And E.V. Enzmann. 1935. The comparative behaviour of mammalian eggs *in vivo* and *in vitro*. Journal of Experimental Medicine. 62: 665-675.

Pintado, B., A. Gutierrez-Adan and B. Perez Llano. 1998. Superovulatory response of Muciana goats to treatments based on PMSG/ anti-PMSG or combined FSH/ PMSG administration. Theriogenology. 50: 357-364.

Piper, L.R., B.M. Bindon, Y.M. Curtis, M.A. Cheers and R.D. Nethery. 1982. Response to PMSG in Merino and Booroola Merino crosses. Proceedings of the Australian Society for Reproductive Biology. 14: 82-89.

Pollard, J.W. and S.P. Leibo. 1994. Chilling sensitivity of mammalian embryos. Theriogenology. 41: 101-106.

Pool, T.B. 2004. Development of culture media for human assisted reproductive technology. Fertility and Sterility. 81: 287-289.

Pope, C.E., C.A. Johnson, M.A. McRae, G.L. Keller and B.L. Dresser. 1998. Development of embryos produced by intracytoplasmic sperm injection of cat oocytes. Animal Reproduction Science. 53: 221-236.

Prakash, N., K. Narayana, P. Pignon and Y. Cognie. 1996. Disposition kinetics of bufflalo pituitary FSH following single intramuscular administration in female buffalo calves (*Bubalus bubalis*). Buffalo Bulletin. 18: 86-91.

Probst, S. and D. Rath. 2003. Production of piglets using intracytoplasmic sperm injection (ICSI) with flowcytometrical sorted boar semen and artificially activated oocytes. Theriogenology. 59: 961-973.

Qian, Y., W.Q. Shi, J.T. Ding, J.H. Sha and B.Q. Fan. 2003. Predictive value of the area of expanded cumulus mass on development of porcine oocytes matured and fertilised *in vitro*. Journal of Rerproduction and Development. 49. 167-174.

Quinn, P. 1995. Enhanced results in mouse and human embryo culture using a modified human tubal fluid medium lacking glucose and phosphate. Journal of Assisted Reproduction and Gamete. 12: 97-105.

Rahman, A.N.M.A, R.B. Abdullah, W.E. Wan Khadijah. 2007. Goat embryo development from *in vitro* matured ooctes of heterogeneous quality through intracytoplasmic sperm injection technique. Biotechnology. 6: 373-382.

Rahman, A.N.M.A. 2008a. Goat embryo production from *in vitro* matured heterogeneous oocytes fertilised by intracytoplasmic sperm injection (ICSI) technique. PhD Thesis. University of Malaya, Kuala Lumpur.

Rahman, A.N.M.A, R.B. Abdullah, W.E. Wan Khadijah. 2008b. Recovery and grading of oocytes with special reference to laparoscopic ovum pick-up technique: A review. Biotechnology. 7: 612-622.

Rajikin, M.H., M. Yusoff and R.B. Abdullah. 1994. Ultrastructural studies of developing goat oocytes *in vitro*. Theriogenology. 42: 1003-1016.

Rajikin, M.H. 1995. *In vitro* studies of maturation and fertilisation of oocytes and subsequent culture of embryos in goats. PhD Thesis. University of Malaya. Kuala Lumpur, Malaysia.

Ramalho-Santos, J., P. Sutovsky, C. Simerly, R. Oko, G.M. Wessel, L. Hewtson and G. Schatten. 2000. ICSI choreography:fate of sperm structures after monospermic rhesus ICSI and first cell cycle implications. Human Reproduction. 15: 2610-2620.

Ratky, J., D. Rath and K.P. Brussow. 2003. *In vitro* fertilization of *in vivo* matured porcine oocytes obtained from prepubertal gilts at different time interval after hCG injection. Acta Veterunaria Hungarica. 51: 91-101.

Redgment, C.J., D. Yang, M. Tsirigotis, N. Yazdani, T. Al Shawaf and I.L. Craft. 1994. Experience with assisted fertilization in severe male factor infertility and unexplained failed fertilization *in vitro*. Human Reproduction. 9: 680-683.

Reiger,D., N.M. Laskutoff and K.J. Betteridge. 1992. Developmentally related changes in the metabolism of glucose and glutamine by cattle embryos produced and co-cultured *in vitro*. Journal of Reproduction and Fertility. 95: 585-595.

Rhind, S.M., W.A.C. McKelvey, S.R. McMillen, R.G. Gunn and D.A. Elston. 1989. Effect of restricted food intake, before and/or after mating, on the reproductive performance of Greyface ewes. Animal Production. 48: 149-155.

Rho, G.J., B. Wu, S. Kawarsky, S.P. Leibo and K.J. Betteridge. 1998. Activation regimes to prepare bovine oocytes for intracytoplasmic sperm injection. Molecular Reproduction and Development. 50: 485-492.

Rho, G.J., A.C. hahnel and K.J. Betteridge. 2001. Comparisons if oocyte maturation times and of three methods of sperm preparation for their effects on the production of goat embryos *in vitro*. Theriogenology. 56: 503-516.

Rienzi, L., F.M. Ubaldi, M. Iacobelli, M.G. Minasi, S. Romano, S. Ferero, F. Sapienza, E. Baroni, K. Litwicka, and E. Greco. 2008. Significance of metaphase II human oocyte morphology on ICSI outcome. 90: 1692-1700.

Ritar, A.J., S. Salamon, P.D. ball and P.J. O'May. 1989. Ovulation and fertility in goats after inravaginal device-PMSG treatment. Small Ruminant Research. 2: 323-331.

Rodriguez-Gonzalez, E., M. Lopez-Bejar, D. Izquierdo and M.T. Paramio. 2003. Developmental competence of prepubertal goat oocytes selected with brillian cresyl

blue and matured with cysteamine supplementation. Reproduction, Nutrition and Development. 43: 179-187.

Rosenbush, B. and K. Sterzik. 1996. Irregular chromosome segregation following ICSI? Human Reproduction. 11: 2337-2338.

Rosenkrans, C.F. and N.L. First. 1991. Culture of bovine zygotes to the blastocyst stage: effect of amino acids and vitamins. Theriogenology. 35: 266.

Rubianes, E. and A. Menchaca. 2003. The pattern and manipulation of ovarian follicular growth in goats. Animal Reproduction Science. 78: 271-287.

Sagirkaya, H., M. Misirlioglu, A. Kaya, N.L. First, J.J. Parish and E. Memili. 2006. Developmental and molecular correlates of bovine preimplantation embryos. Reproduction. 131: 895-904.

Saharrea, A., J. Valencia, A. Balcazar, O. Mejia, J.L. Cerbon, V. Caballero and L. Zarco. 1998. Premature luteal regression in goats superovulated with PMSG: effect of hCG or GnRH administration during the early luteal phase. Theriogenology. 50: 1039-1052.

Sakkas, D., Y. Shoukir, D. Chardonnens, P.G. Bianchi and A. Campana. 1993. Early cleavage of human embryos to the two-cell stage after intracytoplasmic sperm injection as an indicator of embryo viability. Human Reproduction. 13: 182-187.

Salamone, D.F., P. Damiani, R.A. Fissore, J.M. Robl and R.T. Duby. 2001. Biochemical and developmental evidence that ooplasmic maturation of prepubertal bovine oocytes is compromised. Biology of Reproduction. 64: 1761-1768.

Samake, S., E.A. Amoah, S. Mobini, O. gazal and S. Gelaye. 2000. *In vitro* fertilisation of goat oocytes during the non-breeding season. Small Ruminant research. 35: 49-54.

Sasagawa, I. and R. Yanagimachi. 1996. Comparison of methods for activating mouse oocytes for spermatid nucleus transfer. Zygote. 4: 269–274.

Sathananthan, A.H., A. Szell, S.C. Ng, A. Kausche, O. Lacham-Kaplan and A. Trounson, 1997. Is the acrosome reaction a prerequisite for sperm incorporation after intra-cytoplasmic sperm injection (ICSI)? Reproduction, Fertility and Development. 9: 703-709.

Scaramuzzi R.J., J.A. Downing, B.K. Campbell and Y. Cognie. 1988. Control of fertility and fecundity of sheep by means of hormonal manipulation. Australia Journal Biological Science. 41: 37-45.

Scaramuzzi R.J., and J.F. Murray.1994. The nutrient requirements for the optimum production of gametes in assited reproduction in ruminants animals. In Proceeding of the 10th AETE Meeting. pp 85-103. France: Lyon.

Schafer, T.W., A. Pascale, G. Shimonaski and P.E. Came. 1972. Evaluation of gentamicin for use in virology and tissue culture. Applied Microbiology. 23: 565-570.

Schenk, S.L. 1880. As cited by R.L. Brinster. 1969. *In vitro* cultivation of mammalian ova. Advance in Bioscience. 4: 199.

Schuetz, A.W. 1975. Cytoplasmic activation of starfish oocytes by sperm and divalent ionophore A-23187. Journal of Cell Biology. 66: 86-94.

Schultz, R.M. and G.S. Kopf. 1995. Molecular basis of mammalian egg activation. Current Topics in Developmental Biology. 30: 21-61.

Sehral, P., D. Ranier and A. Kinis. 1997. Oocyte morphology predicts outcome of intracytoplasmic sperm injection. Human Reproduction. 12: 1267-1270.

Selgrath, J.P., M.A. Memon, T.E. Smith and K.M. Ebert. 1990. Collection and transfer of microinjectable embryos from dairy goats. Theriogenology. 34: 1195-1205.

Senger, P.L. 2003. Pathways to pregnancy and parturition. Current Conceptions. Washington. pp. 373.

Senthil Kumar, P., D. Saravanan, R.C. Rajasundaram, M. Selvaraju and D. Kathiresan. 2003. Serum oestradiol and progesterone profiles and their relationship with superovulatory responses in Tellicherry goats treated with eCG and FSH. Small Ruminant Research. 49: 69-77.

Sharma, G.T., A.C. Majumdar and S.W. Bonde. 1996. Chronology of maturational events in goat oocytes cultured *in vitro*. Small Ruminant Research. 22: 25-30.

Shea, B.T., J.P.A. Latour and K.N. Bedirian. 1976. Maturation *in vitro* and subsequent penetrability of bovine follicular oocytes. Journal of Animal Science. 43: 809-815.

Shioya, Y., M. Kuwayama, Fukushima, S. Iwasaki and A. Hanada. 1988. *In vitro* fertilization and cleavage capability of bovine follicular oocytes classified by cumulus cells and matured *in vitro*. Theriogenology. 30: 489-496.

Simoes, J., J.C. Almeida, R. Valentim, G. Baril, J. Azevedo, P. Fontes and R. Mascarenhas. 2006. Follicular dynamics in Serrana goats. Animal Reproduction Science. 95: 16-26.

Sirard, M.A., F. Richard, P. Blondin and C. Robert. 2006. Contribution of the oocyte to embryo quality. Theriogenology. 65. 126-136.

Slavik, T., J. Fulka and I. Gall. 1992. Pregnancy rate after the transfer of sheep embryos originated from randomly chosen oocytes matured and fertilised *in vitro*. Theriogenology. 38: 749-756.

Smith, J.F. 1976. Selection for fertility and response to PMSG in Romney ewes. Proceedings of the New Zealand Society of Animal Production. 36: 247-251.

Smith, J.F. 1991. A review of recent developments on the effect of nutrition on ovulation rate (the flushing effect) with particular reference to research at Ruakura. Proceedings of the New Zealand Society for Animal Production. 51; 15-23.

Smith, S., M. Schmidt, B. Purwantara and I. Greve. 1992. Oviduct epithelial cell co-culture of early porcine embryos. Acta Veterinaria Scandinavica. 33: 349-355.

Song, H.B. and A. Iritani. 1987. Studies on *in vitro* maturation of follicular oocytes in the immature goats. Korean Journal of Animal Science. 29: 303-309.

Spearow, J. 1988. Major genes control hormone-induced ovulation rate in mice. Journal of Reproduction and Fertility. 82: 787-797.

Stangl, M., B. Kuhholzer, U. Besenfelder and G. Brem. 1999. Repeated endoscopic ovum pick-up in sheep. Theriogenology. 54: 709-716.

Steinhardt, R.A., D. Epel, E.J. Carroll Jr. and R. Yanagimachi. 1974. Is calcium ionophore a universal activator for unfertilized eggs? Nature. 252: 41-43.

Stice, S.L. and J.M. Robl. 1990. Activation of mammalian oocytes by a factor obtained from rabbit sperm. Mol. Reprod. Dev., 25: 272-280.

Strehler, E., B. Baccetti, K. Sterzik, S. X. Apitani, G. Collodel, D.M. Santo, L. Gambera and P. Piomboni. 1998. Detrimental effects of polyvinylpyrolidone on the ultrastructure of spermatozoa (Notulae seminologicae 13). Human Reproduction. 13: 157-176.

Stricker, S.A. 1999. Comparative biology of calcium signaling during fertilisation and egg activation in animals. Developmental Biology. 211: 157-176.

Susko-Parrish, J.L., M.L. Leibfried-Rutledge, D.L.Northey,V. Schutzkus, N.L. First., 1994. Inhibition of protein kinases after aninduced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion. Development of Biology. 166, 729–739.

Sutovsky, P., G. Manandhar, A. Wu and R. Oko. 2003. Interactions of sperm perinuclear theca with the oocyte: implications for oocyte activation, anti-polyspermy defense and assisted reproduction. Microscopic Research Technique. 61: 362-378.

Suttner, R., V. zakhartchenko, P. Stojkovic, S. Muller, R. Alberio and I. Medjugorac. 2000. Intracytoplasmic sperm injection in bovine: effects of oocyte activation, sperm pretreatment and injection technique. Theriogenology. 54: 935-948.

Swann, K. 1990. A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster oocytes. Development, 110: 1295-1302.

Synder, D.A. 1986. Superovulation of cows and heifers selected for twinning. Theriogenology. 25: 200.

Szczygiel, M.A and M. Kurpisz. 2001. Chromosomal anomalies in human gametes and pre-implantation embryos, and their potential effect on reproduction. Andrologia. 33: 249-265.

Szczygiel, M.A. and W.S. Ward. 2002. Combination of dithiothreitol and detergent treatment of spermatozoa causes paternal chromosomal damage. Biology of Reproduction. 67: 1532-1537.

Szczygiel, M.A., H. Kusakabe, R. Yanagimachi and D.G. Whittingham. 2002. Intracytoplasmic sperm injection is more efficient than in vitro fertilization for generating mouse embryos from cryopreserved spermatozoa. Biology of Reproduction. 67:1278-1284.

Tajikin, M.H. 1995. *In vitro* studies of maturation and fertilisation of oocytes ans subsequent culture of embryos in goats. Department of zoology. PhD Thesis. The University of Malaya, Kuala Lumpur.

Takahashi, Y. And N.L. First. 1992. *In vitro* development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology. 37: 963-978.

Takahashi Y. and N.L. First. 1993. *In vitro* fertilization of bovine oocytes in the presence of theophylline . Animal of Reproductive Science. 34: 1-18.

Takenaka, M, T. Horiuchi and R. Yanagimachi. 2007. Effects of light on development of mammalian zygotes. Proceedings of the National Academy of Sciences. 104: 14289-14293.

Tateno, H. and Y. Kamiguchi. 2007. Evaluation of risk following intracytoplasmic sperm injection in mouse. Biology of Reproduction. 77: 336-342.

Teotia, S. S., K. N., Khattri and P. K. Roy. 1997. Multifractal analysis of seismicity of the Himalyan region. Current Science. 73: 359–366.

Teotia, A., G.T. Sharma and A.C. Majumdar. 2001. Fertilisation and development of caprine oocytes matured over granulose cell monolayers. Small Ruminant Research. 40: 165-177.

Tervit, H.R., D.G. Whittingham and L.E.A. Rowson. 1972. Successful culture *in vitro* of sheep and cattle ova. Journal of reproduction and Fertility. 30: 493-497.

Tervit, H.R. 1996. Laparoscopy/laparotomy oocyte recovery and juvenile breeding. Animal Reproduction Science. 42: 227-238.

Tesarik, J. and V. Kopecny. 1989. Developmental control of the human male pronucleus by ooplasmic factors. Hum. Reprod., 4: 962-968.

Tesarik, J., C. Mendoza and E. Greco. 2002. Paternal effects acting during the first cell cycle of human preimplantation development after ICSI. Human Reproduction. 17: 184-189.

Thibault, C., O. Szollosi and M. Gerard. 1987. Mammalian oocytes maturation. Reproduction Nutrition Development. 27: 865-896.

Thompson, J.G., R.J. Patridge, F.D. Houghton, C.I. Cox, and H.J. Leese. 1996. Oxygen uptake and carbohydrate metabolism by *in vitro* derived bovine embryos. Journal of Reproduction and Fertility. 196: 299-306.

Thompson, J.G. 2000. *In vitro* culture and embryo metabolism of cattle and sheep embryos a decade of achievement. Animal Reproduction Science. 60-61: 263-275.

Thornton, S.J., Chopper. Pantos, A. Speirs and I. Johnston. 1990. Human chorionic gonadotrophin to oocyte retrieval interval in *in vitro* fertilization-how critical is it? Fertility and Sterility. 53: 177-179.

Tibary, A., A.A. Anouassian and H. Khatir. 2005. Update on reproductive biotechnologies in small ruminants and camelids. Theriogenology. 64: 618-638.

Tocharus, C., Y. Kitiyanant and K. Pavasuthipaisit, 1996. Fertilization and subsequent development of bovine embryos after intracytoplasmic sperrn injection (ICSI) using different injection pipettes. Theriogenology, 45: 302-302.

Torres, S., Y. Cognie and G. Colas. 1987. Transfer of superovulated sheep embryos obtained with different FSH-P. Theriogenology. 27: 407-419.

Toyoda, Y., T. Yokoyama and t. Hosi. 1971. Studies on the fertilization of mouse eggs *in vitro. In: In vitro* fertilization of eggs by fresh epididymal spermatozoa. Japanese Journal of Animal Reproduction. 16: 147-151.

Uehara, T. and R. Yanagimachi. 1976. Microsurgical injection of spermatozoa into hamster eggs with subsequent.transformation of sperm nuclei into male pronuclei. Biology of Reproduction. 16: 315-321.

Urdaneta, A., A.R. Jimenez-Macedo, D. Izquierdo and M.T. Paramio. 2003. Supplementation with cycteamine during maturation and embryo culture on embryo development of prepubertal goat oocytes selected by the brilliant cresyl blue test. Zygote. 11: 347-354.

Urdaneta, A., A.R. Jimenez, M.T. Paramio and D. Izquierdo. 2004. Cysteamine, glutathione and ionomycin treatments improve *in vitro* fertilisation of prepubertal goat oocytes. Zygote. 12: 277-284.

Van Blerkom, J., P.W. Davis and J. Merriam. 1994. Fertilisation and early embryology: a retrospective analysis of unfertilised and presumed parthenogenetically activated human oocytes demonstrates a high frequency of sperm penetration. Human Production. 9: 2381-2388.

Van den Bergh, A., E. Bertrand, J. Biramane and Y. Englert. 1995. Importance of breaking a spermatozoon's tail before intracytoplasmic injection: a prospective randomized trial. Human Reproduction. 10: 2819-2820.

Van der Auwera, I., R. Pijnenborg and P.R. Koninckx. 1999. The influence of *in vitro* culture versus stimulated and untreated oviductal environment on mouse development and implantation. Human Reproduction. 14: 2570-2574.

Van Steirteghem, A.C., Z. Nagy, H. Joris, J. Liu and C. Staessen,J. Smitz, A. Wisanto and P. Devroy. 1993. High fertilization and implantation rates after intracytoplasmic sperm injection. Hum. Reprod., 8: 1061-1066.

Van Wagtendonke Leeum, A.M., E. Mullaart, A.P.W. de Roos, J.S. Merton, J.H.G. den Daas. B. Kemp and L. de Ruigh. 2000. Effects of different reproduction techniques: AI, MOET, or IVP on health and welfare of bovine offspring. Theriogenology. 53: 575-597.

Vanderzwalmen, P., G. Bertin, B. Lejeune, M. Nijs, B. Vandamme and R. Schoysman. 1996. Two essential steps for a successful intracytoplasmic sperm injection: Injection of immobilized spermatozoa after rupture of the oolema. Hum. Reprod., 11: 540-547.

Velilla, E., M. Lopez-bejar, E. Rodriguaz-Gonzalez, F. Vidal and M.T. Paramio. 2002. Effect of Hoechst 33342 staining on developmental competence of prepubertal goat oocytes. Zygote. 10: 201-208.

Velilla, E., D. Izquierdo, E. Rodriguez-Gonzalez, M. Lopez-Bejar, F. Vidal and M.T. Paramio. 2004. Distribution of prepubertal and adult goat oocyte cortical granules during meiotic maturation and fertilisation: ultrastructural and cytochemical study. Molecular Reproduction and Development. 68: 507-514.

Vergara, G.J., M.J. Irwin, R.J. Moffatt and C.A Pinkert. 1997. *In vitro* fertilization in mice: strain differences in response to superovulation protocols and effects of cumulus cells removal. Theriogenology. 47: 1245-1252.

Vermeiden, J.P.W. and A. Bast. 1995. Antioxidants in IVF culture media. Human Reproduction. 10: 697-698.

Vivanco, H.M., K.B. Greaney and H. Varela. 1994. Explaining the viability in superovulatory responses and yield of transferable embryos in sheep embryo transfer. Theriogenology. 41: 329 (abstract).

Voronina, E. And G.M. Wessel. 2003. The regulation of oocyte maturation. Current Topics in Developmental Biology. 58: 53-110.

Wahid, H., I. Gordon, H. Sharif, P. Lonergan, P. Monaghan and M. Gallagher. 1992. Effect and efficiency of recovery method for obtaining ovine follicular oocytes for *in vitro* procedures. Theriogenology. 37: 318 (abstract).

Wakayama, T., D.G. Whittingham and R. Yanagimachi, 1998. Production of normal offspring from mouse oocytes. Journal of Reproduction and Fertility., 112: 11-17.

Walton, E.A., and D.T. Amstrong. 1981. Ovarian function and early embryo development in immature rats given a superovulatory dose of PMSG, later neutralized by antiserum. Biology of Reproduction. 25: 272-280.

Wang, B., H. Baldassarre, T. Tao, M. Gauthier, N. Neveu, J.F. zhou, M. Leduc, F. Duguay, A.S. Bilodeau, A. Lazaris, C. Keefer and K.N. Karatzas. 2002. Transgenic goat produced by DNA pronuclear microinjection of *in vitro* derived zygotes. Molecualr Reproduction and Development. 63: 437-443.

Wang, B., H. Baldassarre, J. Pierson, F. Cote, K.M. Rao and C.N. Karatzas. 2003. The *in vitro* and *in vivo* development of goat embryos produced by intracytoplasmic sperm injection using tail-cut spermatozoa. Zygote. 11: 219-227.

Wang W.H. and Q.Y. Sun. 2007. Evaluation of oocyte quality: morphological, cellular and molecular predictors. Reproduction, Fertility and Development. 19: 1-12.

Wani, N.A., G.M. Wani, M.Z. Khan and M.A. Sidiqi. 1999. Effect of different factors on the recovery rate of oocytes for *in vitro* maturation and *in vitro* fertilisation

Wani, N.A. 2002. *In vitro* maturation and *in vitro* fertilization of sheep oocytes. Small Ruminant Research, 44: 89-95.

Fprocedures in sheep. Small Ruminant Research. 34: 71-76.

Warriach, H.M. and K.R. Chohan. 2004. Thickness of cumulus cell layer is a significant factor in meiotic competence of buffalo oocytes. Journal of Veterinary Science. 5: 247-251.

Watson, A.J., P.H. Watson, D. Warnes, S.K. Walker, D.T. Armstrong and R.F. Seamark. 1994. Preimplantation development of *in vitro* matured and *in vitro* fertilised ovine zygotes: comparison between co-culture on oviduct epithelial cell monolayer and culture under low oxygen atmosphere. Biology of Reproduction. 50: 715-724.

Watson, A.J. 2007. Oocyte cytoplasmic maturation: A key mediator of oocyte and embryo developmental competence. Journal of Animal Science. 85( Supplement E): E1-E3.

Wei, H. and Y. Fukui. 1999. Effects of bull, sperm type and sperm pretreatment on pronuclear formation after intracytoplasmic sperm injection in cattle. Reproduction, Fertility and Development. 11: 59-65.

Wei, H. and Y. Fukui. 2002. Births of calves derived from embryos produced by intracytoplasmic sperm injection without exogenous oocyte activation. Zygote. 10: 149-153.

Whitten, W.K. 1956. Modification of the oestrus cycle of the mouse by external stimuli associated with the male. Journal of Endocrinology. 13: 399-404.

Whitten W.K. 1971. Nutrient requirements for the culture of preimplantation embryos *in vitro*. Advancement in Bioscience. 6: 129-141.

Wilcox, A.J., C.R. Weinberg, D.D Baird. 1998. Post-ovulatory ageing of human oocyte and embryo failure. Human Reproduction. 13: 394-397.

Wildeus, S. 2000. Current concepts in synchronnisation of oestrus: sheep and goats. Joural of Animal Science. 77: 1-14.

Williams, C.J. 2002. Signaling mechanisms of mammalian oocyte activation. Human Reproduction Update. 8: 313-321.

Wollen, T.S., R.H. Schultz and H.L. Newkirk. 1985. Use and handling of drugs and biological in embryo transfer. Theriogenology. 23: 31-43.

Wrenzycki, C., D. Herrmann, A. Lucas-Hahn, K. Korsawe, E. Lemme and H. Niemann. 2005. Messenger RNA expression patterns in bovine embryos derived from *in vitro* procedures and their implications for development. Reproduction, Fertility and Development. 17: 23-35.

Wyllie, A.H. 1981. Cell death: a new classification separating apoptosis from necrosis. *In*: Cell Death in Biology and Pathology. *Editors*: Bowen, I.D. and R.A. Lockshm. Chapman & Hall, New York. Pp 9-13.

Xia, P. 1997. Intracytoplasmic sperm injection: correlation of oocyte grade based on polar body, preivatelline space and cytoplasmic inclusions with fertilization rate and embryo quality. Human Reproductive. 12: 1750-1755.

Yadav, B.R., P.K. Katiyar, M.S. Chauhan and M.L. Madan. 1997. Chromosome configuration during *in vitro* maturation of goat, sheep and buffalo oocytes. Theriogenology. 47: 943-951.

Yamauchi Y., R. Yanagimachi and T. Horiuchi. 2002. Full-term development of golden hamster oocytes following intracytoplasmic sperm head injection. Biology of Reproduction. 67: 534-539.

Yanagida, K., H. Katayose, H. Yazawa, Y. Kimura and A. Sato, H. Yanagimachi and R. Yanagimachi.1999. Successful fertilization and pregnancy following ICSI and electrical oocyte activation. Human Reproduction. 14: 1307-1311.

Yanagida, K., Y. Fujikura and H. Katayose. 2008. The present status of artificial oocyte activation is assited reproductive technology. Reproductive Biomedicine Online. 13 (abstract).

Yanagimach, R. 2005. Intracytoplasmic injection of spermatozoa and spermatogenic cells: its biology and applications in human and animals. Reproductive Biomedicine Online. 10: 247-188.

Yanagimachi, R. 1994. Mammalian fertilisation. *In*: The physiology of reproduction. *Editors*: E. Knobil and J. Neill. Raven Press, New York. pp 189-317.

Yazawa, H., K. Yanagida and A. Sato. 2001. Oocyte activation and $Ca^{2+}$ oscillation-inducing abilities of mouse round/elongated spermatids and the developmental capacities of embryos from spermatid injection. Human Reproduction. 16: 1221-1228.

Yoo., J.G., C.G. Hur, M.R. Park, J.Y. Park, K.C. Hwang, J.H. Kim, J.H. Kim and S.K. Cho. 2011. Electrical activation enhances pre-implantation embryo development following sperm injection into *in vitro* matured pig oocytes. Theriogenology. 74: 429-434.

Yoshida, M., K. Ishigaki, T. Nagai, M. Chikyo and V.G. Pursel. 1993. Glutathione concentration during maturation and after fertilisation in pig oocytes: relevance to the ability of oocytes to form male pronucleus. Biology of Reproduction. 44: 1177-1182.

Yoshioko K., C. Suzuki, S. Itoh, K. Kikuchi, S. Iwamura and H. Rodriguez-Martinez. 2003. Production of piglets derived from *in vitro*-produced blastocyst fertilized and cultured *in* chemically defined media: Effects of theophylline, adenosine, and cysteine during *in vitro* fertilization. Biology of Reproduction. 69: 2092-2099.

Younis, A. I., K.A. Zuelke, K.M. Harper, M.A.L. Oliveira and B.G. Brackett. 1991. *In vitro fertilization* of goat oocytes. Biology of reproduction. 44: 1177-1182.

Yu, Y., J. Yan, Z.C. Liu, L.Y.Yan, M. Li, Q. Zhou, and J. Qiao. 2011. Optimal timing of oocyte maturation and its relationship with the spindle assembly and developmental competence of *in vitro* matured human oocytes. 96: 73-78.

Yuan, Y.Q., A, Van Soom, J.L. M.R. Leroy, J. Dewulf, A. Van Zeveren, A. De Kruif and L.J. Peelman. 2005. Apoptosis in cumulus cells, but not in oocytes, may influence bovine embryonic developmental competence. Theriogenology. 63: 2147-2163.

Yuswiati, E. And W. Holtz. 1996. Superovulation with different exogenous gonadotrophins in prepubertal goats. Indian Journal of Animal Science. 66: 131-133.

Zhang R, D.P. Hurst, J. Barnett-Norris, P.H. Reggio, and Z.H. Song. 2005. Cysteine 2.59 (89) in the second transmembrane domain of human CB2 receptor is accessible within the ligand binding crevice: evidence for possible CB2 deviation from a rhodopsin template. Molecular Pharmacology. 68 :69–83.

Zernicka-Goetz, M., M.H. Verlhac, G. Geraud and J.Z. Kubiak. 1997. Protein phosphatise control MAP kinase activation and microtubule organisation during rat oocyte maturation. European Jpurnal of cell Biology. 72: 30-38.

Zheng, Y.L., M.X. Jiang, Y.L. Zhang, Q.Y. Sun and D.Y. Chen. 2004. Effects of oocyte age, cumulus cells and injection methods on *in vitro* development of intracytoplasmic sperm injection rabbit embryos. Zygote. 12: 75-80.

Zhou, H., S.H. McKiernan, W. Ji and B.D. Bavister. 2000. Effect of antibiotics on development *in vitro* of hamster pronuclear ova. Theriogenology. 54: 999-1006.

Zhou, J.B., Y.G. Wu, D. Han, L.Q. Liu, X.W. Tan, N. Liu, M.J. Luo, Z.L. Chang and J.H. Tan. 2004. Effects of sperm and oocytes quality control on intracytoplasmic sperm injection (ICSI) in goats. Shi Yan Sheng Wu Xue Bao. (Journal of Experimental Biology). 37: 367-374.

Zimmermann, U., and J. Vienken. 1982. Electric field-induced cell-to-cell fusion. Journal of Membrane Biology. 67: 165-182.

Zudova, D., A.J. Wyrobek, J. Bishop and F. Marchetti. 2004. Impaired fertility in T-stock female after superovulation. Journal of Reproduction. 128: 573-581.

Zuelke, K.A. and B.G. Brackett. 1990. Luteinizing hormones enhanced *in vitro* maturation of bovine oocytes with and without protein supplementation. Biology of Reproduction. 43: 784-787.

Zuerner, A., K. Muller, K. Reguszynski and K. Jewgenow. 2003. Apoptosis within bovine follicular cell and its effect on oocyte development during *in vitro* maturation. Theriogenology. 59: 1421-1433.

# 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, Switzland |
| Digital camera (X-Cam-α) | - | microLAMBDA Sdn Bhd, Malaysia |
| Digital timer | - | Seiko, Japan |
| Dissecting board | - | - |
| 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 |
| Impulser sealer | KF-300H | Khind, Taiwan |
| Inverted microscope | IX71 | Olympus, Japan |
| Laminar flow cabinet | HLF-120 | German Sciences, Australia |
| Laporoscopic camera system | | |
| (a) Endoscopic camera system | PV431 | Aesculap$^{®}$, Germany |
| (b) CCD camera | PV430 | Aesculap$^{®}$, Germany |
| (c) Pediatric Storz laparoscope (7mm) | PE688A | Aesculap$^{®}$, Germany |
| (d) Light probe with fibre optic cable | OP913 | Aesculap$^{®}$, Germany |
| (e) Light system (300 W) | OP927 | Aesculap$^{®}$, Germany |
| Liquid nitrogent tank (small) | SC2/1V | MVE, USA |
| Microforge | - | Technical Products Internationals, USA |
| Microgrinder | EG-4 | Narishige, Japan |
| Micropipette dispenser | - | Eppendorf, Germany |
| Micropuller dispenser | P-97 | Sutter Instrument Co., USA |
| Narishige hydraulic manipulators | ON3-99D | Narishige, Japan |

| | | |
|---|---|---|
| Oocyte pick-up needle | FAS set C2 | Gynetics Medical Products, Belgium |
| Oven | 40050-IP20 | Memmert GmbH, Germany |
| pH meter | AL204 | Metler Toledo |
| 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 | Milipore, USA |
| Vapour pressure osmometer | 5520 | Vapor Wescor, USA |
| 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) | ET 150-50 | System ChemAR®, Poland |
| Hibiscrub (antiseptic) | HK-06770 | SSL International Plc, UK |
| BME amino acids solution (50X) | B6766 | Sigma-Aldrich, USA |
| Calcium chloride | C7902 | Sigma-Aldrich, USA |
| Calcium ionophore | I0634 | Sigma-Aldrich, USA |
| Calcium lactate | 2376 | Ajax Chem. Pty.Ltd. Australia |
| 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 pellet feed | - | KMM Berhad, Malaysia |
| Heparin | H0777 | Sigma-Aldrich, USA |
| HEPES | H7006 | Sigma-Aldrich, USA |
| Hydrochloric acids | HY450-70 | System ChemAR®, Poland |
| Hydrofluoric acids | 1301030 | Hmbg Chemicals, Germany |
| Hyaluronidase (from bovine testes) | H4272 | Sigma-Aldrich, USA |
| Intravaginal progesterone release (CIDR) 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 | - | In-house prepared |
| 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 |
| Photassium chloride | P5405 | Sigma-Aldrich, USA |
| Potassium hydrogen orthophosphate | Prod29068 | BDH Laboratory Supplies, England |
| Polyvinylpyrollidone (PVP) 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 |
| 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, USA |

**Appendix Table 1.3**: List of labware and disposable items

| Labwares and disposables | Manufacturer |
|---|---|
| Aluminium foil | Reynolds Consumer Products, USA |
| Autoclave disposable bag | Megalab supplies, Malaysia |
| Blades (Super Nacet) | Gillette, USA |
| Borosilicate glass tubing (Microcaps®) | Drummond Scientific Company, USA |
| Chromic catgut | Aesculap®, Germany |
| Culture dish (35 mm, 60 mm) | Nunc, Denmark |
| Disposable glass Pasteur pipette | Hirshmann® 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, measuring cyclinder 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 coverslip | Hirshmann® Laborgerate, Germany |
| Millex®-GS syringe driven filter | Scheicher and Schuell, Germany |
| Needle | Terumocorporation, Japan |
| Parafilm | Pechiney Plastic Packaging, USA |
| Schott bottle | Duran, Germany |
| Serogical pipette | LP Italiana SPA, Italy |
| Streile glove | Ansell International, Malaysia |
| Terumo venojector holder | Terumo Corporation, Japan |
| Vacutainer® blood collection tubes | Becton Dickinson, USA |
| Vacutainer® needle | Becton Dickinson, USA |

# APPENDICES

## APPENDIX 2: SUPPLEMENTARY FIGURES

**a) Mouse experiment**
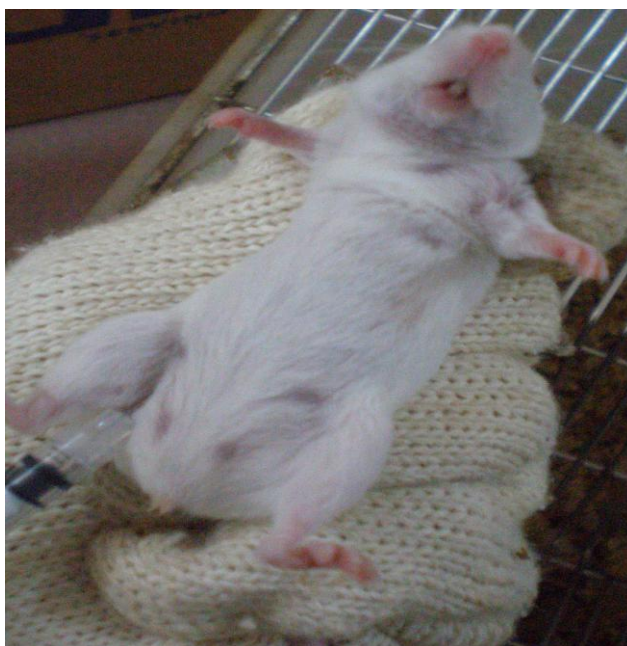


**Appendix Figure 2.1**: ICR mouse: hormone injection through intraperitoneal injection (i.p.).
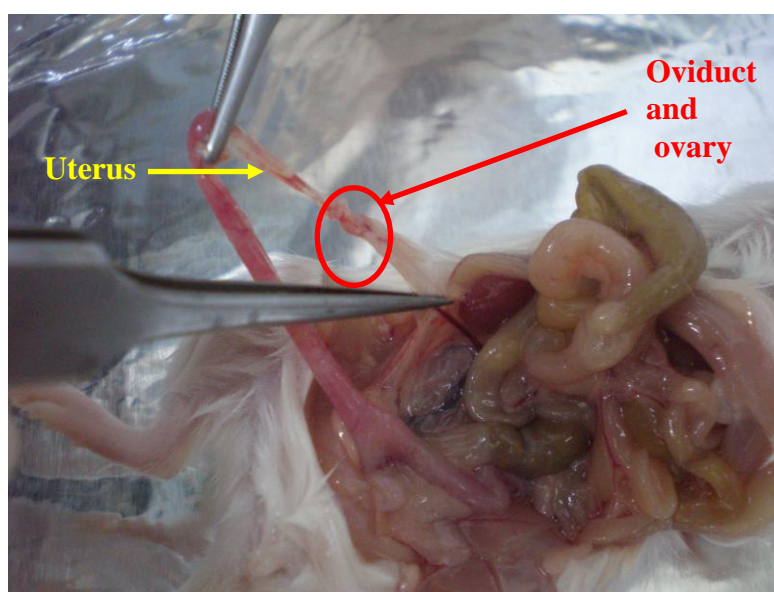


**Appendix Figure 2.2**: Dissection area for obtain oviduct.
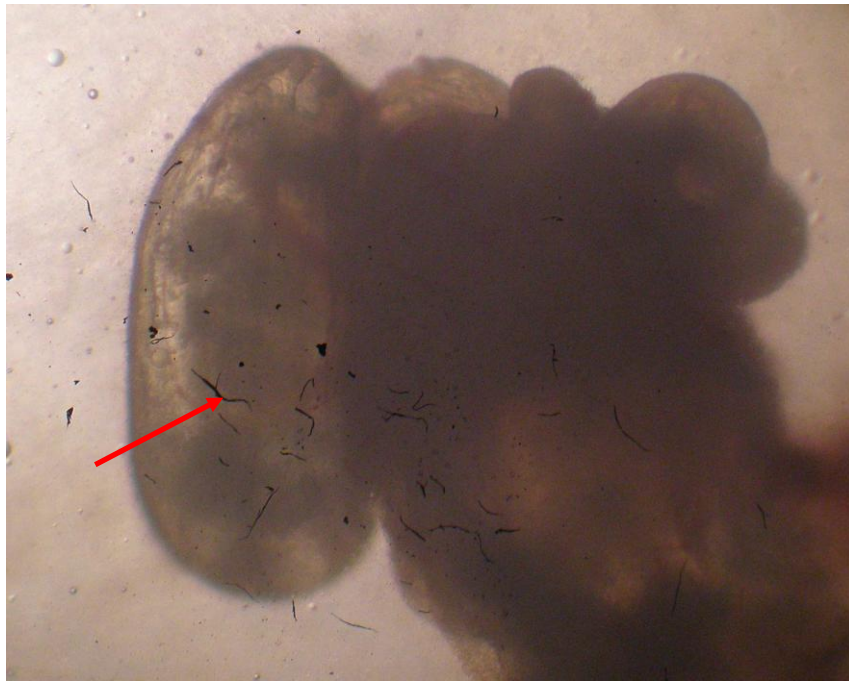
**Appendix Figure 2.3**: Arrow showed swollen area at oviduct that contained cumulus oocytes complexes (COCs).
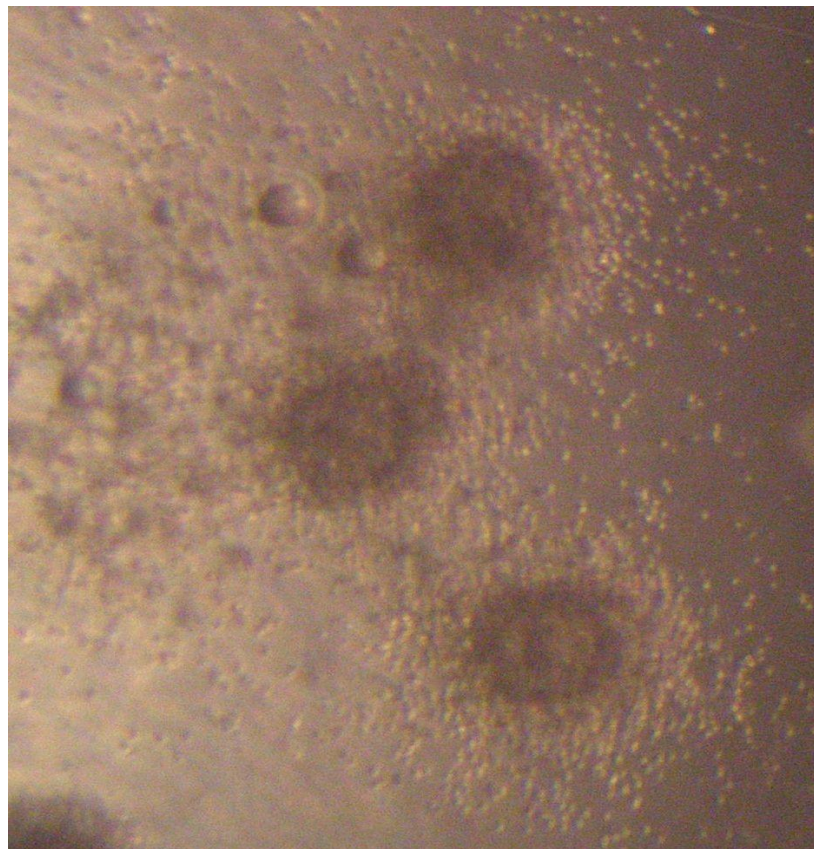


**Appendix Figure 2.4**:  Cumulus oocytes complexes (COCs).

**b) LOPU in goat**



**Appendix Figure 2.5**: Surgical instruments and consumables that involved during LOPU procedure.



**Appendix Figure 2.6**: Conducting LOPU procedure.
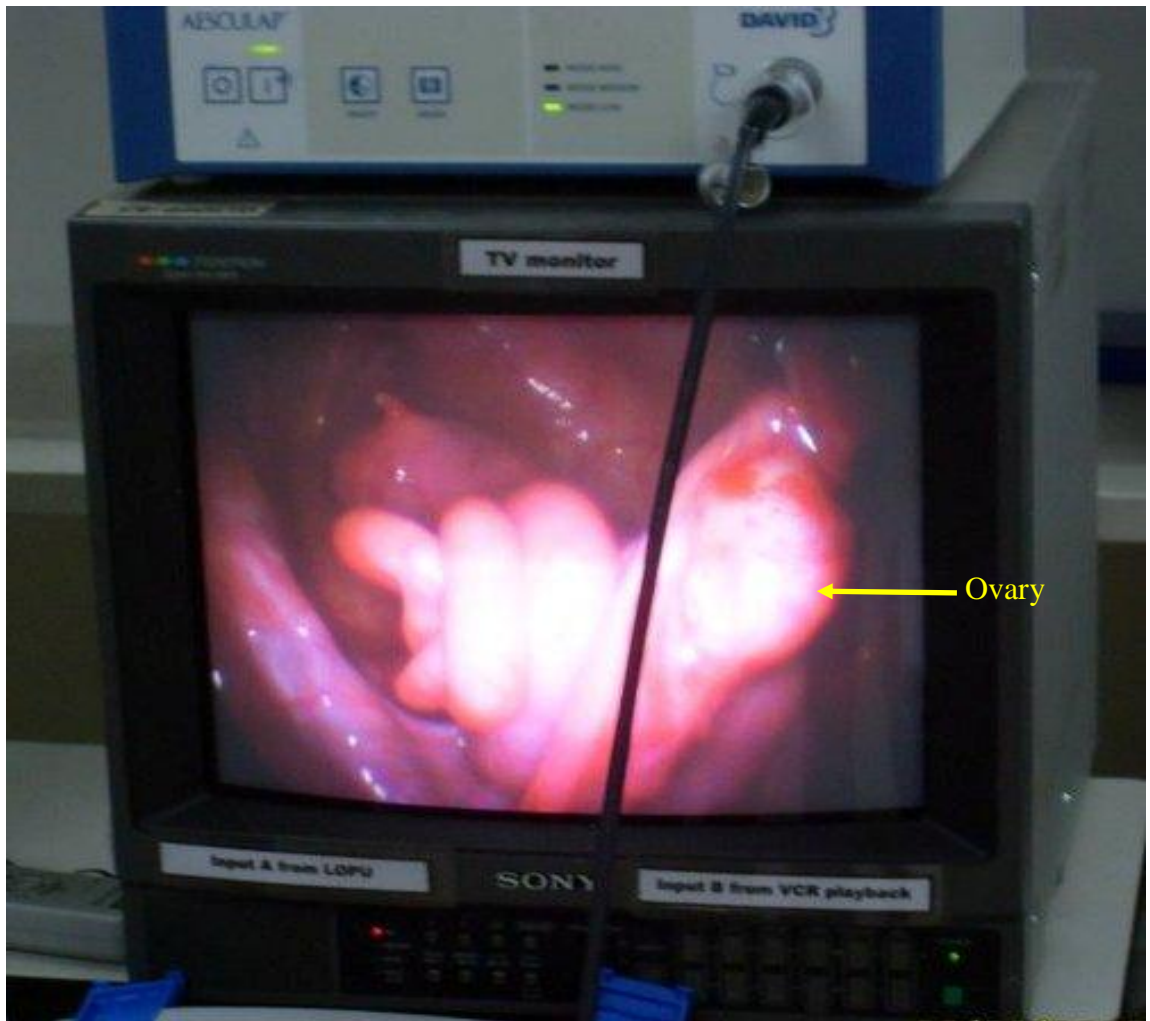
**Appendix Figure 2.7**: Ovary morphology observations through the LOPU monitor system.

**c) Microneedles preparation equipment**



**Appendix Figure 2.8**: Micropuller.
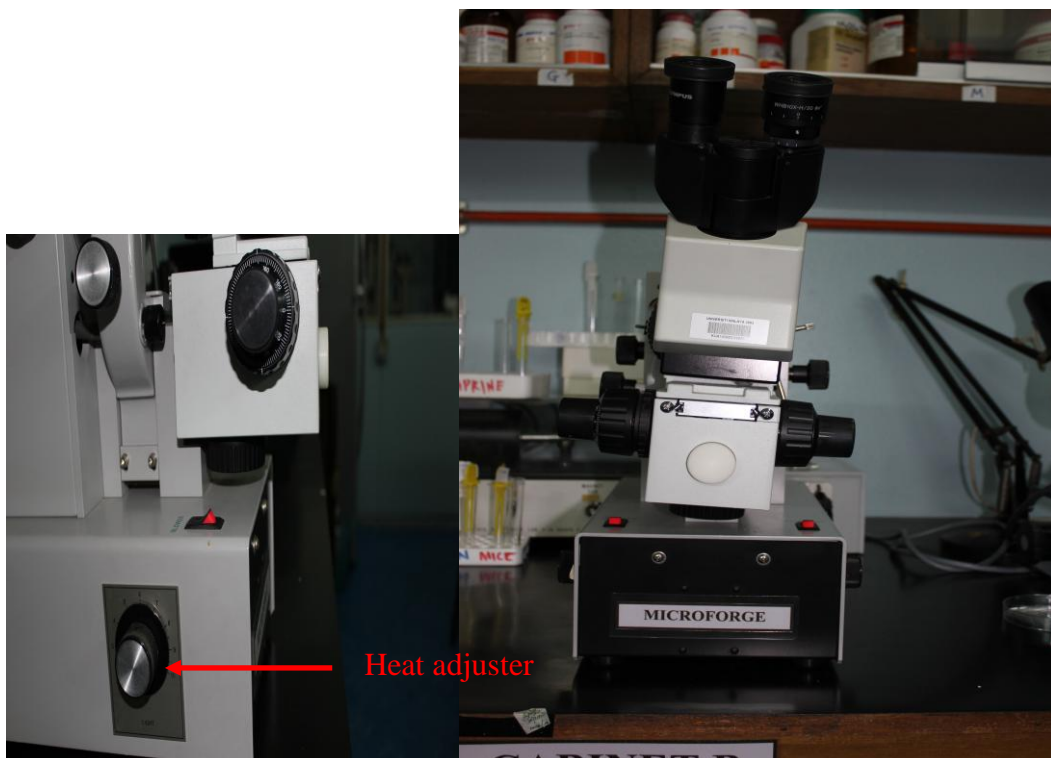


**Appendix Figure 2.9**: Microphorge.
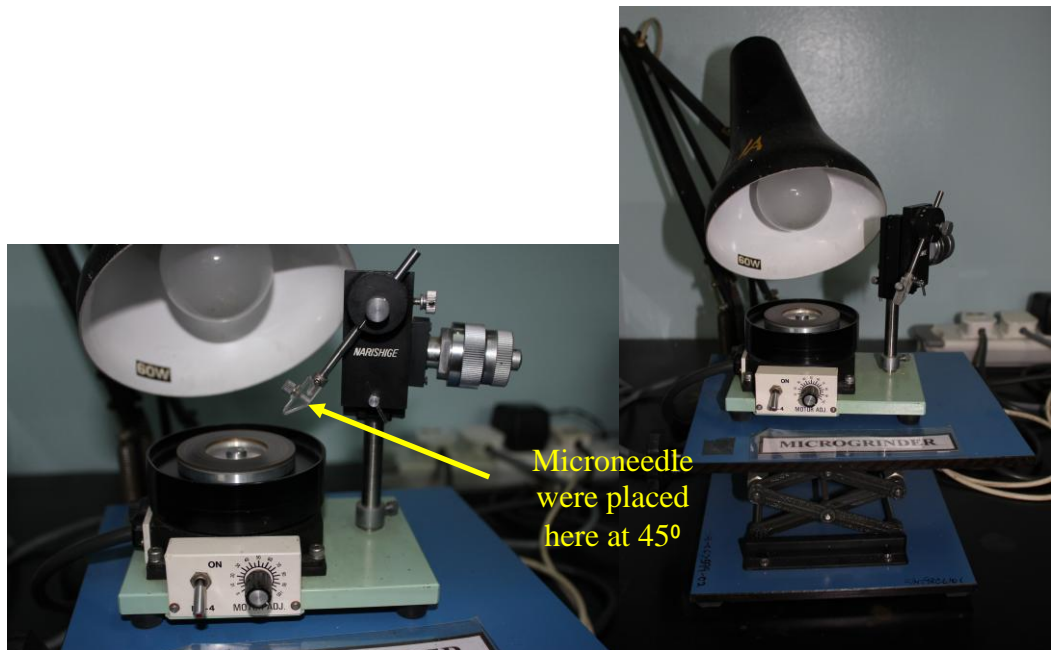
**Appendix Figure 2.10**: Microgrinder for making bevel at the tips of ICSI microneedle.

## APPENDIX 3: STATISTISTICAL DATA

**EFFECT OF SPERM FACTORS ON ICSI PERFORMANCE IN MICE (EXPERIMENT 1)**

**Appendix Table 3.1**: Effect of  sperm movement with Heparin on mouse embryo cleavage rate

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | 95% Confidence Interval for Mean Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| two_cell | rapid | 19 | 65.4312 | 8.28457 | 1.90061 | 61.4382 | 69.4243 | 50.00 | 79.17 |
| | slow | 18 | 63.8568 | 12.22267 | 2.88091 | 57.7787 | 69.9350 | 28.57 | 81.82 |
| | Total | 37 | 64.6653 | 10.27134 | 1.68860 | 61.2407 | 68.0900 | 28.57 | 81.82 |
| four_cell | rapid | 19 | 52.3226 | 8.52431 | 1.95561 | 48.2140 | 56.4312 | 33.33 | 68.75 |
| | slow | 18 | 48.6736 | 15.21706 | 3.58670 | 41.1063 | 56.2408 | .00 | 67.27 |
| | Total | 37 | 50.5474 | 12.21057 | 2.00741 | 46.4762 | 54.6186 | .00 | 68.75 |
| eight_cell | rapid | 19 | 33.7849 | 10.06451 | 2.30896 | 28.9340 | 38.6359 | 11.11 | 52.17 |
| | slow | 18 | 29.8327 | 14.27167 | 3.36386 | 22.7356 | 36.9299 | .00 | 50.00 |
| | Total | 37 | 31.8622 | 12.28170 | 2.01910 | 27.7673 | 35.9572 | .00 | 52.17 |
| morula | rapid | 19 | 12.6066 | 6.59350 | 1.51265 | 9.4287 | 15.7846 | .00 | 26.09 |
| | slow | 18 | 10.7822 | 7.40944 | 1.74642 | 7.0975 | 14.4668 | .00 | 21.82 |
| | Total | 37 | 11.7191 | 6.96539 | 1.14510 | 9.3967 | 14.0414 | .00 | 26.09 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| two_cell | Between Groups | 22.911 | 1 | 22.911 | .212 | .648 |
| | Within Groups | 3775.108 | 35 | 107.860 | | |
| | Total | 3798.019 | 36 | | | |
| four_cell | Between Groups | 123.079 | 1 | 123.079 | .821 | .371 |
| | Within Groups | 5244.450 | 35 | 149.841 | | |
| | Total | 5367.530 | 36 | | | |
| eight_cell | Between Groups | 144.378 | 1 | 144.378 | .956 | .335 |
| | Within Groups | 5285.865 | 35 | 151.025 | | |
| | Total | 5430.243 | 36 | | | |
| morula | Between Groups | 30.767 | 1 | 30.767 | .628 | .434 |
| | Within Groups | 1715.831 | 35 | 49.024 | | |
| | Total | 1746.599 | 36 | | | |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| rapid | 2-cell | 19 | 65.4312 | 8.28457 | 1.90061 | 61.4382 | 69.4243 | 50.00 | 79.17 |
| | 4-cell | 19 | 52.3226 | 8.52431 | 1.95561 | 48.2140 | 56.4312 | 33.33 | 68.75 |
| | 8-cell | 19 | 33.7849 | 10.06451 | 2.30896 | 28.9340 | 38.6359 | 11.11 | 52.17 |
| | morula | 19 | 12.6066 | 6.59350 | 1.51265 | 9.4287 | 15.7846 | .00 | 26.09 |
| | Total | 76 | 41.0364 | 21.67404 | 2.48618 | 36.0836 | 45.9891 | .00 | 79.17 |
| slow | 2-cell | 18 | 63.8568 | 12.22267 | 2.88091 | 57.7787 | 69.9350 | 28.57 | 81.82 |
| | 4-cell | 18 | 48.6736 | 15.21706 | 3.58670 | 41.1063 | 56.2408 | .00 | 67.27 |
| | 8-cell | 18 | 29.8327 | 14.27167 | 3.36386 | 22.7356 | 36.9299 | .00 | 50.00 |
| | morula | 18 | 10.7822 | 7.40944 | 1.74642 | 7.0975 | 14.4668 | .00 | 21.82 |
| | Total | 72 | 38.2863 | 23.58277 | 2.77926 | 32.7446 | 43.8280 | .00 | 81.82 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| rapid | Between Groups | 30083.121 | 3 | 10027.707 | 140.215 | .000 |
| | Within Groups | 5149.195 | 72 | 71.517 | | |
| | Total | 35232.316 | 75 | | | |
| slow | Between Groups | 28614.373 | 3 | 9538.124 | 59.657 | .000 |
| | Within Groups | 10872.059 | 68 | 159.883 | | |
| | Total | 39486.432 | 71 | | | |

**rapid**

Duncan[a]

| development | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| morula | 19 | 12.6066 | | | |
| 8-cell | 19 | | 33.7849 | | |
| 4-cell | 19 | | | 52.3226 | |
| 2-cell | 19 | | | | 65.4312 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 |

**slow**

Duncan[a]

| development | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| morula | 18 | 10.7822 | | | |
| 8-cell | 18 | | 29.8327 | | |
| 4-cell | 18 | | | 48.6736 | |
| 2-cell | 18 | | | | 63.8568 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 |

**Appendix Table 3.2**: Effect of sperm movement with Theophylline on mouse embryo cleavage rate

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | 95% Confidence Interval for Mean Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| two_cell | rapid | 20 | 61.3940 | 6.10987 | 1.36621 | 58.5345 | 64.2535 | 50.00 | 72.41 |
| | slow | 18 | 59.5086 | 8.75687 | 2.06401 | 55.1539 | 63.8633 | 44.44 | 75.00 |
| | Total | 38 | 60.5009 | 7.43724 | 1.20648 | 58.0564 | 62.9455 | 44.44 | 75.00 |
| four_cell | rapid | 20 | 45.8739 | 8.31214 | 1.85865 | 41.9837 | 49.7641 | 25.00 | 58.82 |
| | slow | 18 | 44.2284 | 11.53303 | 2.71836 | 38.4932 | 49.9636 | 11.11 | 60.00 |
| | Total | 38 | 45.0945 | 9.86336 | 1.60005 | 41.8525 | 48.3365 | 11.11 | 60.00 |
| eight_cell | rapid | 20 | 31.4178 | 10.35380 | 2.31518 | 26.5721 | 36.2635 | 12.50 | 48.28 |
| | slow | 18 | 28.4271 | 12.95566 | 3.05368 | 21.9844 | 34.8698 | .00 | 42.86 |
| | Total | 38 | 30.0011 | 11.59566 | 1.88106 | 26.1897 | 33.8125 | .00 | 48.28 |
| morula | rapid | 20 | 13.8555 | 6.92781 | 1.54911 | 10.6131 | 17.0978 | .00 | 24.14 |
| | slow | 18 | 11.9063 | 6.87241 | 1.61984 | 8.4888 | 15.3239 | .00 | 23.81 |
| | Total | 38 | 12.9322 | 6.87887 | 1.11590 | 10.6712 | 15.1932 | .00 | 24.14 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| two_cell | Between Groups | 33.678 | 1 | 33.678 | .602 | .443 |
| | Within Groups | 2012.887 | 36 | 55.914 | | |
| | Total | 2046.565 | 37 | | | |
| four_cell | Between Groups | 25.653 | 1 | 25.653 | .258 | .614 |
| | Within Groups | 3573.927 | 36 | 99.276 | | |
| | Total | 3599.580 | 37 | | | |
| eight_cell | Between Groups | 84.737 | 1 | 84.737 | .624 | .435 |
| | Within Groups | 4890.258 | 36 | 135.840 | | |
| | Total | 4974.994 | 37 | | | |
| morula | Between Groups | 35.991 | 1 | 35.991 | .756 | .390 |
| | Within Groups | 1714.807 | 36 | 47.634 | | |
| | Total | 1750.798 | 37 | | | |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| rapid | 2-cell | 20 | 61.3940 | 6.10987 | 1.36621 | 58.5345 | 64.2535 | 50.00 | 72.41 |
| | 4-cell | 20 | 45.8739 | 8.31214 | 1.85865 | 41.9837 | 49.7641 | 25.00 | 58.82 |
| | 8-cell | 20 | 31.4178 | 10.35380 | 2.31518 | 26.5721 | 36.2635 | 12.50 | 48.28 |
| | morula | 20 | 13.8555 | 6.92781 | 1.54911 | 10.6131 | 17.0978 | .00 | 24.14 |
| | Total | 80 | 38.1353 | 19.38306 | 2.16709 | 33.8218 | 42.4488 | .00 | 72.41 |
| slow | 2-cell | 18 | 59.5086 | 8.75687 | 2.06401 | 55.1539 | 63.8633 | 44.44 | 75.00 |
| | 4-cell | 18 | 44.2284 | 11.53303 | 2.71836 | 38.4932 | 49.9636 | 11.11 | 60.00 |
| | 8-cell | 18 | 28.4271 | 12.95566 | 3.05368 | 21.9844 | 34.8698 | .00 | 42.86 |
| | morula | 18 | 11.9063 | 6.87241 | 1.61984 | 8.4888 | 15.3239 | .00 | 23.81 |
| | Total | 72 | 36.0176 | 20.51071 | 2.41721 | 31.1978 | 40.8374 | .00 | 75.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| rapid | Between Groups | 24709.797 | 3 | 8236.599 | 125.933 | .000 |
| | Within Groups | 4970.745 | 76 | 65.405 | | |
| | Total | 29680.542 | 79 | | | |
| slow | Between Groups | 22647.815 | 3 | 7549.272 | 71.090 | .000 |
| | Within Groups | 7221.134 | 68 | 106.193 | | |
| | Total | 29868.949 | 71 | | | |

**rapid**

Duncan[a]

| development | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| morula | 20 | 13.8555 | | | |
| 8-cell | 20 | | 31.4178 | | |
| 4-cell | 20 | | | 45.8739 | |
| 2-cell | 20 | | | | 61.3940 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 |

**slow**

| development | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| morula | 18 | 11.9063 | | | |
| 8-cell | 18 | | 28.4271 | | |
| 4-cell | 18 | | | 44.2284 | |
| 2-cell | 18 | | | | 59.5086 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 |

**Appendix Table 3.3**: Effect of sperm capacitation chemical treatments regardless of sperm movement on mouse embryo cleavage rate

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| two_cell | heparin | 37 | 64.6653 | 10.27134 | 1.68860 | 61.2407 | 68.0900 | 28.57 | 81.82 |
| | theophylline | 38 | 60.5009 | 7.43724 | 1.20648 | 58.0564 | 62.9455 | 44.44 | 75.00 |
| | Total | 75 | 62.5553 | 9.13095 | 1.05435 | 60.4545 | 64.6562 | 28.57 | 81.82 |
| four_cell | heparin | 37 | 50.5474 | 12.21057 | 2.00741 | 46.4762 | 54.6186 | .00 | 68.75 |
| | theophylline | 38 | 45.0945 | 9.86336 | 1.60005 | 41.8525 | 48.3365 | 11.11 | 60.00 |
| | Total | 75 | 47.7846 | 11.34504 | 1.31001 | 45.1743 | 50.3948 | .00 | 68.75 |
| eight_cell | heparin | 37 | 31.8622 | 12.28170 | 2.01910 | 27.7673 | 35.9572 | .00 | 52.17 |
| | theophylline | 38 | 30.0011 | 11.59566 | 1.88106 | 26.1897 | 33.8125 | .00 | 48.28 |
| | Total | 75 | 30.9193 | 11.89491 | 1.37351 | 28.1825 | 33.6561 | .00 | 52.17 |
| morula | heparin | 37 | 11.7191 | 6.96539 | 1.14510 | 9.3967 | 14.0414 | .00 | 26.09 |
| | theophylline | 38 | 12.9322 | 6.87887 | 1.11590 | 10.6712 | 15.1932 | .00 | 24.14 |
| | Total | 75 | 12.3337 | 6.90181 | .79695 | 10.7457 | 13.9217 | .00 | 26.09 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| two_cell | Between Groups | 325.109 | 1 | 325.109 | 4.061 | .048 |
| | Within Groups | 5844.584 | 73 | 80.063 | | |
| | Total | 6169.693 | 74 | | | |

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| four_cell | Between Groups | 557.420 | 1 | 557.420 | 4.538 | .037 |
| | Within Groups | 8967.110 | 73 | 122.837 | | |
| | Total | 9524.529 | 74 | | | |
| eight_cell | Between Groups | 64.934 | 1 | 64.934 | .456 | .502 |
| | Within Groups | 10405.237 | 73 | 142.537 | | |
| | Total | 10470.171 | 74 | | | |
| morula | Between Groups | 27.590 | 1 | 27.590 | .576 | .450 |
| | Within Groups | 3497.397 | 73 | 47.910 | | |
| | Total | 3524.987 | 74 | | | |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| Heparin | 2-cell | 37 | 64.6653 | 10.27134 | 1.68860 | 61.2407 | 68.0900 | 28.57 | 81.82 |
| | 4-cell | 37 | 50.5474 | 12.21057 | 2.00741 | 46.4762 | 54.6186 | .00 | 68.75 |
| | 8-cell | 37 | 31.8622 | 12.28170 | 2.01910 | 27.7673 | 35.9572 | .00 | 52.17 |
| | morula | 37 | 11.7191 | 6.96539 | 1.14510 | 9.3967 | 14.0414 | .00 | 26.09 |
| | Total | 148 | 39.6985 | 22.58745 | 1.85668 | 36.0293 | 43.3677 | .00 | 81.82 |
| Theophylline | 2-cell | 38 | 60.5009 | 7.43724 | 1.20648 | 58.0564 | 62.9455 | 44.44 | 75.00 |
| | 4-cell | 38 | 45.0945 | 9.86336 | 1.60005 | 41.8525 | 48.3365 | 11.11 | 60.00 |
| | 8-cell | 38 | 30.0011 | 11.59566 | 1.88106 | 26.1897 | 33.8125 | .00 | 48.28 |
| | morula | 38 | 12.9322 | 6.87887 | 1.11590 | 10.6712 | 15.1932 | .00 | 24.14 |
| | Total | 152 | 37.1322 | 19.88700 | 1.61305 | 33.9451 | 40.3192 | .00 | 75.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Heparin | Between Groups | 58655.971 | 3 | 19551.990 | 172.281 | .000 |
| | Within Groups | 16342.390 | 144 | 113.489 | | |
| | Total | 74998.361 | 147 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Theophylline | Between Groups | 47347.499 | 3 | 15782.500 | 188.799 | .000 |
| | Within Groups | 12371.937 | 148 | 83.594 | | |
| | Total | 59719.436 | 151 | | | |

**Heparin**

Duncan[a]

| developm ent | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| morula | 37 | 11.7191 | | | |
| 8-cell | 37 | | 31.8622 | | |
| 4-cell | 37 | | | 50.5474 | |
| 2-cell | 37 | | | | 64.6653 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 |

**Theophylline**

Duncan[a]

| developm ent | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| morula | 38 | 12.9322 | | | |
| 8-cell | 38 | | 30.0011 | | |
| 4-cell | 38 | | | 45.0945 | |
| 2-cell | 38 | | | | 60.5009 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 |

225

**Appendix Table 3.4**: Effect of sperm movement regardless of sperm capacitation chemicals treatments on mouse embryo cleavage rate

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| two_cell | rapid | 39 | 63.3609 | 7.44013 | 1.19137 | 60.9491 | 65.7727 | 50.00 | 79.17 |
| | slow | 36 | 61.6827 | 10.70843 | 1.78474 | 58.0595 | 65.3059 | 28.57 | 81.82 |
| | Total | 75 | 62.5553 | 9.13095 | 1.05435 | 60.4545 | 64.6562 | 28.57 | 81.82 |
| four_cell | rapid | 39 | 49.0156 | 8.92348 | 1.42890 | 46.1229 | 51.9083 | 25.00 | 68.75 |
| | slow | 36 | 46.4510 | 13.49658 | 2.24943 | 41.8844 | 51.0176 | .00 | 67.27 |
| | Total | 75 | 47.7846 | 11.34504 | 1.31001 | 45.1743 | 50.3948 | .00 | 68.75 |
| eight_cell | rapid | 39 | 32.5710 | 10.14982 | 1.62527 | 29.2808 | 35.8612 | 11.11 | 52.17 |
| | slow | 36 | 29.1299 | 13.45233 | 2.24206 | 24.5783 | 33.6815 | .00 | 50.00 |
| | Total | 75 | 30.9193 | 11.89491 | 1.37351 | 28.1825 | 33.6561 | .00 | 52.17 |
| morula | rapid | 39 | 13.2471 | 6.70747 | 1.07406 | 11.0727 | 15.4214 | .00 | 26.09 |
| | slow | 36 | 11.3443 | 7.06618 | 1.17770 | 8.9534 | 13.7351 | .00 | 23.81 |
| | Total | 75 | 12.3337 | 6.90181 | .79695 | 10.7457 | 13.9217 | .00 | 26.09 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| two_cell | Between Groups | 52.719 | 1 | 52.719 | .629 | .430 |
| | Within Groups | 6116.974 | 73 | 83.794 | | |
| | Total | 6169.693 | 74 | | | |
| four_cell | Between Groups | 123.127 | 1 | 123.127 | .956 | .331 |
| | Within Groups | 9401.402 | 73 | 128.786 | | |
| | Total | 9524.529 | 74 | | | |
| eight_cell | Between Groups | 221.668 | 1 | 221.668 | 1.579 | .213 |
| | Within Groups | 10248.503 | 73 | 140.390 | | |
| | Total | 10470.171 | 74 | | | |
| morula | Between Groups | 67.778 | 1 | 67.778 | 1.431 | .235 |
| | Within Groups | 3457.209 | 73 | 47.359 | | |
| | Total | 3524.987 | 74 | | | |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| rapid | 2-cell | 39 | 63.3609 | 7.44013 | 1.19137 | 60.9491 | 65.7727 | 50.00 | 79.17 |
| | 4-cell | 39 | 49.0156 | 8.92348 | 1.42890 | 46.1229 | 51.9083 | 25.00 | 68.75 |
| | 8-cell | 39 | 32.5710 | 10.14982 | 1.62527 | 29.2808 | 35.8612 | 11.11 | 52.17 |
| | morula | 39 | 13.2471 | 6.70747 | 1.07406 | 11.0727 | 15.4214 | .00 | 26.09 |
| | Total | 156 | 39.5486 | 20.51606 | 1.64260 | 36.3039 | 42.7934 | .00 | 79.17 |
| slow | 2-cell | 36 | 61.6827 | 10.70843 | 1.78474 | 58.0595 | 65.3059 | 28.57 | 81.82 |
| | 4-cell | 36 | 46.4510 | 13.49658 | 2.24943 | 41.8844 | 51.0176 | .00 | 67.27 |
| | 8-cell | 36 | 29.1299 | 13.45233 | 2.24206 | 24.5783 | 33.6815 | .00 | 50.00 |
| | morula | 36 | 11.3443 | 7.06618 | 1.17770 | 8.9534 | 13.7351 | .00 | 23.81 |
| | Total | 144 | 37.1520 | 22.05218 | 1.83768 | 33.5194 | 40.7845 | .00 | 81.82 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| rapid | Between Groups | 54487.134 | 3 | 18162.378 | 256.718 | .000 |
| | Within Groups | 10753.736 | 152 | 70.748 | | |
| | Total | 65240.869 | 155 | | | |
| slow | Between Groups | 51070.326 | 3 | 17023.442 | 129.033 | .000 |
| | Within Groups | 18470.352 | 140 | 131.931 | | |
| | Total | 69540.678 | 143 | | | |

**rapid**

Duncan[a]

| development | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| morula | 39 | 13.2471 | | | |
| 8-cell | 39 | | 32.5710 | | |
| 4-cell | 39 | | | 49.0156 | |
| 2-cell | 39 | | | | 63.3609 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 |

**slow**

Duncan[a]

| developm ent | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| morula | 36 | 11.3443 | | | |
| 8-cell | 36 | | 29.1299 | | |
| 4-cell | 36 | | | 46.4510 | |
| 2-cell | 36 | | | | 61.6827 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 |

# EFFECT OF OOCYTE FACTORS ON ICSI PERFORMANCE   IN MICE (EXPERIMENT 2)

**Appendix Table 3.5**: Effect of  post-hCG duration administration duration on mouse quality of oocytes retrieval

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | 95% Confidence Interval for Mean Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| N_O_R | 13-15 hours | 112 | 13.9107 | 6.47317 | .61166 | 12.6987 | 15.1228 | 2.00 | 30.00 |
| | 16-18 hours | 112 | 13.5714 | 5.75117 | .54343 | 12.4946 | 14.6483 | 2.00 | 30.00 |
| | Total | 224 | 13.7411 | 6.11144 | .40834 | 12.9364 | 14.5458 | 2.00 | 30.00 |
| P_PB | 13-15 hours | 112 | 75.6145 | 17.32656 | 1.63721 | 72.3703 | 78.8587 | .00 | 100.00 |
| | 16-18 hours | 112 | 67.3746 | 15.88152 | 1.50066 | 64.4009 | 70.3482 | 25.00 | 100.00 |
| | Total | 224 | 71.4945 | 17.08882 | 1.14179 | 69.2444 | 73.7446 | .00 | 100.00 |
| P_W_PB | 13-15 hours | 112 | 12.5006 | 16.45386 | 1.55474 | 9.4197 | 15.5814 | .00 | 100.00 |
| | 16-18 hours | 112 | 21.8209 | 15.74322 | 1.48759 | 18.8731 | 24.7687 | .00 | 61.54 |
| | Total | 224 | 17.1607 | 16.73144 | 1.11792 | 14.9577 | 19.3638 | .00 | 100.00 |
| P_Dysmorphic | 13-15 hours | 112 | 11.8849 | 12.26514 | 1.15895 | 9.5884 | 14.1815 | .00 | 55.56 |
| | 16-18 hours | 112 | 10.7620 | 13.14980 | 1.24254 | 8.2999 | 13.2242 | .00 | 75.00 |
| | Total | 224 | 11.3235 | 12.69910 | .84849 | 9.6514 | 12.9956 | .00 | 75.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| N_O_R | Between Groups | 6.446 | 1 | 6.446 | .172 | .679 |
| | Within Groups | 8322.536 | 222 | 37.489 | | |
| | Total | 8328.982 | 223 | | | |
| P_PB | Between Groups | 3802.216 | 1 | 3802.216 | 13.765 | .000 |
| | Within Groups | 61319.995 | 222 | 276.216 | | |
| | Total | 65122.211 | 223 | | | |
| P_W_PB | Between Groups | 4864.649 | 1 | 4864.649 | 18.761 | .000 |
| | Within Groups | 57562.215 | 222 | 259.289 | | |
| | Total | 62426.864 | 223 | | | |

| | | | | | |
|---|---|---|---|---|---|
| P_Dysmorphic | Between Groups | 70.612 | 1 | 70.612 | .437 | .509 |
| | Within Groups | 35891.952 | 222 | 161.675 | | |
| | Total | 35962.564 | 223 | | | |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| 13-15 hr | PB | 112 | 75.6145 | 17.32656 | 1.63721 | 72.3703 | 78.8587 | .00 | 100.00 |
| | W/PB | 112 | 12.5006 | 16.45386 | 1.55474 | 9.4197 | 15.5814 | .00 | 100.00 |
| | dysmorphic | 112 | 11.8849 | 12.26514 | 1.15895 | 9.5884 | 14.1815 | .00 | 55.56 |
| | Total | 336 | 33.3333 | 33.69869 | 1.83841 | 29.7170 | 36.9496 | .00 | 100.00 |
| 16-18 hr | PB | 112 | 67.3746 | 15.88152 | 1.50066 | 64.4009 | 70.3482 | 25.00 | 100.00 |
| | W/PB | 112 | 21.8209 | 15.74322 | 1.48759 | 18.8731 | 24.7687 | .00 | 61.54 |
| | dysmorphic | 112 | 10.7620 | 13.14980 | 1.24254 | 8.2999 | 13.2242 | .00 | 75.00 |
| | Total | 336 | 33.3192 | 28.72369 | 1.56701 | 30.2368 | 36.4016 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| 13-15 hr | Between Groups | 300354.310 | 2 | 150177.155 | 624.547 | .000 |
| | Within Groups | 80072.373 | 333 | 240.458 | | |
| | Total | 380426.683 | 335 | | | |
| 16-18 hr | Between Groups | 201690.035 | 2 | 100845.018 | 449.539 | .000 |
| | Within Groups | 74701.789 | 333 | 224.330 | | |
| | Total | 276391.824 | 335 | | | |

**13-15 hr**

Duncan[a]

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| oocyte_quality | N | 1 | 2 |
| dysmorphic | 112 | 11.8849 | |
| W/PB | 112 | 12.5006 | |
| PB | 112 | | 75.6145 |
| Sig. | | .767 | 1.000 |

230

**16-18 hr**

| oocyte_quality | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| dysmorphic | 112 | 10.7620 | | |
| W/PB | 112 | | 21.8209 | |
| PB | 112 | | | 67.3746 |
| Sig. | | 1.000 | 1.000 | 1.000 |

**Appendix Table 3.6**: Percentage of mouse oocyte quality retrievalregardless post-hCG duration administration duration

**Descriptives**

P_ALLduration

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| PB | 224 | 71.4945 | 17.08882 | 1.14179 | 69.2444 | 73.7446 | .00 | 100.00 |
| W/PB | 224 | 17.1607 | 16.73144 | 1.11792 | 14.9577 | 19.3638 | .00 | 100.00 |
| Dysmorphic | 224 | 11.3235 | 12.69910 | .84849 | 9.6514 | 12.9956 | .00 | 75.00 |
| Total | 672 | 33.3262 | 31.28682 | 1.20692 | 30.9565 | 35.6960 | .00 | 100.00 |

**ANOVA**

P_ALLduration

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 493306.902 | 2 | 246653.451 | 1009.171 | .000 |
| Within Groups | 163511.639 | 669 | 244.412 | | |
| Total | 656818.541 | 671 | | | |

**P_ALLduration**

Duncan[a]

| quality_Oocyte | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| Dysmorphic | 224 | 11.3235 | | |
| W/PB | 224 | | 17.1607 | |
| PB | 224 | | | 71.4945 |
| Sig. | | 1.000 | 1.000 | 1.000 |

# EFFECTS OF SPERM FACTORS ON ICSI PERFORMANCE IN GOAT (EXPERIMENT 3)

**Appendix Table 3.7**: Effect of oocyte qualities on number of goat oocyte recovery from LOPU

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | 95% Confidence Interval for Mean Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| N_OR | A | 63 | 1.8889 | 1.84138 | .23199 | 1.4251 | 2.3526 | .00 | 8.00 |
| | B | 64 | 1.4375 | 1.50000 | .18750 | 1.0628 | 1.8122 | .00 | 5.00 |
| | C | 64 | 2.3906 | 2.73494 | .34187 | 1.7075 | 3.0738 | .00 | 11.00 |
| | D | 64 | .9844 | 1.74112 | .21764 | .5495 | 1.4193 | .00 | 8.00 |
| | E | 64 | .2813 | .62915 | .07864 | .1241 | .4384 | .00 | 3.00 |
| | Total | 319 | 1.3950 | 1.94922 | .10914 | 1.1803 | 1.6097 | .00 | 11.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| N_OR | Between Groups | 169.104 | 4 | 42.276 | 12.775 | .000 |
| | Within Groups | 1039.128 | 314 | 3.309 | | |
| | Total | 1208.232 | 318 | | | |

**N_OR**

Duncan[a,,b]

| Grade | N | Subset for alpha = 0.05 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| E | 64 | .2813 | | | |
| D | 64 | | .9844 | | |
| B | 64 | | 1.4375 | 1.4375 | |
| A | 63 | | | 1.8889 | 1.8889 |
| C | 64 | | | | 2.3906 |
| Sig. | | 1.000 | .160 | .162 | .120 |

**Appendix Table 3.8**: Effect of goat oocyte qualities on percentage of oocyte recovery from LOPU procedure

**Descriptives**

P_matured

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| A | 8 | 96.3480 | 5.97769 | 2.11343 | 91.3506 | 101.3455 | 83.33 | 100.00 |
| B | 8 | 87.5919 | 12.92024 | 4.56800 | 76.7903 | 98.3935 | 62.50 | 100.00 |
| C | 8 | 84.3974 | 7.84889 | 2.77500 | 77.8356 | 90.9592 | 75.00 | 100.00 |
| D | 8 | 51.0872 | 27.13202 | 9.59262 | 28.4042 | 73.7701 | .00 | 80.95 |
| E | 8 | 4.1667 | 11.78511 | 4.16667 | -5.6859 | 14.0193 | .00 | 33.33 |
| Total | 40 | 64.7182 | 37.23828 | 5.88789 | 52.8089 | 76.6276 | .00 | 100.00 |

**ANOVA**

P_matured

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 46105.741 | 4 | 11526.435 | 50.585 | .000 |
| Within Groups | 7975.141 | 35 | 227.861 | | |
| Total | 54080.882 | 39 | | | |

**P_matured**

Duncan[a]

| grades | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| E | 8 | 4.1667 | | |
| D | 8 | | 51.0872 | |
| C | 8 | | | 84.3974 |
| B | 8 | | | 87.5919 |
| A | 8 | | | 96.3480 |
| Sig. | | 1.000 | 1.000 | .143 |

**Appendix Table 3.9**: Effect of sperm movement with Heparin on goat embryo cleavage rate for Grade A

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| two_cell | Rapid | 15 | 62.8889 | 36.46300 | 9.41471 | 42.6964 | 83.0814 | .00 | 100.00 |
| | Slow | 10 | 84.6667 | 21.49935 | 6.79869 | 69.2870 | 100.0464 | 50.00 | 100.00 |
| | Total | 25 | 71.6000 | 32.67219 | 6.53444 | 58.1136 | 85.0864 | .00 | 100.00 |
| four_cell | Rapid | 15 | 49.5556 | 36.72326 | 9.48191 | 29.2189 | 69.8922 | .00 | 100.00 |
| | Slow | 10 | 55.5000 | 42.45586 | 13.42572 | 25.1289 | 85.8711 | .00 | 100.00 |
| | Total | 25 | 51.9333 | 38.35953 | 7.67191 | 36.0993 | 67.7674 | .00 | 100.00 |
| eight_cell | Rapid | 15 | 30.7778 | 30.86624 | 7.96963 | 13.6846 | 47.8709 | .00 | 100.00 |
| | Slow | 10 | 43.5000 | 41.23442 | 13.03947 | 14.0027 | 72.9973 | .00 | 100.00 |
| | Total | 25 | 35.8667 | 35.12583 | 7.02517 | 21.3674 | 50.3659 | .00 | 100.00 |
| morula | Rapid | 15 | 14.5556 | 27.46185 | 7.09062 | -.6523 | 29.7634 | .00 | 100.00 |
| | Slow | 10 | 8.2500 | 16.24679 | 5.13769 | -3.3723 | 19.8723 | .00 | 50.00 |
| | Total | 25 | 12.0333 | 23.42749 | 4.68550 | 2.3629 | 21.7037 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| two_cell | Between Groups | 2845.630 | 1 | 2845.630 | 2.874 | .104 |
| | Within Groups | 22773.704 | 23 | 990.161 | | |
| | Total | 25619.333 | 24 | | | |
| four_cell | Between Groups | 212.019 | 1 | 212.019 | .139 | .713 |
| | Within Groups | 35102.870 | 23 | 1526.212 | | |
| | Total | 35314.889 | 24 | | | |
| eight_cell | Between Groups | 971.130 | 1 | 971.130 | .780 | .386 |
| | Within Groups | 28640.648 | 23 | 1245.246 | | |
| | Total | 29611.778 | 24 | | | |
| morula | Between Groups | 238.560 | 1 | 238.560 | .424 | .521 |
| | Within Groups | 12933.773 | 23 | 562.338 | | |
| | Total | 13172.333 | 24 | | | |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| Rapid | 2-cell | 15 | 62.8889 | 36.46300 | 9.41471 | 42.6964 | 83.0814 | .00 | 100.00 |
| | 4-cell | 15 | 49.5556 | 36.72326 | 9.48191 | 29.2189 | 69.8922 | .00 | 100.00 |
| | 8-cell | 15 | 30.7778 | 30.86624 | 7.96963 | 13.6846 | 47.8709 | .00 | 100.00 |
| | morula | 15 | 14.5556 | 27.46185 | 7.09062 | -.6523 | 29.7634 | .00 | 100.00 |
| | Total | 60 | 39.4444 | 37.18644 | 4.80075 | 29.8382 | 49.0507 | .00 | 100.00 |
| Slow | 2-cell | 10 | 84.6667 | 21.49935 | 6.79869 | 69.2870 | 100.0464 | 50.00 | 100.00 |
| | 4-cell | 10 | 55.5000 | 42.45586 | 13.42572 | 25.1289 | 85.8711 | .00 | 100.00 |
| | 8-cell | 10 | 43.5000 | 41.23442 | 13.03947 | 14.0027 | 72.9973 | .00 | 100.00 |
| | morula | 10 | 8.2500 | 16.24679 | 5.13769 | -3.3723 | 19.8723 | .00 | 50.00 |
| | Total | 40 | 47.9792 | 41.77801 | 6.60568 | 34.6179 | 61.3404 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Rapid | Between Groups | 20196.667 | 3 | 6732.222 | 6.141 | .001 |
| | Within Groups | 61390.370 | 56 | 1096.257 | | |
| | Total | 81587.037 | 59 | | | |
| Slow | Between Groups | 30010.052 | 3 | 10003.351 | 9.462 | .000 |
| | Within Groups | 38060.625 | 36 | 1057.240 | | |
| | Total | 68070.677 | 39 | | | |

**Rapid**

Duncan[a]

| Development A | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| morula | 15 | 14.5556 | | |
| 8-cell | 15 | 30.7778 | 30.7778 | |
| 4-cell | 15 | | 49.5556 | 49.5556 |
| 2-cell | 15 | | | 62.8889 |
| Sig. | | .185 | .126 | .275 |

**Slow**

Duncan[a]

| Development A | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| morula | 10 | 8.2500 | | |
| 8-cell | 10 | | 43.5000 | |
| 4-cell | 10 | | 55.5000 | 55.5000 |
| 2-cell | 10 | | | 84.6667 |
| Sig. | | 1.000 | .415 | .052 |

**Appendix Table 3.10**: Effect of sperm movement with Heparin on goat embryo cleavage rate for Grade B

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | 95% Confidence Interval for Mean Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| two_cell | Rapid | 7 | 52.3810 | 41.30798 | 15.61295 | 14.1774 | 90.5845 | .00 | 100.00 |
| | Slow | 10 | 70.1667 | 32.37941 | 10.23927 | 47.0038 | 93.3295 | .00 | 100.00 |
| | Total | 17 | 62.8431 | 36.20814 | 8.78176 | 44.2266 | 81.4596 | .00 | 100.00 |
| four_cell | Rapid | 7 | 47.6190 | 41.30798 | 15.61295 | 14.1774 | 90.5845 | .00 | 100.00 |
| | Slow | 10 | 43.5000 | 34.73400 | 10.98385 | 18.6528 | 68.3472 | .00 | 100.00 |
| | Total | 17 | 47.1569 | 36.58971 | 8.87431 | 28.3442 | 65.9696 | .00 | 100.00 |
| eight_cell | Rapid | 7 | 29.7619 | 36.59625 | 13.83208 | -4.0840 | 63.6078 | .00 | 100.00 |
| | Slow | 10 | 26.6667 | 37.01851 | 11.70628 | .1852 | 53.1481 | .00 | 100.00 |
| | Total | 17 | 27.9412 | 35.71458 | 8.66206 | 9.5784 | 46.3039 | .00 | 100.00 |
| morula | Rapid | 7 | .0000 | .00000 | .00000 | .0000 | .0000 | .00 | .00 |
| | Slow | 10 | .0000 | .00000 | .00000 | .0000 | .0000 | .00 | .00 |
| | Total | 17 | .0000 | .00000 | .00000 | .0000 | .0000 | .00 | .00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| two_cell | Between Groups | 1302.542 | 1 | 1302.542 | .993 | .335 |
| | Within Groups | 19673.929 | 15 | 1311.595 | | |
| | Total | 20976.471 | 16 | | | |
| four_cell | Between Groups | 324.764 | 1 | 324.764 | .231 | .638 |
| | Within Groups | 21096.151 | 15 | 1406.410 | | |
| | Total | 21420.915 | 16 | | | |
| eight_cell | Between Groups | 39.449 | 1 | 39.449 | .029 | .867 |
| | Within Groups | 20369.048 | 15 | 1357.937 | | |
| | Total | 20408.497 | 16 | | | |
| morula | Between Groups | .000 | 1 | .000 | . | . |
| | Within Groups | .000 | 15 | .000 | | |
| | Total | .000 | 16 | | | |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| Rapid | 2-cell | 7 | 52.3810 | 41.30798 | 15.61295 | 14.1774 | 90.5845 | .00 | 100.00 |
| | 4-cell | 7 | 47.6190 | 41.30798 | 15.61295 | 9.4155 | 85.8226 | .00 | 100.00 |
| | 8-cell | 7 | 29.7619 | 36.59625 | 13.83208 | -4.0840 | 63.6078 | .00 | 100.00 |
| | Morula | 7 | .0000 | .00000 | .00000 | .0000 | .0000 | .00 | .00 |
| | Total | 28 | 32.4405 | 38.64602 | 7.30341 | 17.4551 | 47.4258 | .00 | 100.00 |
| Slow | 2-cell | 10 | 70.1667 | 32.37941 | 10.23927 | 47.0038 | 93.3295 | .00 | 100.00 |
| | 4-cell | 10 | 43.5000 | 34.73400 | 10.98385 | 18.6528 | 68.3472 | .00 | 100.00 |
| | 8-cell | 10 | 26.6667 | 37.01851 | 11.70628 | .1852 | 53.1481 | .00 | 100.00 |
| | Morula | 10 | .0000 | .00000 | .00000 | .0000 | .0000 | .00 | .00 |
| | Total | 40 | 35.0833 | 38.78304 | 6.13214 | 22.6799 | 47.4868 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Rapid | Between Groups | 11812.996 | 3 | 3937.665 | 3.315 | .037 |
| | Within Groups | 28511.905 | 24 | 1187.996 | | |
| | Total | 40324.901 | 27 | | | |
| Slow | Between Groups | 26033.611 | 3 | 8677.870 | 9.575 | .000 |
| | Within Groups | 32627.222 | 36 | 906.312 | | |
| | Total | 58660.833 | 39 | | | |

**Rapid**

Duncan[a]

| Development B | N | Subset for alpha = 0.05 | |
| | | 1 | 2 |
|---|---|---|---|
| Morula | 7 | .0000 | |
| 8-cell | 7 | 29.7619 | 29.7619 |
| 4-cell | 7 | | 47.6190 |
| 2-cell | 7 | | 52.3810 |
| Sig. | | .119 | .258 |

**Slow**

Duncan[a]

| Development B | N | Subset for alpha = 0.05 | | |
| | | 1 | 2 | 3 |
|---|---|---|---|---|
| Morula | 10 | .0000 | | |
| 8-cell | 10 | 26.6667 | 26.6667 | |
| 4-cell | 10 | | 43.5000 | 43.5000 |
| 2-cell | 10 | | | 70.1667 |
| Sig. | | .055 | .219 | .055 |

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**Appendix Table 3.11**: Effect of sperm movement with Heparin on goat embryo cleavage rate for Grade C

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | 95% Confidence Interval for Mean Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| two_cell | Rapid | 12 | 63.1845 | 30.15315 | 8.70446 | 44.0261 | 82.3429 | .00 | 100.00 |
| | Slow | 11 | 45.5844 | 34.20565 | 10.31339 | 22.6047 | 68.5641 | .00 | 100.00 |
| | Total | 23 | 54.7671 | 32.66871 | 6.81190 | 40.6401 | 68.8941 | .00 | 100.00 |
| four_cell | Rapid | 12 | 41.1607 | 31.56291 | 9.11143 | 21.1066 | 61.2148 | .00 | 100.00 |
| | Slow | 11 | 26.2554 | 27.11446 | 8.17532 | 8.0397 | 44.4712 | .00 | 66.67 |
| | Total | 23 | 34.0321 | 29.83697 | 6.22144 | 21.1296 | 46.9346 | .00 | 100.00 |
| eight_cell | Rapid | 12 | 20.2480 | 22.53862 | 6.50634 | 5.9277 | 34.5684 | .00 | 57.14 |
| | Slow | 11 | 12.8355 | 18.33193 | 5.52728 | .5199 | 25.1511 | .00 | 42.86 |
| | Total | 23 | 16.7029 | 20.52030 | 4.27878 | 7.8293 | 25.5765 | .00 | 57.14 |
| morula | Rapid | 12 | 4.8115 | 9.40284 | 2.71437 | -1.1628 | 10.7858 | .00 | 28.57 |
| | Slow | 11 | 3.1169 | 7.05136 | 2.12607 | -1.6203 | 7.8541 | .00 | 20.00 |
| | Total | 23 | 4.0010 | 8.21928 | 1.71384 | .4468 | 7.5553 | .00 | 28.57 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| two_cell | Between Groups | 1777.775 | 1 | 1777.775 | 1.720 | .204 |
| | Within Groups | 21701.604 | 21 | 1033.410 | | |
| | Total | 23479.379 | 22 | | | |
| four_cell | Between Groups | 1275.051 | 1 | 1275.051 | 1.462 | .240 |
| | Within Groups | 18310.331 | 21 | 871.921 | | |
| | Total | 19585.383 | 22 | | | |
| eight_cell | Between Groups | 315.339 | 1 | 315.339 | .740 | .399 |
| | Within Groups | 8948.477 | 21 | 426.118 | | |
| | Total | 9263.816 | 22 | | | |
| morula | Between Groups | 16.481 | 1 | 16.481 | .235 | .633 |
| | Within Groups | 1469.764 | 21 | 69.989 | | |
| | Total | 1486.245 | 22 | | | |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| Rapid | 2-cell | 12 | 63.1845 | 30.15315 | 8.70446 | 44.0261 | 82.3429 | .00 | 100.00 |
| | 4-cell | 12 | 41.1607 | 31.56291 | 9.11143 | 21.1066 | 61.2148 | .00 | 100.00 |
| | 8-cell | 12 | 20.2480 | 22.53862 | 6.50634 | 5.9277 | 34.5684 | .00 | 57.14 |
| | morula | 12 | 4.8115 | 9.40284 | 2.71437 | -1.1628 | 10.7858 | .00 | 28.57 |
| | Total | 48 | 32.3512 | 32.85003 | 4.74149 | 22.8125 | 41.8898 | .00 | 100.00 |
| Slow | 2-cell | 11 | 45.5844 | 34.20565 | 10.31339 | 22.6047 | 68.5641 | .00 | 100.00 |
| | 4-cell | 11 | 26.2554 | 27.11446 | 8.17532 | 8.0397 | 44.4712 | .00 | 66.67 |
| | 8-cell | 11 | 12.8355 | 18.33193 | 5.52728 | .5199 | 25.1511 | .00 | 42.86 |
| | morula | 11 | 3.1169 | 7.05136 | 2.12607 | -1.6203 | 7.8541 | .00 | 20.00 |
| | Total | 44 | 21.9481 | 28.14982 | 4.24374 | 13.3897 | 30.5064 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Rapid | Between Groups | 23198.677 | 3 | 7732.892 | 12.364 | .000 |
| | Within Groups | 27520.158 | 44 | 625.458 | | |
| | Total | 50718.835 | 47 | | | |
| Slow | Between Groups | 11163.709 | 3 | 3721.236 | 6.497 | .001 |
| | Within Groups | 22910.019 | 40 | 572.750 | | |
| | Total | 34073.727 | 43 | | | |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| Rapid | 2-cell | 12 | 63.1845 | 30.15315 | 8.70446 | 44.0261 | 82.3429 | .00 | 100.00 |
| | 4-cell | 12 | 41.1607 | 31.56291 | 9.11143 | 21.1066 | 61.2148 | .00 | 100.00 |
| | 8-cell | 12 | 20.2480 | 22.53862 | 6.50634 | 5.9277 | 34.5684 | .00 | 57.14 |
| | morula | 12 | 4.8115 | 9.40284 | 2.71437 | -1.1628 | 10.7858 | .00 | 28.57 |
| | Total | 48 | 32.3512 | 32.85003 | 4.74149 | 22.8125 | 41.8898 | .00 | 100.00 |
| Slow | 2-cell | 11 | 45.5844 | 34.20565 | 10.31339 | 22.6047 | 68.5641 | .00 | 100.00 |
| | 4-cell | 11 | 26.2554 | 27.11446 | 8.17532 | 8.0397 | 44.4712 | .00 | 66.67 |
| | 8-cell | 11 | 12.8355 | 18.33193 | 5.52728 | .5199 | 25.1511 | .00 | 42.86 |
| | morula | 11 | 3.1169 | 7.05136 | 2.12607 | -1.6203 | 7.8541 | .00 | 20.00 |
| | Total | 44 | 21.9481 | 28.14982 | 4.24374 | 13.3897 | 30.5064 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Rapid | Between Groups | 23198.677 | 3 | 7732.892 | 12.364 | .000 |
| | Within Groups | 27520.158 | 44 | 625.458 | | |
| | Total | 50718.835 | 47 | | | |
| Slow | Between Groups | 11163.709 | 3 | 3721.236 | 6.497 | .001 |
| | Within Groups | 22910.019 | 40 | 572.750 | | |
| | Total | 34073.727 | 43 | | | |

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**Rapid**

Duncan[a]

| Developement C | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| morula | 12 | 4.8115 | | |
| 8-cell | 12 | 20.2480 | | |
| 4-cell | 12 | | 41.1607 | |
| 2-cell | 12 | | | 63.1845 |
| Sig. | | .138 | 1.000 | 1.000 |

**Slow**

Duncan[a]

| Developement C | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| morula | 11 | 3.1169 | | |
| 8-cell | 11 | 12.8355 | 12.8355 | |
| 4-cell | 11 | | 26.2554 | 26.2554 |
| 2-cell | 11 | | | 45.5844 |
| Sig. | | .347 | .196 | .065 |

**Appendix Table 3.12**: Effect of sperm movement with Heparin on goat embryo cleavage rate for Combined Grades (A, B and C)

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | 95% Confidence Interval for Mean Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| two_cell | Rapid | 34 | 60.8298 | 34.58978 | 5.93210 | 48.7609 | 72.8988 | .00 | 100.00 |
| | Slow | 31 | 66.1214 | 33.43574 | 6.00524 | 53.8570 | 78.3857 | .00 | 100.00 |
| | Total | 65 | 63.3535 | 33.88294 | 4.20266 | 54.9577 | 71.7493 | .00 | 100.00 |
| four_cell | Rapid | 34 | 46.1940 | 35.06037 | 6.01280 | 33.9608 | 58.4271 | .00 | 100.00 |
| | Slow | 31 | 41.2519 | 36.04948 | 6.47468 | 28.0289 | 54.4750 | .00 | 100.00 |
| | Total | 65 | 43.8370 | 35.34375 | 4.38385 | 35.0792 | 52.5947 | .00 | 100.00 |
| eight_cell | Rapid | 34 | 26.8522 | 29.01178 | 4.97548 | 16.7296 | 36.9749 | .00 | 100.00 |
| | Slow | 31 | 27.1889 | 34.60535 | 6.21530 | 14.4956 | 39.8823 | .00 | 100.00 |
| | Total | 65 | 27.0128 | 31.54939 | 3.91322 | 19.1953 | 34.8304 | .00 | 100.00 |
| morula | Rapid | 34 | 8.1197 | 19.65215 | 3.37032 | 1.2628 | 14.9767 | .00 | 100.00 |
| | Slow | 31 | 3.7673 | 10.36077 | 1.86085 | -.0331 | 7.5676 | .00 | 50.00 |
| | Total | 65 | 6.0440 | 15.94541 | 1.97778 | 2.0929 | 9.9950 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| two_cell | Between Groups | 454.034 | 1 | 454.034 | .392 | .534 |
| | Within Groups | 73021.408 | 63 | 1159.070 | | |
| | Total | 73475.441 | 64 | | | |
| four_cell | Between Groups | 396.043 | 1 | 396.043 | .314 | .577 |
| | Within Groups | 79551.541 | 63 | 1262.723 | | |
| | Total | 79947.584 | 64 | | | |
| eight_cell | Between Groups | 1.838 | 1 | 1.838 | .002 | .966 |
| | Within Groups | 63701.442 | 63 | 1011.134 | | |
| | Total | 63703.280 | 64 | | | |
| morula | Between Groups | 307.184 | 1 | 307.184 | 1.212 | .275 |
| | Within Groups | 15965.202 | 63 | 253.416 | | |
| | Total | 16272.386 | 64 | | | |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| Rapid | 2-cell | 34 | 60.8298 | 34.58978 | 5.93210 | 48.7609 | 72.8988 | .00 | 100.00 |
| | 4-cell | 34 | 46.1940 | 35.06037 | 6.01280 | 33.9608 | 58.4271 | .00 | 100.00 |
| | 8-cell | 34 | 26.8522 | 29.01178 | 4.97548 | 16.7296 | 36.9749 | .00 | 100.00 |
| | morula | 34 | 8.1197 | 19.65215 | 3.37032 | 1.2628 | 14.9767 | .00 | 100.00 |
| | Total | 136 | 35.4989 | 35.93227 | 3.08117 | 29.4053 | 41.5925 | .00 | 100.00 |
| Slow | 2-cell | 31 | 66.1214 | 33.43574 | 6.00524 | 53.8570 | 78.3857 | .00 | 100.00 |
| | 4-cell | 31 | 41.2519 | 36.04948 | 6.47468 | 28.0289 | 54.4750 | .00 | 100.00 |
| | 8-cell | 31 | 27.1889 | 34.60535 | 6.21530 | 14.4956 | 39.8823 | .00 | 100.00 |
| | morula | 31 | 3.7673 | 10.36077 | 1.86085 | -.0331 | 7.5676 | .00 | 50.00 |
| | Total | 124 | 34.5824 | 37.72146 | 3.38749 | 27.8770 | 41.2877 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Rapid | Between Groups | 53734.398 | 3 | 17911.466 | 19.610 | .000 |
| | Within Groups | 120567.912 | 132 | 913.393 | | |
| | Total | 174302.311 | 135 | | | |
| Slow | Between Groups | 63346.106 | 3 | 21115.369 | 22.690 | .000 |
| | Within Groups | 111671.680 | 120 | 930.597 | | |
| | Total | 175017.786 | 123 | | | |

Rapid

Duncan[a]

| Development Com Grade | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| morula | 34 | 8.1197 | | | |
| 8-cell | 34 | | 26.8522 | | |
| 4-cell | 34 | | | 46.1940 | |
| 2-cell | 34 | | | | 60.8298 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 |

**Slow**

Duncan[a]

| Development Com Grade | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| morula | 31 | 3.7673 | | |
| 8-cell | 31 | | 27.1889 | |
| 4-cell | 31 | | 41.2519 | |
| 2-cell | 31 | | | 66.1214 |
| Sig. | | 1.000 | .072 | 1.000 |

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**Appendix Table 3.13**: Effect of sperm movement with Theophylline on goat embryo cleavage rate for Grade A

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| two_cell | Rapid | 8 | 95.8333 | 11.78511 | 4.16667 | 85.9807 | 105.6859 | 66.67 | 100.00 |
| | Slow | 14 | 63.2653 | 34.03839 | 9.09714 | 43.6121 | 82.9185 | .00 | 100.00 |
| | Total | 22 | 75.1082 | 31.94786 | 6.81131 | 60.9433 | 89.2731 | .00 | 100.00 |
| four_cell | Rapid | 8 | 83.3333 | 35.63483 | 12.59882 | 53.5419 | 113.1248 | .00 | 100.00 |
| | Slow | 14 | 47.9592 | 37.10782 | 9.91748 | 26.5338 | 69.3846 | .00 | 100.00 |
| | Total | 22 | 60.8225 | 39.73734 | 8.47203 | 43.2040 | 78.4411 | .00 | 100.00 |
| eight_cell | Rapid | 8 | 77.0833 | 36.66396 | 12.96267 | 46.4315 | 107.7352 | .00 | 100.00 |
| | Slow | 14 | 20.1531 | 31.87025 | 8.51768 | 1.7517 | 38.5544 | .00 | 100.00 |
| | Total | 22 | 40.8550 | 43.15747 | 9.20120 | 21.7200 | 59.9899 | .00 | 100.00 |
| morula | Rapid | 8 | 18.7500 | 37.20119 | 13.15261 | -12.3510 | 49.8510 | .00 | 100.00 |
| | Slow | 14 | 1.0204 | 3.81802 | 1.02041 | -1.1840 | 3.2249 | .00 | 14.29 |
| | Total | 22 | 7.4675 | 23.37813 | 4.98423 | -2.8977 | 17.8328 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| two_cell | Between Groups | 5399.807 | 1 | 5399.807 | 6.735 | .017 |
| | Within Groups | 16034.176 | 20 | 801.709 | | |
| | Total | 21433.983 | 21 | | | |
| four_cell | Between Groups | 6370.410 | 1 | 6370.410 | 4.756 | .041 |
| | Within Groups | 26789.764 | 20 | 1339.488 | | |
| | Total | 33160.173 | 21 | | | |
| eight_cell | Between Groups | 16499.921 | 1 | 16499.921 | 14.593 | .001 |
| | Within Groups | 22613.986 | 20 | 1130.699 | | |
| | Total | 39113.907 | 21 | | | |
| morula | Between Groups | 1600.268 | 1 | 1600.268 | 3.240 | .087 |
| | Within Groups | 9877.004 | 20 | 493.850 | | |
| | Total | 11477.273 | 21 | | | |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| Rapid | 2-cell | 8 | 95.8333 | 11.78511 | 4.16667 | 85.9807 | 105.6859 | 66.67 | 100.00 |
| | 4-cell | 8 | 83.3333 | 35.63483 | 12.59882 | 53.5419 | 113.1248 | .00 | 100.00 |
| | 8-cell | 8 | 77.0833 | 36.66396 | 12.96267 | 46.4315 | 107.7352 | .00 | 100.00 |
| | morula | 8 | 18.7500 | 37.20119 | 13.15261 | -12.3510 | 49.8510 | .00 | 100.00 |
| | Total | 32 | 68.7500 | 42.91151 | 7.58576 | 53.2788 | 84.2212 | .00 | 100.00 |
| Slow | 2-cell | 14 | 63.2653 | 34.03839 | 9.09714 | 43.6121 | 82.9185 | .00 | 100.00 |
| | 4-cell | 14 | 47.9592 | 37.10782 | 9.91748 | 26.5338 | 69.3846 | .00 | 100.00 |
| | 8-cell | 14 | 20.1531 | 31.87025 | 8.51768 | 1.7517 | 38.5544 | .00 | 100.00 |
| | morula | 14 | 1.0204 | 3.81802 | 1.02041 | -1.1840 | 3.2249 | .00 | 14.29 |
| | Total | 56 | 33.0995 | 37.88526 | 5.06263 | 22.9538 | 43.2452 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Rapid | Between Groups | 28125.000 | 3 | 9375.000 | 9.065 | .000 |
| | Within Groups | 28958.333 | 28 | 1034.226 | | |
| | Total | 57083.333 | 31 | | | |
| Slow | Between Groups | 32584.503 | 3 | 10861.501 | 12.184 | .000 |
| | Within Groups | 46356.596 | 52 | 891.473 | | |
| | Total | 78941.099 | 55 | | | |

**Rapid**

Duncan[a]

| Development A | N | Subset for alpha = 0.05 | |
| | | 1 | 2 |
|---|---|---|---|
| morula | 8 | 18.7500 | |
| 8-cell | 8 | | 77.0833 |
| 4-cell | 8 | | 83.3333 |
| 2-cell | 8 | | 95.8333 |
| Sig. | | 1.000 | .281 |

**Slow**

Duncan[a]

| Development A | N | Subset for alpha = 0.05 | |
| | | 1 | 2 |
|---|---|---|---|
| morula | 14 | 1.0204 | |
| 8-cell | 14 | 20.1531 | |
| 4-cell | 14 | | 47.9592 |
| 2-cell | 14 | | 63.2653 |
| Sig. | | .096 | .181 |

**Appendix Table 3.14**: Effect of sperm movement with Theophylline on goat embryo
cleavage rate for Grade B

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| two_cell | Rapid | 8 | 84.3750 | 16.92508 | 5.98392 | 70.2253 | 98.5247 | 66.67 | 100.00 |
| | Slow | 11 | 50.0000 | 39.44053 | 11.89177 | 23.5035 | 76.4965 | .00 | 100.00 |
| | Total | 19 | 64.4737 | 35.77214 | 8.20669 | 47.2321 | 81.7153 | .00 | 100.00 |
| four_cell | Rapid | 8 | 63.5417 | 33.31473 | 11.77853 | 35.6899 | 91.3935 | .00 | 100.00 |
| | Slow | 11 | 28.0303 | 39.48852 | 11.90624 | 1.5016 | 54.5591 | .00 | 100.00 |
| | Total | 19 | 42.9825 | 40.27904 | 9.24065 | 23.5686 | 62.3963 | .00 | 100.00 |
| eight_cell | Rapid | 8 | 59.3750 | 34.91415 | 12.34402 | 30.1860 | 88.5640 | .00 | 100.00 |
| | Slow | 11 | 25.0000 | 40.31129 | 12.15431 | -2.0815 | 52.0815 | .00 | 100.00 |
| | Total | 19 | 39.4737 | 40.99854 | 9.40571 | 19.7130 | 59.2343 | .00 | 100.00 |
| morula | Rapid | 8 | 7.2917 | 13.68400 | 4.83803 | -4.1484 | 18.7318 | .00 | 33.33 |
| | Slow | 11 | .0000 | .00000 | .00000 | .0000 | .0000 | .00 | .00 |
| | Total | 19 | 3.0702 | 9.30059 | 2.13370 | -1.4126 | 7.5529 | .00 | 33.33 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| two_cell | Between Groups | 5472.862 | 1 | 5472.862 | 5.298 | .034 |
| | Within Groups | 17560.764 | 17 | 1032.986 | | |
| | Total | 23033.626 | 18 | | | |
| four_cell | Between Groups | 5840.685 | 1 | 5840.685 | 4.250 | .055 |
| | Within Groups | 23362.532 | 17 | 1374.267 | | |
| | Total | 29203.216 | 18 | | | |
| eight_cell | Between Groups | 5472.862 | 1 | 5472.862 | 3.754 | .069 |
| | Within Groups | 24782.986 | 17 | 1457.823 | | |
| | Total | 30255.848 | 18 | | | |
| morula | Between Groups | 246.254 | 1 | 246.254 | 3.194 | .092 |
| | Within Groups | 1310.764 | 17 | 77.104 | | |
| | Total | 1557.018 | 18 | | | |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| rapid | 2-cell | 8 | 84.3750 | 16.92508 | 5.98392 | 70.2253 | 98.5247 | 66.67 | 100.00 |
| | 4-cell | 8 | 63.5417 | 33.31473 | 11.77853 | 35.6899 | 91.3935 | .00 | 100.00 |
| | 8-cell | 8 | 59.3750 | 34.91415 | 12.34402 | 30.1860 | 88.5640 | .00 | 100.00 |
| | morula | 8 | 7.2917 | 13.68400 | 4.83803 | -4.1484 | 18.7318 | .00 | 33.33 |
| | Total | 32 | 53.6458 | 38.27236 | 6.76566 | 39.8472 | 67.4445 | .00 | 100.00 |
| slow | 2-cell | 11 | 50.0000 | 39.44053 | 11.89177 | 23.5035 | 76.4965 | .00 | 100.00 |
| | 4-cell | 11 | 28.0303 | 39.48852 | 11.90624 | 1.5016 | 54.5591 | .00 | 100.00 |
| | 8-cell | 11 | 25.0000 | 40.31129 | 12.15431 | -2.0815 | 52.0815 | .00 | 100.00 |
| | morula | 11 | .0000 | .00000 | .00000 | .0000 | .0000 | .00 | .00 |
| | Total | 44 | 25.7576 | 37.73369 | 5.68857 | 14.2855 | 37.2297 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| rapid | Between Groups | 25789.931 | 3 | 8596.644 | 12.270 | .000 |
| | Within Groups | 19618.056 | 28 | 700.645 | | |
| | Total | 45407.986 | 31 | | | |
| slow | Between Groups | 13825.758 | 3 | 4608.586 | 3.889 | .016 |
| | Within Groups | 47398.990 | 40 | 1184.975 | | |
| | Total | 61224.747 | 43 | | | |

**rapid**

Duncan[a]

| Development B | N | Subset for alpha = 0.05 | |
|---|---|---|---|
| | | 1 | 2 |
| morula | 8 | 7.2917 | |
| 8-cell | 8 | | 59.3750 |
| 4-cell | 8 | | 63.5417 |
| 2-cell | 8 | | 84.3750 |
| Sig. | | 1.000 | .084 |

**slow**

Duncan[a]

| Development B | N | Subset for alpha = 0.05 | |
|---|---|---|---|
| | | 1 | 2 |
| morula | 11 | .0000 | |
| 8-cell | 11 | 25.0000 | 25.0000 |
| 4-cell | 11 | 28.0303 | 28.0303 |
| 2-cell | 11 | | 50.0000 |
| Sig. | | .078 | .115 |

**Appendix Table 3.15**: Effect of sperm movement with Theophylline on goat embryo cleavage rate for Grade C

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| two_cell | Rapid | 8 | 76.0417 | 26.87327 | 9.50114 | 53.5751 | 98.5083 | 20.00 | 100.00 |
| | Slow | 10 | 46.6667 | 38.32931 | 12.12079 | 19.2475 | 74.0858 | .00 | 100.00 |
| | Total | 18 | 59.7222 | 36.06571 | 8.50077 | 41.7872 | 77.6573 | .00 | 100.00 |
| four_cell | Rapid | 8 | 67.2917 | 26.15487 | 9.24714 | 45.4256 | 89.1577 | 20.00 | 100.00 |
| | Slow | 10 | 50.0000 | 34.24674 | 10.82977 | 25.5014 | 74.4986 | .00 | 100.00 |
| | Total | 18 | 57.6852 | 31.31716 | 7.38152 | 42.1115 | 73.2588 | .00 | 100.00 |
| eight_cell | Rapid | 8 | 35.8333 | 32.40370 | 11.45644 | 8.7432 | 62.9235 | .00 | 100.00 |
| | Slow | 10 | 20.0000 | 21.94269 | 6.93889 | 4.3031 | 35.6969 | .00 | 50.00 |
| | Total | 18 | 27.0370 | 27.43713 | 6.46699 | 13.3929 | 40.6812 | .00 | 100.00 |
| morula | Rapid | 8 | 6.2500 | 17.67767 | 6.25000 | -8.5289 | 21.0289 | .00 | 50.00 |
| | Slow | 10 | 1.6667 | 5.27046 | 1.66667 | -2.1036 | 5.4369 | .00 | 16.67 |
| | Total | 18 | 3.7037 | 12.20141 | 2.87590 | -2.3639 | 9.7713 | .00 | 50.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| two_cell | Between Groups | 3835.069 | 1 | 3835.069 | 3.357 | .086 |
| | Within Groups | 18277.431 | 16 | 1142.339 | | |
| | Total | 22112.500 | 17 | | | |
| four_cell | Between Groups | 1328.897 | 1 | 1328.897 | 1.386 | .256 |
| | Within Groups | 15344.097 | 16 | 959.006 | | |
| | Total | 16672.994 | 17 | | | |
| eight_cell | Between Groups | 1114.198 | 1 | 1114.198 | 1.526 | .235 |
| | Within Groups | 11683.333 | 16 | 730.208 | | |
| | Total | 12797.531 | 17 | | | |
| morula | Between Groups | 93.364 | 1 | 93.364 | .613 | .445 |
| | Within Groups | 2437.500 | 16 | 152.344 | | |
| | Total | 2530.864 | 17 | | | |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| rapid | 2-cell | 8 | 76.0417 | 26.87327 | 9.50114 | 53.5751 | 98.5083 | 20.00 | 100.00 |
| | 4-cell | 8 | 67.2917 | 26.15487 | 9.24714 | 45.4256 | 89.1577 | 20.00 | 100.00 |
| | 8-cell | 8 | 35.8333 | 32.40370 | 11.45644 | 8.7432 | 62.9235 | .00 | 100.00 |
| | morula | 8 | 6.2500 | 17.67767 | 6.25000 | -8.5289 | 21.0289 | .00 | 50.00 |
| | Total | 32 | 46.3542 | 37.54194 | 6.63654 | 32.8189 | 59.8895 | .00 | 100.00 |
| slow | 2-cell | 10 | 46.6667 | 38.32931 | 12.12079 | 19.2475 | 74.0858 | .00 | 100.00 |
| | 4-cell | 10 | 50.0000 | 34.24674 | 10.82977 | 25.5014 | 74.4986 | .00 | 100.00 |
| | 8-cell | 10 | 20.0000 | 21.94269 | 6.93889 | 4.3031 | 35.6969 | .00 | 50.00 |
| | morula | 10 | 1.6667 | 5.27046 | 1.66667 | -2.1036 | 5.4369 | .00 | 16.67 |
| | Total | 40 | 29.5833 | 33.64967 | 5.32048 | 18.8216 | 40.3450 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| rapid | Between Groups | 24310.069 | 3 | 8103.356 | 11.707 | .000 |
| | Within Groups | 19381.250 | 28 | 692.187 | | |
| | Total | 43691.319 | 31 | | | |
| slow | Between Groups | 15798.611 | 3 | 5266.204 | 6.685 | .001 |
| | Within Groups | 28361.111 | 36 | 787.809 | | |
| | Total | 44159.722 | 39 | | | |

**rapid**

Duncan[a]

| Development C | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| morula | 8 | 6.2500 | | |
| 8-cell | 8 | | 35.8333 | |
| 4-cell | 8 | | | 67.2917 |
| 2-cell | 8 | | | 76.0417 |
| Sig. | | 1.000 | 1.000 | .511 |

**slow**

Duncan[a]

| Development C | N | Subset for alpha = 0.05 | |
|---|---|---|---|
| | | 1 | 2 |
| morula | 10 | 1.6667 | |
| 8-cell | 10 | 20.0000 | |
| 2-cell | 10 | | 46.6667 |
| 4-cell | 10 | | 50.0000 |
| Sig. | | .153 | .792 |

**Appendix Table 3.16**: Effect of sperm movement with Theophylline on goat embryo cleavage rate for Combine Grades (A, B and C)

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | 95% Confidence Interval for Mean Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| two_cell | Rapid | 24 | 85.4167 | 20.44345 | 4.17300 | 76.7842 | 94.0492 | 20.00 | 100.00 |
| | Slow | 35 | 54.3537 | 36.68265 | 6.20050 | 41.7528 | 66.9547 | .00 | 100.00 |
| | Total | 59 | 66.9895 | 34.51664 | 4.49368 | 57.9944 | 75.9846 | .00 | 100.00 |
| four_cell | Rapid | 24 | 71.3889 | 31.77011 | 6.48505 | 57.9735 | 84.8042 | .00 | 100.00 |
| | Slow | 35 | 42.2789 | 37.31069 | 6.30666 | 29.4622 | 55.0956 | .00 | 100.00 |
| | Total | 59 | 54.1203 | 37.74012 | 4.91334 | 44.2851 | 63.9554 | .00 | 100.00 |
| eight_cell | Rapid | 24 | 57.4306 | 37.38421 | 7.63102 | 41.6446 | 73.2165 | .00 | 100.00 |
| | Slow | 35 | 21.6327 | 31.60865 | 5.34284 | 10.7747 | 32.4906 | .00 | 100.00 |
| | Total | 59 | 36.1945 | 38.13743 | 4.96507 | 26.2558 | 46.1332 | .00 | 100.00 |
| morula | Rapid | 24 | 10.7639 | 24.63246 | 5.02808 | .3625 | 21.1653 | .00 | 100.00 |
| | Slow | 35 | .8844 | 3.65613 | .61800 | -.3716 | 2.1403 | .00 | 16.67 |
| | Total | 59 | 4.9031 | 16.50473 | 2.14873 | .6020 | 9.2043 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| two_cell | Between Groups | 13737.635 | 1 | 13737.635 | 14.144 | .000 |
| | Within Groups | 55363.472 | 57 | 971.289 | | |
| | Total | 69101.107 | 58 | | | |
| four_cell | Between Groups | 12064.547 | 1 | 12064.547 | 9.748 | .003 |
| | Within Groups | 70545.800 | 57 | 1237.646 | | |
| | Total | 82610.346 | 58 | | | |
| eight_cell | Between Groups | 18244.940 | 1 | 18244.940 | 15.730 | .000 |
| | Within Groups | 66113.959 | 57 | 1159.894 | | |
| | Total | 84358.899 | 58 | | | |
| morula | Between Groups | 1389.634 | 1 | 1389.634 | 5.497 | .023 |
| | Within Groups | 14409.926 | 57 | 252.806 | | |
| | Total | 15799.560 | 58 | | | |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| Rapid | 2-cell | 24 | 85.4167 | 20.44345 | 4.17300 | 76.7842 | 94.0492 | 20.00 | 100.00 |
| | 4-cell | 24 | 71.3889 | 31.77011 | 6.48505 | 57.9735 | 84.8042 | .00 | 100.00 |
| | 8-cell | 24 | 57.4306 | 37.38421 | 7.63102 | 41.6446 | 73.2165 | .00 | 100.00 |
| | morula | 24 | 10.7639 | 24.63246 | 5.02808 | .3625 | 21.1653 | .00 | 100.00 |
| | Total | 96 | 56.2500 | 40.33196 | 4.11636 | 48.0780 | 64.4220 | .00 | 100.00 |
| Slow | 2-cell | 35 | 54.3537 | 36.68265 | 6.20050 | 41.7528 | 66.9547 | .00 | 100.00 |
| | 4-cell | 35 | 42.2789 | 37.31069 | 6.30666 | 29.4622 | 55.0956 | .00 | 100.00 |
| | 8-cell | 35 | 21.6327 | 31.60865 | 5.34284 | 10.7747 | 32.4906 | .00 | 100.00 |
| | morula | 35 | .8844 | 3.65613 | .61800 | -.3716 | 2.1403 | .00 | 16.67 |
| | Total | 140 | 29.7874 | 36.54662 | 3.08875 | 23.6804 | 35.8944 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Rapid | Between Groups | 75606.250 | 3 | 25202.083 | 29.376 | .000 |
| | Within Groups | 78927.083 | 92 | 857.903 | | |
| | Total | 154533.333 | 95 | | | |
| Slow | Between Groups | 58150.014 | 3 | 19383.338 | 20.675 | .000 |
| | Within Groups | 127506.074 | 136 | 937.545 | | |
| | Total | 185656.088 | 139 | | | |

**Rapid**

Duncan[a]

| Development ABC | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| morula | 24 | 10.7639 | | |
| 8-cell | 24 | | 57.4306 | |
| 4-cell | 24 | | 71.3889 | 71.3889 |
| 2-cell | 24 | | | 85.4167 |
| Sig. | | 1.000 | .102 | .101 |

**Slow**

Duncan[a]

| Development ABC | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| morula | 35 | .8844 | | |
| 8-cell | 35 | | 21.6327 | |
| 4-cell | 35 | | | 42.2789 |
| 2-cell | 35 | | | 54.3537 |
| Sig. | | 1.000 | 1.000 | .101 |

**Appendix Table 3.17**: Effect of sperm movement on goat embryo cleavage rate regardless sperm capacitation chemical treatment

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| two_cell | Rapid | 58 | 71.0037 | 31.78886 | 4.17408 | 62.6452 | 79.3622 | .00 | 100.00 |
| | Slow | 66 | 59.8810 | 35.42400 | 4.36039 | 51.1726 | 68.5893 | .00 | 100.00 |
| | Total | 124 | 65.0835 | 34.09521 | 3.06184 | 59.0228 | 71.1442 | .00 | 100.00 |
| four_cell | Rapid | 58 | 56.6195 | 35.71578 | 4.68971 | 47.2285 | 66.0104 | .00 | 100.00 |
| | Slow | 66 | 41.7965 | 36.44496 | 4.48606 | 32.8372 | 50.7558 | .00 | 100.00 |
| | Total | 124 | 48.7298 | 36.71782 | 3.29736 | 42.2029 | 55.2568 | .00 | 100.00 |
| eight_cell | Rapid | 58 | 39.5053 | 35.80523 | 4.70146 | 30.0908 | 48.9198 | .00 | 100.00 |
| | Slow | 66 | 24.2424 | 32.91085 | 4.05105 | 16.1519 | 32.3329 | .00 | 100.00 |
| | Total | 124 | 31.3815 | 34.99936 | 3.14304 | 25.1601 | 37.6030 | .00 | 100.00 |
| morula | Rapid | 58 | 9.2139 | 21.68298 | 2.84711 | 3.5126 | 14.9151 | .00 | 100.00 |
| | Slow | 66 | 2.2385 | 7.65756 | .94258 | .3560 | 4.1209 | .00 | 50.00 |
| | Total | 124 | 5.5012 | 16.15781 | 1.45101 | 2.6290 | 8.3733 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| two_cell | Between Groups | 3819.214 | 1 | 3819.214 | 3.348 | .070 |
| | Within Groups | 139166.216 | 122 | 1140.707 | | |
| | Total | 142985.430 | 123 | | | |
| four_cell | Between Groups | 6782.938 | 1 | 6782.938 | 5.203 | .024 |
| | Within Groups | 159045.427 | 122 | 1303.651 | | |
| | Total | 165828.365 | 123 | | | |
| eight_cell | Between Groups | 7191.592 | 1 | 7191.592 | 6.115 | .015 |
| | Within Groups | 143477.875 | 122 | 1176.048 | | |
| | Total | 150669.468 | 123 | | | |
| morula | Between Groups | 1502.072 | 1 | 1502.072 | 5.987 | .016 |
| | Within Groups | 30610.124 | 122 | 250.903 | | |
| | Total | 32112.196 | 123 | | | |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| Rapid | 2-cell | 58 | 71.0037 | 31.78886 | 4.17408 | 62.6452 | 79.3622 | .00 | 100.00 |
| | 4-cell | 58 | 56.6195 | 35.71578 | 4.68971 | 47.2285 | 66.0104 | .00 | 100.00 |
| | 8-cell | 58 | 39.5053 | 35.80523 | 4.70146 | 30.0908 | 48.9198 | .00 | 100.00 |
| | morula | 58 | 9.2139 | 21.68298 | 2.84711 | 3.5126 | 14.9151 | .00 | 100.00 |
| | Total | 232 | 44.0856 | 39.09520 | 2.56673 | 39.0284 | 49.1428 | .00 | 100.00 |
| Slow | 2-cell | 66 | 59.8810 | 35.42400 | 4.36039 | 51.1726 | 68.5893 | .00 | 100.00 |
| | 4-cell | 66 | 41.7965 | 36.44496 | 4.48606 | 32.8372 | 50.7558 | .00 | 100.00 |
| | 8-cell | 66 | 24.2424 | 32.91085 | 4.05105 | 16.1519 | 32.3329 | .00 | 100.00 |
| | morula | 66 | 2.2385 | 7.65756 | .94258 | .3560 | 4.1209 | .00 | 50.00 |
| | Total | 264 | 32.0396 | 37.10973 | 2.28395 | 27.5424 | 36.5367 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Rapid | Between Groups | 122884.450 | 3 | 40961.483 | 40.573 | .000 |
| | Within Groups | 230183.923 | 228 | 1009.579 | | |
| | Total | 353068.373 | 231 | | | |
| Slow | Between Groups | 120070.028 | 3 | 40023.343 | 42.980 | .000 |
| | Within Groups | 242115.719 | 260 | 931.214 | | |
| | Total | 362185.748 | 263 | | | |

**Rapid**

Duncan[a]

| | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| morula | 58 | 9.2139 | | | |
| 8-cell | 58 | | 39.5053 | | |
| 4-cell | 58 | | | 56.6195 | |
| 2-cell | 58 | | | | 71.0037 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 |

**Slow**

Duncan[a]

| | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| morula | 66 | 2.2385 | | | |
| 8-cell | 66 | | 24.2424 | | |
| 4-cell | 66 | | | 41.7965 | |
| 2-cell | 66 | | | | 59.8810 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 |

# EFFECT OF OOCYTE FACTORS ON ICSI PERFORMANCE  IN GOAT (EXPERIMENT 4)

## 4.4.1 The Effect of LOPU Cycle on Yield of Oocytes

**Appendix Table 3.18**:  Number of oocyte qualities  in different cycles of LOPU

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| A | C1 | 32 | 1.5000 | 1.90076 | .33601 | .8147 | 2.1853 | .00 | 9.00 |
| | C2 | 32 | 1.2500 | 1.16398 | .20576 | .8303 | 1.6697 | .00 | 4.00 |
| | C3 | 32 | .5625 | .75935 | .13424 | .2887 | .8363 | .00 | 3.00 |
| | Total | 96 | 1.1042 | 1.40285 | .14318 | .8199 | 1.3884 | .00 | 9.00 |
| B | C1 | 32 | 1.2500 | 1.52400 | .26941 | .7005 | 1.7995 | .00 | 6.00 |
| | C2 | 32 | .9375 | 1.24272 | .21968 | .4895 | 1.3855 | .00 | 5.00 |
| | C3 | 32 | .6563 | 1.12478 | .19883 | .2507 | 1.0618 | .00 | 5.00 |
| | Total | 96 | .9479 | 1.31685 | .13440 | .6811 | 1.2147 | .00 | 6.00 |
| C | C1 | 32 | 2.4063 | 2.06131 | .36439 | 1.6631 | 3.1494 | .00 | 8.00 |
| | C2 | 32 | 1.2500 | 1.31982 | .23331 | .7742 | 1.7258 | .00 | 5.00 |
| | C3 | 32 | 1.0625 | 1.75862 | .31088 | .4285 | 1.6965 | .00 | 8.00 |
| | Total | 96 | 1.5729 | 1.82235 | .18599 | 1.2037 | 1.9422 | .00 | 8.00 |
| D | C1 | 32 | .2813 | .68318 | .12077 | .0349 | .5276 | .00 | 3.00 |
| | C2 | 32 | .2813 | .45680 | .08075 | .1166 | .4459 | .00 | 1.00 |
| | C3 | 32 | .4063 | .87471 | .15463 | .0909 | .7216 | .00 | 4.00 |
| | Total | 96 | .3229 | .68817 | .07024 | .1835 | .4624 | .00 | 4.00 |
| E | C1 | 32 | .0313 | .17678 | .03125 | -.0325 | .0950 | .00 | 1.00 |
| | C2 | 32 | .2188 | .42001 | .07425 | .0673 | .3702 | .00 | 1.00 |
| | C3 | 32 | .3438 | .74528 | .13175 | .0750 | .6125 | .00 | 3.00 |
| | Total | 96 | .1979 | .51544 | .05261 | .0935 | .3024 | .00 | 3.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| A | Between Groups | 15.083 | 2 | 7.542 | 4.081 | .020 |
| | Within Groups | 171.875 | 93 | 1.848 | | |
| | Total | 186.958 | 95 | | | |
| B | Between Groups | 5.646 | 2 | 2.823 | 1.650 | .198 |
| | Within Groups | 159.094 | 93 | 1.711 | | |
| | Total | 164.740 | 95 | | | |
| C | Between Groups | 33.896 | 2 | 16.948 | 5.597 | .005 |
| | Within Groups | 281.594 | 93 | 3.028 | | |
| | Total | 315.490 | 95 | | | |
| D | Between Groups | .333 | 2 | .167 | .347 | .708 |
| | Within Groups | 44.656 | 93 | .480 | | |
| | Total | 44.990 | 95 | | | |
| E | Between Groups | 1.583 | 2 | .792 | 3.112 | .049 |
| | Within Groups | 23.656 | 93 | .254 | | |
| | Total | 25.240 | 95 | | | |

**A**

Duncan[a]

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| OR_cycle | N | 1 | 2 |
| C3 | 32 | .5625 | |
| C2 | 32 | | 1.2500 |
| C1 | 32 | | 1.5000 |
| Sig. | | 1.000 | .464 |

**B**

Duncan[a]

| OR_cycle | N | Subset for alpha = 0.05 |
|---|---|---|
| | | 1 |
| C3 | 32 | .6563 |
| C2 | 32 | .9375 |
| C1 | 32 | 1.2500 |
| Sig. | | .089 |

**C**

Duncan[a]

| OR_cycle | N | Subset for alpha = 0.05 | |
|---|---|---|---|
| | | 1 | 2 |
| C3 | 32 | 1.0625 | |
| C2 | 32 | 1.2500 | |
| C1 | 32 | | 2.4063 |
| Sig. | | .667 | 1.000 |

**D**

Duncan[a]

| OR_cycle | N | Subset for alpha = 0.05 |
|---|---|---|
| | | 1 |
| C1 | 32 | .2813 |
| C2 | 32 | .2813 |
| C3 | 32 | .4063 |
| Sig. | | .501 |

**E**

Duncan[a]

| OR_cycle | N | Subset for alpha = 0.05 | |
|---|---|---|---|
| | | 1 | 2 |
| C1 | 32 | .0313 | |
| C2 | 32 | .2188 | .2188 |
| C3 | 32 | | .3438 |
| Sig. | | .140 | .324 |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| OR1 | A | 32 | 1.5000 | 1.90076 | .33601 | .8147 | 2.1853 | .00 | 9.00 |
| | B | 32 | 1.2500 | 1.52400 | .26941 | .7005 | 1.7995 | .00 | 6.00 |
| | C | 32 | 2.4063 | 2.06131 | .36439 | 1.6631 | 3.1494 | .00 | 8.00 |
| | D | 32 | .2813 | .68318 | .12077 | .0349 | .5276 | .00 | 3.00 |
| | E | 32 | .0313 | .17678 | .03125 | -.0325 | .0950 | .00 | 1.00 |
| | Total | 160 | 1.0938 | 1.68156 | .13294 | .8312 | 1.3563 | .00 | 9.00 |
| OR2 | A | 32 | 1.2500 | 1.16398 | .20576 | .8303 | 1.6697 | .00 | 4.00 |
| | B | 32 | .9375 | 1.24272 | .21968 | .4895 | 1.3855 | .00 | 5.00 |
| | C | 32 | 1.2500 | 1.31982 | .23331 | .7742 | 1.7258 | .00 | 5.00 |
| | D | 32 | .2813 | .45680 | .08075 | .1166 | .4459 | .00 | 1.00 |
| | E | 32 | .2188 | .42001 | .07425 | .0673 | .3702 | .00 | 1.00 |
| | Total | 160 | .7875 | 1.08962 | .08614 | .6174 | .9576 | .00 | 5.00 |
| OR3 | A | 32 | .5625 | .75935 | .13424 | .2887 | .8363 | .00 | 3.00 |
| | B | 32 | .6563 | 1.12478 | .19883 | .2507 | 1.0618 | .00 | 5.00 |
| | C | 32 | 1.0625 | 1.75862 | .31088 | .4285 | 1.6965 | .00 | 8.00 |
| | D | 32 | .4063 | .87471 | .15463 | .0909 | .7216 | .00 | 4.00 |
| | E | 32 | .3438 | .74528 | .13175 | .0750 | .6125 | .00 | 3.00 |
| | Total | 160 | .6063 | 1.13324 | .08959 | .4293 | .7832 | .00 | 8.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| OR1 | Between Groups | 118.438 | 4 | 29.609 | 13.859 | .000 |
| | Within Groups | 331.156 | 155 | 2.136 | | |
| | Total | 449.594 | 159 | | | |
| OR2 | Between Groups | 32.963 | 4 | 8.241 | 8.198 | .000 |
| | Within Groups | 155.813 | 155 | 1.005 | | |
| | Total | 188.775 | 159 | | | |
| OR3 | Between Groups | 10.288 | 4 | 2.572 | 2.056 | .089 |
| | Within Groups | 193.906 | 155 | 1.251 | | |
| | Total | 204.194 | 159 | | | |

**OR1**

Duncan[a]

| | | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| Grades | N | 1 | 2 | 3 |
| E | 32 | .0313 | | |
| D | 32 | .2813 | | |
| B | 32 | | 1.2500 | |
| A | 32 | | 1.5000 | |
| C | 32 | | | 2.4063 |
| Sig. | | .495 | .495 | 1.000 |

**OR2**

Duncan[a]

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| Grades | N | 1 | 2 |
| E | 32 | .2188 | |
| D | 32 | .2813 | |
| B | 32 | | .9375 |
| A | 32 | | 1.2500 |
| C | 32 | | 1.2500 |
| Sig. | | .803 | .243 |

**OR3**

| Grades | N | Subset for alpha = 0.05 | |
| | | 1 | 2 |
|---|---|---|---|
| E | 32 | .3438 | |
| D | 32 | .4063 | |
| A | 32 | .5625 | .5625 |
| B | 32 | .6563 | .6563 |
| C | 32 | | 1.0625 |
| Sig. | | .315 | .093 |

**Appendix Table 3.19**: Number of different oocyte qualities regardless cycles of LOPU procedure

**Descriptives**

OR123

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
| | | | | | Lower Bound | Upper Bound | | |
|---|---|---|---|---|---|---|---|---|
| A | 96 | 1.1042 | 1.40285 | .14318 | .8199 | 1.3884 | .00 | 9.00 |
| B | 96 | .9479 | 1.31685 | .13440 | .6811 | 1.2147 | .00 | 6.00 |
| C | 96 | 1.5729 | 1.82235 | .18599 | 1.2037 | 1.9422 | .00 | 8.00 |
| D | 96 | .3229 | .68817 | .07024 | .1835 | .4624 | .00 | 4.00 |
| E | 96 | .1979 | .51544 | .05261 | .0935 | .3024 | .00 | 3.00 |
| Total | 480 | .8292 | 1.34148 | .06123 | .7089 | .9495 | .00 | 9.00 |

**ANOVA**

OR123

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 124.575 | 4 | 31.144 | 20.061 | .000 |
| Within Groups | 737.417 | 475 | 1.552 | | |
| Total | 861.992 | 479 | | | |

**OR 123**

Duncan[a]

| Grades | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| E | 96 | .1979 | | |
| D | 96 | .3229 | | |
| B | 96 | | .9479 | |
| A | 96 | | 1.1042 | |
| C | 96 | | | 1.5729 |
| Sig. | | .487 | .385 | 1.000 |

**Appendix Table 3.20**: Percentage of different  oocyte qualities from LOPU procedure

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| OR1 | A | 32 | 24.7764 | 26.75673 | 4.72997 | 15.1295 | 34.4232 | .00 | 100.00 |
| | B | 32 | 21.3915 | 24.35372 | 4.30517 | 12.6110 | 30.1719 | .00 | 100.00 |
| | C | 32 | 40.0390 | 29.50933 | 5.21656 | 29.3997 | 50.6782 | .00 | 100.00 |
| | D | 32 | 4.1578 | 10.77477 | 1.90473 | .2731 | 8.0425 | .00 | 42.86 |
| | E | 32 | .2604 | 1.47314 | .26042 | -.2707 | .7915 | .00 | 8.33 |
| | Total | 160 | 18.1250 | 25.67581 | 2.02985 | 14.1161 | 22.1339 | .00 | 100.00 |
| OR2 | A | 32 | 29.3552 | 29.78853 | 5.26592 | 18.6152 | 40.0951 | .00 | 100.00 |
| | B | 32 | 21.9469 | 28.36754 | 5.01472 | 11.7193 | 32.1745 | .00 | 100.00 |
| | C | 32 | 26.4980 | 24.70578 | 4.36741 | 17.5906 | 35.4054 | .00 | 100.00 |
| | D | 32 | 7.6860 | 13.57287 | 2.39937 | 2.7925 | 12.5796 | .00 | 50.00 |
| | E | 32 | 8.2639 | 20.63448 | 3.64769 | .8244 | 15.7034 | .00 | 100.00 |
| | Total | 160 | 18.7500 | 25.52210 | 2.01770 | 14.7651 | 22.7349 | .00 | 100.00 |
| OR3 | A | 32 | 23.3774 | 36.07166 | 6.37663 | 10.3722 | 36.3826 | .00 | 100.00 |
| | B | 32 | 18.9503 | 36.19381 | 6.39822 | 5.9011 | 31.9996 | .00 | 166.67 |
| | C | 32 | 24.0064 | 31.46690 | 5.56261 | 12.6614 | 35.3514 | .00 | 100.00 |
| | D | 32 | 13.3681 | 30.35891 | 5.36675 | 2.4225 | 24.3136 | .00 | 100.00 |
| | E | 32 | 10.3472 | 24.69356 | 4.36525 | 1.4442 | 19.2502 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| OR1 | Between Groups | 33579.457 | 4 | 8394.864 | 18.265 | .000 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Within Groups | 71240.869 | 155 | 459.619 | | |
| | Total | 104820.326 | 159 | | | |
| OR2 | Between Groups | 13282.938 | 4 | 3320.735 | 5.701 | .000 |
| | Within Groups | 90286.084 | 155 | 582.491 | | |
| | Total | 103569.022 | 159 | | | |
| OR3 | Between Groups | 4669.311 | 4 | 1167.328 | 1.137 | .341 |
| | Within Groups | 159115.493 | 155 | 1026.552 | | |
| | Total | 163784.804 | 159 | | | |

**OR1**

Duncan[a]

| | | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| Grades | N | 1 | 2 | 3 |
| E | 32 | .2604 | | |
| D | 32 | 4.1578 | | |
| B | 32 | | 21.3915 | |
| A | 32 | | 24.7764 | |
| C | 32 | | | 40.0390 |
| Sig. | | .468 | .529 | 1.000 |

**OR2**

Duncan[a]

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| Grades | N | 1 | 2 |
| D | 32 | 7.6860 | |
| E | 32 | 8.2639 | |
| B | 32 | | 21.9469 |
| C | 32 | | 26.4980 |
| A | 32 | | 29.3552 |
| Sig. | | .924 | .251 |

**OR3**

Duncan[a]

| | | Subset for alpha = 0.05 |
|---|---|---|
| Grades | N | |

|  |  | 1 |
|---|---|---|
| E | 32 | 10.3472 |
| D | 32 | 13.3681 |
| B | 32 | 18.9503 |
| A | 32 | 23.3774 |
| C | 32 | 24.0064 |
| Sig. |  | .133 |

**Appendix Table 3.21**: Percentage of different oocyte qualities regardless cycles of LOPU procedure

**Descriptives**

|  |  | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
|  |  |  |  |  |  | Lower Bound | Upper Bound |  |  |
| A | OR1 | 32 | 24.7764 | 26.75673 | 4.72997 | 15.1295 | 34.4232 | .00 | 100.00 |
|  | OR2 | 32 | 29.3552 | 29.78853 | 5.26592 | 18.6152 | 40.0951 | .00 | 100.00 |
|  | OR3 | 32 | 23.3774 | 36.07166 | 6.37663 | 10.3722 | 36.3826 | .00 | 100.00 |
|  | Total | 96 | 25.8363 | 30.89259 | 3.15296 | 19.5769 | 32.0957 | .00 | 100.00 |
| B | OR1 | 32 | 21.3915 | 24.35372 | 4.30517 | 12.6110 | 30.1719 | .00 | 100.00 |
|  | OR2 | 32 | 21.9469 | 28.36754 | 5.01472 | 11.7193 | 32.1745 | .00 | 100.00 |
|  | OR3 | 32 | 18.9503 | 36.19381 | 6.39822 | 5.9011 | 31.9996 | .00 | 166.67 |
|  | Total | 96 | 20.7629 | 29.75423 | 3.03678 | 14.7341 | 26.7917 | .00 | 166.67 |
| C | OR1 | 32 | 40.0390 | 29.50933 | 5.21656 | 29.3997 | 50.6782 | .00 | 100.00 |
|  | OR2 | 32 | 26.4980 | 24.70578 | 4.36741 | 17.5906 | 35.4054 | .00 | 100.00 |
|  | OR3 | 32 | 24.0064 | 31.46690 | 5.56261 | 12.6614 | 35.3514 | .00 | 100.00 |
|  | Total | 96 | 30.1811 | 29.26744 | 2.98710 | 24.2510 | 36.1113 | .00 | 100.00 |
| D | OR1 | 32 | 4.1578 | 10.77477 | 1.90473 | .2731 | 8.0425 | .00 | 42.86 |
|  | OR2 | 32 | 7.6860 | 13.57287 | 2.39937 | 2.7925 | 12.5796 | .00 | 50.00 |
|  | OR3 | 32 | 13.3681 | 30.35891 | 5.36675 | 2.4225 | 24.3136 | .00 | 100.00 |
|  | Total | 96 | 8.4040 | 20.32977 | 2.07490 | 4.2848 | 12.5231 | .00 | 100.00 |
| E | OR1 | 32 | .2604 | 1.47314 | .26042 | -.2707 | .7915 | .00 | 8.33 |
|  | OR2 | 32 | 7.6860 | 13.57287 | 2.39937 | 2.7925 | 12.5796 | .00 | 50.00 |

| | N | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| OR3 | 32 | 10.3472 | 24.69356 | 4.36525 | 1.4442 | 19.2502 | .00 | 100.00 |
| Total | 96 | 6.0979 | 16.67967 | 1.70236 | 2.7183 | 9.4775 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| A | Between Groups | 625.664 | 2 | 312.832 | .323 | .725 |
| | Within Groups | 90037.767 | 93 | 968.148 | | |
| | Total | 90663.431 | 95 | | | |
| B | Between Groups | 162.638 | 2 | 81.319 | .090 | .914 |
| | Within Groups | 83942.212 | 93 | 902.604 | | |
| | Total | 84104.850 | 95 | | | |
| C | Between Groups | 4763.814 | 2 | 2381.907 | 2.891 | .061 |
| | Within Groups | 76611.591 | 93 | 823.781 | | |
| | Total | 81375.405 | 95 | | | |
| D | Between Groups | 1382.003 | 2 | 691.002 | 1.696 | .189 |
| | Within Groups | 37881.441 | 93 | 407.327 | | |
| | Total | 39263.445 | 95 | | | |
| E | Between Groups | 1748.962 | 2 | 874.481 | 3.295 | .041 |
| | Within Groups | 24681.117 | 93 | 265.388 | | |
| | Total | 26430.079 | 95 | | | |

**A**

Duncan[a]

| | | Subset for alpha = 0.05 |
|---|---|---|
| cycle | N | 1 |
| OR3 | 32 | 23.3774 |
| OR1 | 32 | 24.7764 |
| OR2 | 32 | 29.3552 |

| Sig. | | .474 |
|---|---|---|

**B**

Duncan[a]

| cycle | N | Subset for alpha = 0.05 1 |
|---|---|---|
| OR3 | 32 | 18.9503 |
| OR1 | 32 | 21.3915 |
| OR2 | 32 | 21.9469 |
| Sig. | | .710 |

**C**

Duncan[a]

| cycle | N | Subset for alpha = 0.05 1 | 2 |
|---|---|---|---|
| OR3 | 32 | 24.0064 | |
| OR2 | 32 | 26.4980 | 26.4980 |
| OR1 | 32 | | 40.0390 |
| Sig. | | .729 | .062 |

**D**

Duncan[a]

| cycle | N | Subset for alpha = 0.05 |
|---|---|---|
| | | 1 |
| OR1 | 32 | 4.1578 |
| OR2 | 32 | 7.6860 |
| OR3 | 32 | 13.3681 |
| Sig. | | .087 |

**E**

Duncan[a]

| cycle | N | Subset for alpha = 0.05 | |
|---|---|---|---|
| | | 1 | 2 |
| OR1 | 32 | .2604 | |
| OR2 | 32 | 7.6860 | 7.6860 |
| OR3 | 32 | | 10.3472 |
| Sig. | | .071 | .515 |

## 4.4.2 The Effects of IVM Duration on Maturation Rate and ICSI- Derived Embryonic Development

**Appendix Table 3.22**: Effect of 18 to 21 hours IVM duration from different grades of oocytes on goat embryo cleavage rate

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | 95% Confidence Interval for Mean Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| A | 2-cell | 29 | 59.1954 | 38.91796 | 7.22688 | 44.3918 | 73.9990 | .00 | 100.00 |
| | 4-cell | 29 | 47.4138 | 38.51815 | 7.15264 | 32.7623 | 62.0653 | .00 | 100.00 |
| | 8-cell | 29 | 33.0460 | 38.67105 | 7.18103 | 18.3363 | 47.7557 | .00 | 100.00 |
| | morula | 29 | 11.9253 | 28.12167 | 5.22206 | 1.2284 | 22.6222 | .00 | 100.00 |
| | Total | 116 | 37.8951 | 39.99916 | 3.71383 | 30.5387 | 45.2515 | .00 | 100.00 |
| B | 2-cell | 29 | 31.3793 | 42.62719 | 7.91567 | 15.1648 | 47.5938 | .00 | 100.00 |
| | 4-cell | 29 | 20.4598 | 33.22272 | 6.16930 | 7.8225 | 33.0970 | .00 | 100.00 |
| | 8-cell | 29 | 10.9195 | 27.55797 | 5.11739 | .4370 | 21.4020 | .00 | 100.00 |
| | morula | 29 | .0000 | .00000 | .00000 | .0000 | .0000 | .00 | .00 |
| | Total | 116 | 15.6897 | 32.12000 | 2.98227 | 9.7824 | 21.5970 | .00 | 100.00 |
| C | 2-cell | 29 | 35.9483 | 39.53457 | 7.34139 | 20.9101 | 50.9864 | .00 | 100.00 |
| | 4-cell | 29 | 24.4540 | 31.69820 | 5.88621 | 12.3967 | 36.5114 | .00 | 100.00 |
| | 8-cell | 29 | 9.2816 | 17.27423 | 3.20774 | 2.7108 | 15.8524 | .00 | 50.00 |
| | morula | 29 | 1.0057 | 3.80175 | .70597 | -.4404 | 2.4519 | .00 | 16.67 |
| | Total | 116 | 17.6724 | 29.74885 | 2.76211 | 12.2012 | 23.1436 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| A | Between Groups | 36025.353 | 3 | 12008.451 | 9.090 | .000 |
| | Within Groups | 147966.954 | 112 | 1321.134 | | |
| | Total | 183992.307 | 115 | | | |
| B | Between Groups | 15597.318 | 3 | 5199.106 | 5.651 | .001 |
| | Within Groups | 103047.510 | 112 | 920.067 | | |
| | Total | 118644.828 | 115 | | | |
| C | Between Groups | 21117.241 | 3 | 7039.080 | 9.774 | .000 |
| | Within Groups | 80657.088 | 112 | 720.153 | | |
| | Total | 101774.330 | 115 | | | |

**A**

Duncan[a]

| development | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| morula | 29 | 11.9253 | | |
| 8-cell | 29 | | 33.0460 | |
| 4-cell | 29 | | 47.4138 | 47.4138 |
| 2-cell | 29 | | | 59.1954 |
| Sig. | | 1.000 | .135 | .220 |

**B**

Duncan[a]

| development | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| morula | 29 | .0000 | | |
| 8-cell | 29 | 10.9195 | 10.9195 | |
| 4-cell | 29 | | 20.4598 | 20.4598 |
| 2-cell | 29 | | | 31.3793 |
| Sig. | | .173 | .234 | .173 |

**C**

Duncan[a]

| development | N | Subset for alpha = 0.05 | |
|---|---|---|---|
| | | 1 | 2 |
| morula | 29 | 1.0057 | |
| 8-cell | 29 | 9.2816 | |
| 4-cell | 29 | | 24.4540 |
| 2-cell | 29 | | 35.9483 |
| Sig. | | .243 | .106 |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| two_cell | A | 29 | 59.1954 | 38.91796 | 7.22688 | 44.3918 | 73.9990 | .00 | 100.00 |
| | B | 29 | 31.3793 | 42.62719 | 7.91567 | 15.1648 | 47.5938 | .00 | 100.00 |
| | C | 29 | 35.9483 | 39.53457 | 7.34139 | 20.9101 | 50.9864 | .00 | 100.00 |
| | Total | 87 | 42.1743 | 41.75733 | 4.47686 | 33.2746 | 51.0740 | .00 | 100.00 |
| four_cell | A | 29 | 47.4138 | 38.51815 | 7.15264 | 32.7623 | 62.0653 | .00 | 100.00 |
| | B | 29 | 20.4598 | 33.22272 | 6.16930 | 7.8225 | 33.0970 | .00 | 100.00 |
| | C | 29 | 24.4540 | 31.69820 | 5.88621 | 12.3967 | 36.5114 | .00 | 100.00 |
| | Total | 87 | 30.7759 | 36.22505 | 3.88373 | 23.0553 | 38.4965 | .00 | 100.00 |
| eight_cell | A | 29 | 33.0460 | 38.67105 | 7.18103 | 18.3363 | 47.7557 | .00 | 100.00 |
| | B | 29 | 10.9195 | 27.55797 | 5.11739 | .4370 | 21.4020 | .00 | 100.00 |
| | C | 29 | 9.2816 | 17.27423 | 3.20774 | 2.7108 | 15.8524 | .00 | 50.00 |
| | Total | 87 | 17.7490 | 30.82393 | 3.30467 | 11.1796 | 24.3185 | .00 | 100.00 |
| morula | A | 29 | 11.9253 | 28.12167 | 5.22206 | 1.2284 | 22.6222 | .00 | 100.00 |
| | B | 29 | .0000 | .00000 | .00000 | .0000 | .0000 | .00 | .00 |
| | C | 29 | 1.0057 | 3.80175 | .70597 | -.4404 | 2.4519 | .00 | 16.67 |
| | Total | 87 | 4.3103 | 17.07882 | 1.83104 | .6704 | 7.9503 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| two_cell | Between Groups | 12905.380 | 2 | 6452.690 | 3.955 | .023 |
| | Within Groups | 137050.670 | 84 | 1631.556 | | |
| | Total | 149956.050 | 86 | | | |
| four_cell | Between Groups | 12273.036 | 2 | 6136.518 | 5.125 | .008 |
| | Within Groups | 100580.843 | 84 | 1197.391 | | |
| | Total | 112853.879 | 86 | | | |
| eight_cell | Between Groups | 10217.736 | 2 | 5108.868 | 6.003 | .004 |
| | Within Groups | 71492.146 | 84 | 851.097 | | |
| | Total | 81709.882 | 86 | | | |
| morula | Between Groups | 2537.117 | 2 | 1268.558 | 4.726 | .011 |
| | Within Groups | 22547.893 | 84 | 268.427 | | |
| | Total | 25085.010 | 86 | | | |

**two_cell**

Duncan[a]

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| Grades | N | 1 | 2 |
| B | 29 | 31.3793 | |
| C | 29 | 35.9483 | |
| A | 29 | | 59.1954 |
| Sig. | | .668 | 1.000 |

**four_cell**

Duncan[a]

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| Grades | N | 1 | 2 |
| B | 29 | 20.4598 | |
| C | 29 | 24.4540 | |
| A | 29 | | 47.4138 |
| Sig. | | .661 | 1.000 |

**eight_cell**

Duncan[a]

| Grades | N | Subset for alpha = 0.05 | |
| | | 1 | 2 |
|---|---|---|---|
| C | 29 | 9.2816 | |
| B | 29 | 10.9195 | |
| A | 29 | | 33.0460 |
| Sig. | | .831 | 1.000 |

**morula**

Duncan[a]

| Grades | N | Subset for alpha = 0.05 | |
| | | 1 | 2 |
|---|---|---|---|
| B | 29 | .0000 | |
| C | 29 | 1.0057 | |
| A | 29 | | 11.9253 |
| Sig. | | .816 | 1.000 |

**Appendix Table 3.23**: Effect of 22 to 25 hours IVM duration from different grades of oocytes on goat embryo cleavage rate

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean Lower Bound | 95% Confidence Interval for Mean Upper Bound | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| two_cell | A | 30 | 57.5238 | 43.95424 | 8.02491 | 41.1110 | 73.9366 | .00 | 100.00 |
| | B | 30 | 41.1111 | 38.27790 | 6.98856 | 26.8179 | 55.4043 | .00 | 100.00 |
| | C | 30 | 46.4048 | 38.47910 | 7.02529 | 32.0364 | 60.7731 | .00 | 100.00 |
| | Total | 90 | 48.3466 | 40.45624 | 4.26446 | 39.8732 | 56.8200 | .00 | 100.00 |
| four_cell | A | 30 | 43.7143 | 43.83507 | 8.00315 | 27.3460 | 60.0826 | .00 | 100.00 |
| | B | 30 | 31.3889 | 37.18310 | 6.78867 | 17.5045 | 45.2733 | .00 | 100.00 |
| | C | 30 | 37.0635 | 35.28518 | 6.44216 | 23.8878 | 50.2392 | .00 | 100.00 |
| | Total | 90 | 37.3889 | 38.83248 | 4.09330 | 29.2556 | 45.5222 | .00 | 100.00 |
| eight_cell | A | 30 | 31.2381 | 39.30024 | 7.17521 | 16.5631 | 45.9130 | .00 | 100.00 |
| | B | 30 | 26.9444 | 35.73820 | 6.52487 | 13.5996 | 40.2893 | .00 | 100.00 |
| | C | 30 | 13.7103 | 17.87208 | 3.26298 | 7.0368 | 20.3839 | .00 | 57.14 |
| | Total | 90 | 23.9643 | 32.86011 | 3.46376 | 17.0819 | 30.8467 | .00 | 100.00 |
| morula | A | 30 | 3.9762 | 9.74640 | 1.77944 | .3368 | 7.6156 | .00 | 40.00 |
| | B | 30 | 1.9444 | 7.48028 | 1.36571 | -.8487 | 4.7376 | .00 | 33.33 |
| | C | 30 | 4.3175 | 11.20187 | 2.04517 | .1346 | 8.5003 | .00 | 50.00 |
| | Total | 90 | 3.4127 | 9.54892 | 1.00654 | 1.4127 | 5.4127 | .00 | 50.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| two_cell | Between Groups | 4210.326 | 2 | 2105.163 | 1.295 | .279 |
| | Within Groups | 141456.608 | 87 | 1625.938 | | |
| | Total | 145666.934 | 89 | | | |
| four_cell | Between Groups | 2283.496 | 2 | 1141.748 | .753 | .474 |
| | Within Groups | 131925.059 | 87 | 1516.380 | | |
| | Total | 134208.554 | 89 | | | |
| eight_cell | Between Groups | 5008.005 | 2 | 2504.003 | 2.391 | .097 |
| | Within Groups | 91093.028 | 87 | 1047.046 | | |
| | Total | 96101.033 | 89 | | | |
| morula | Between Groups | 98.757 | 2 | 49.378 | .536 | .587 |
| | Within Groups | 8016.436 | 87 | 92.143 | | |
| | Total | 8115.193 | 89 | | | |

**two_cell**

Duncan[a]

| | | Subset for alpha = 0.05 |
|---|---|---|
| grades | N | 1 |
| B | 30 | 41.1111 |
| C | 30 | 46.4048 |
| A | 30 | 57.5238 |
| Sig. | | .140 |

**four_cell**

Duncan[a]

| | | Subset for alpha = 0.05 |
|---|---|---|
| grades | N | 1 |
| B | 30 | 31.3889 |
| C | 30 | 37.0635 |
| A | 30 | 43.7143 |
| Sig. | | .252 |

**eight_cell**

282

Duncan[a]

| grades | N | Subset for alpha = 0.05 | |
| --- | --- | --- | --- |
| | | 1 | 2 |
| C | 30 | 13.7103 | |
| B | 30 | 26.9444 | 26.9444 |
| A | 30 | | 31.2381 |
| Sig. | | .117 | .609 |

**morula**

Duncan[a]

| grades | N | Subset for alpha = 0.05 |
| --- | --- | --- |
| | | 1 |
| B | 30 | 1.9444 |
| A | 30 | 3.9762 |
| C | 30 | 4.3175 |
| Sig. | | .372 |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| A | 2-cell | 30 | 57.5238 | 43.95424 | 8.02491 | 41.1110 | 73.9366 | .00 | 100.00 |
| | 4-cell | 30 | 43.7143 | 43.83507 | 8.00315 | 27.3460 | 60.0826 | .00 | 100.00 |
| | 8-cell | 30 | 31.2381 | 39.30024 | 7.17521 | 16.5631 | 45.9130 | .00 | 100.00 |
| | morula | 30 | 3.9762 | 9.74640 | 1.77944 | .3368 | 7.6156 | .00 | 40.00 |
| | Total | 120 | 34.1131 | 41.60630 | 3.79812 | 26.5924 | 41.6337 | .00 | 100.00 |
| B | 2-cell | 30 | 41.1111 | 38.27790 | 6.98856 | 26.8179 | 55.4043 | .00 | 100.00 |
| | 4-cell | 30 | 31.3889 | 37.18310 | 6.78867 | 17.5045 | 45.2733 | .00 | 100.00 |
| | 8-cell | 30 | 26.9444 | 35.73820 | 6.52487 | 13.5996 | 40.2893 | .00 | 100.00 |
| | morula | 30 | 1.9444 | 7.48028 | 1.36571 | -.8487 | 4.7376 | .00 | 33.33 |
| | Total | 120 | 25.3472 | 35.06356 | 3.20085 | 19.0092 | 31.6852 | .00 | 100.00 |
| C | 2-cell | 30 | 46.4048 | 38.47910 | 7.02529 | 32.0364 | 60.7731 | .00 | 100.00 |
| | 4-cell | 30 | 37.0635 | 35.28518 | 6.44216 | 23.8878 | 50.2392 | .00 | 100.00 |
| | 8-cell | 30 | 13.7103 | 17.87208 | 3.26298 | 7.0368 | 20.3839 | .00 | 57.14 |
| | morula | 30 | 4.3175 | 11.20187 | 2.04517 | .1346 | 8.5003 | .00 | 50.00 |
| | Total | 120 | 25.3740 | 32.62950 | 2.97865 | 19.4760 | 31.2720 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| A | Between Groups | 46702.292 | 3 | 15567.431 | 11.336 | .000 |
| | Within Groups | 159296.684 | 116 | 1373.247 | | |
| | Total | 205998.975 | 119 | | | |
| B | Between Groups | 25057.292 | 3 | 8352.431 | 7.991 | .000 |
| | Within Groups | 121247.685 | 116 | 1045.239 | | |
| | Total | 146304.977 | 119 | | | |
| C | Between Groups | 34750.696 | 3 | 11583.565 | 14.614 | .000 |
| | Within Groups | 91946.762 | 116 | 792.644 | | |
| | Total | 126697.457 | 119 | | | |

**A**

Duncan[a]

| developemnt | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| morula | 30 | 3.9762 | | |
| 8-cell | 30 | | 31.2381 | |
| 4-cell | 30 | | 43.7143 | 43.7143 |
| 2-cell | 30 | | | 57.5238 |
| Sig. | | 1.000 | .195 | .152 |

**B**

Duncan[a]

| developemnt | N | Subset for alpha = 0.05 | |
|---|---|---|---|
| | | 1 | 2 |
| morula | 30 | 1.9444 | |
| 8-cell | 30 | | 26.9444 |
| 4-cell | 30 | | 31.3889 |
| 2-cell | 30 | | 41.1111 |
| Sig. | | 1.000 | .112 |

**C**

Duncan[a]

| developemnt | N | Subset for alpha = 0.05 | |
|---|---|---|---|
| | | 1 | 2 |
| morula | 30 | 4.3175 | |
| 8-cell | 30 | 13.7103 | |
| 4-cell | 30 | | 37.0635 |
| 2-cell | 30 | | 46.4048 |
| Sig. | | .199 | .201 |

**Appendix Table 3.24**: Effect of different IVM duration from on goat embryo clevage rate regardless grades of oocytes

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| hours_18 | 2-cell | 87 | 42.1743 | 41.75733 | 4.47686 | 33.2746 | 51.0740 | .00 | 100.00 |
| | 4-cell | 87 | 30.7759 | 36.22505 | 3.88373 | 23.0553 | 38.4965 | .00 | 100.00 |
| | 8-cell | 87 | 17.7490 | 30.82393 | 3.30467 | 11.1796 | 24.3185 | .00 | 100.00 |
| | morula | 87 | 4.3103 | 17.07882 | 1.83104 | .6704 | 7.9503 | .00 | 100.00 |
| | Total | 348 | 23.7524 | 35.58658 | 1.90764 | 20.0004 | 27.5044 | .00 | 100.00 |
| hours_22 | 2-cell | 90 | 48.3466 | 40.45624 | 4.26446 | 39.8732 | 56.8200 | .00 | 100.00 |
| | 4-cell | 90 | 37.3889 | 38.83248 | 4.09330 | 29.2556 | 45.5222 | .00 | 100.00 |
| | 8-cell | 90 | 23.9643 | 32.86011 | 3.46376 | 17.0819 | 30.8467 | .00 | 100.00 |
| | morula | 90 | 3.4127 | 9.54892 | 1.00654 | 1.4127 | 5.4127 | .00 | 50.00 |
| | Total | 360 | 28.2781 | 36.76053 | 1.93745 | 24.4679 | 32.0883 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| hours_18 | Between Groups | 69837.538 | 3 | 23279.179 | 21.666 | .000 |
| | Within Groups | 369604.821 | 344 | 1074.433 | | |
| | Total | 439442.359 | 347 | | | |
| hours_22 | Between Groups | 101038.211 | 3 | 33679.404 | 31.216 | .000 |
| | Within Groups | 384091.715 | 356 | 1078.909 | | |
| | Total | 485129.925 | 359 | | | |

**18-21 hours**

Duncan[a]

| development | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| morula | 87 | 4.3103 | | | |
| 8-cell | 87 | | 17.7490 | | |
| 4-cell | 87 | | | 30.7759 | |
| 2-cell | 87 | | | | 42.1743 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 |

**22-25 hours**

Duncan[a]

| development | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| morula | 90 | 3.4127 | | | |
| 8-cell | 90 | | 23.9643 | | |
| 4-cell | 90 | | | 37.3889 | |
| 2-cell | 90 | | | | 48.3466 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 |

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| two_cell | 18-21 hours | 87 | 42.1743 | 41.75733 | 4.47686 | 33.2746 | 51.0740 | .00 | 100.00 |
| | 22-25 hours | 90 | 48.3466 | 40.45624 | 4.26446 | 39.8732 | 56.8200 | .00 | 100.00 |
| | Total | 177 | 45.3128 | 41.10051 | 3.08930 | 39.2159 | 51.4096 | .00 | 100.00 |
| four_cell | 18-21 hours | 87 | 30.7759 | 36.22505 | 3.88373 | 23.0553 | 38.4965 | .00 | 100.00 |
| | 22-25 hours | 90 | 37.3889 | 38.83248 | 4.09330 | 29.2556 | 45.5222 | .00 | 100.00 |
| | Total | 177 | 34.1384 | 37.61324 | 2.82718 | 28.5589 | 39.7180 | .00 | 100.00 |

| | | N | Mean | Std. Deviation | Std. Error | | | Min | Max |
|---|---|---|---|---|---|---|---|---|---|
| eight_cell | 18-21 hours | 87 | 17.7490 | 30.82393 | 3.30467 | 11.1796 | 24.3185 | .00 | 100.00 |
| | 22-25 hours | 90 | 23.9643 | 32.86011 | 3.46376 | 17.0819 | 30.8467 | .00 | 100.00 |
| | Total | 177 | 20.9093 | 31.93742 | 2.40056 | 16.1717 | 25.6469 | .00 | 100.00 |
| morula | 18-21 hours | 87 | 4.3103 | 17.07882 | 1.83104 | .6704 | 7.9503 | .00 | 100.00 |
| | 22-25 hours | 90 | 3.4127 | 9.54892 | 1.00654 | 1.4127 | 5.4127 | .00 | 50.00 |
| | Total | 177 | 3.8539 | 13.74191 | 1.03291 | 1.8154 | 5.8924 | .00 | 100.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| two_cell | Between Groups | 1685.283 | 1 | 1685.283 | .998 | .319 |
| | Within Groups | 295622.985 | 175 | 1689.274 | | |
| | Total | 297308.268 | 176 | | | |
| four_cell | Between Groups | 1934.591 | 1 | 1934.591 | 1.370 | .243 |
| | Within Groups | 247062.434 | 175 | 1411.785 | | |
| | Total | 248997.024 | 176 | | | |
| eight_cell | Between Groups | 1708.853 | 1 | 1708.853 | 1.682 | .196 |
| | Within Groups | 177810.915 | 175 | 1016.062 | | |
| | Total | 179519.768 | 176 | | | |
| morula | Between Groups | 35.645 | 1 | 35.645 | .188 | .665 |
| | Within Groups | 33200.202 | 175 | 189.715 | | |
| | Total | 33235.847 | 176 | | | |

# APPENDIX 4: PROCEEDING POSTER

4.1: Nor Farizah, A.H., W.E. Wan Khadijah and R.B. Abdulllah. 2011. Effect of sperm movement on ICSI derived embryo development in goat. Proc. 32nd Malaysian Society of Animal Production. Tawau, Malaysia. pp 101-102.

## EFFECTS OF SPERM MOVEMENT ON ICSI-DERIVED EMBRYO DEVELOPMENT IN GOAT

A.H. Nor Farizah, W.E. Wan Khadijah and *R.B. Abdullah

*Animal Biotechnology-Embryo Laboratory (ABEL), Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia*
*Email: ramli@um.edu.my

### INTRODUCTION

Sperm motility is correlated with intracytoplasmic sperm injection (ICSI) outcomes as motility reflects sperm vitality to result in successful fertilisation and subsequent developmental competence (Goto, 1997). Due to lack of information as well as controversial issues regarding the sperm and ICSI performance relationship, therefore, it is timely to study factors such as sperm motility characteristics relating to ICSI performance in order to clarify the underlying factors so that this technique can be applied integrative with other technologies in advance farm management for the goat industry.

Therefore, the objective of this study was to produce embryos via ICSI technique using slow and rapid movement of sperm in goats.

### MATERIALS & METHODS



Fig. 1: LOPU procedure to obtain the oocytes.

*In vitro* maturation (IVM) for 24 hours

Sperm washing and capacitation with heparin for 60 minutes



Fig. 3: ICSI procedure.

ICSI was conducted using two different categories of the sperm movement:
1) Rapid movement = identified by fast movement (>70%) and progressively motility (forward movement)
2) Slow movement = identified by slow movement (<50%) and non-progressively motility (undirected movement)

*In vitro* culture
(observed the development for 7 consecutive days

### RESULTS & DISCUSSION



Fig. 2: Different grades of oocyte (a – c) based on cumulus cells complexes (COCs) layers criteria.

Table 1: Embryo development rate from different sperm movement

| Sperm movement | Grade | No. of matured oocytes (injected oocytes) | Embryo development rate (n) | | | | |
|---|---|---|---|---|---|---|---|
| | | | Cleavage rate (2-cells stage) | 4-cells | 8-cells | 16-cells | Morula |
| Rapid | A | 24 | 70.83* (17) | 70.83 (17) | 45.83 (11) | 25.00 (6) | 16.67 (4) |
| | B | 8 | 50.00 (4) | 50.00 (4) | 50.00 (4) | 25.00 (2) | 0.00 (0) |
| | C | 20 | 74.00 (15) | 75.00 (11) | 30.00 (6) | 20.00 (4) | 15.00 (3) |
| Total | | 52 | 69.23** (36) | 61.53 (32) | 40.38 (21) | 23.07 (12) | 13.40 (7) |
| Slow | A | 10 | 90.00 (9) | 70.00 (7) | 30.00 (3) | 20.00 (2) | 10.00 (1) |
| | B | 13 | 53.85 (7) | 46.15 (6) | 23.08 (3) | 7.69 (1) | 0.00 (0) |
| | C | 22 | 59.09 (13) | 45.45 (10) | 27.27 (6) | 9.09 (2) | 0.00 (0) |
| Total | | 45 | 64.44 (29) | 51.11 (23) | 26.67 (12) | 11.11 (5) | 2.22 (1) |

* mean percentage of embryo development was based on total no. of oocytes of each grade per treatment.
**mean percentage of embryo development was based on total no. of matured oocytes per treatment.

The results have shown that the percentage of cleaved oocytes in rapid sperm movement was higher than that of slow movement group (69.23% vs. 64.44%, respectively). The embryos developed *in vitro* until the morula stage (13.46% vs 2.22%, respectively). There was one embryo successfully developed until blastocysts stage from the rapid movement sperm (1.92%). The sperm that been used are in good morphology (no morphology defect). Therefore, both rapid and slow sperm movement shows the embryos development.

In this study, the immobilisation technique prior to ICSI procedure will brake the plasma membrane and is important for oocyte activation, lead the sperm nuclear decondensation (Montag *et al.,* 1992). Normally sperm are considered when their plasma membranes are severely damaged or lost. However, there has been reported that viable embryos were obtained from dead sperm from a number of species by using frozen-thawed without cryoprotectant (Wakayama *et al.,* 1998) and also together preserved and treated with Triton-X 100 by Zhou *et al.,* (2004). Even those reports are regarding the immotile sperm, via these findings, the slow sperm movement was been focused in this study. There has been shows that the slow sperm also may produce the viable embryos but the survival rate to morula is lower than using the rapid sperm movement.

### CONCLUSION

In conclusion, both rapid and slow movement sperm could be used to produce goat embryos using ICSI procedure with rapid sperm movement gives better ICSI performance than slow movement.

### REFERENCES

1) Montag, M., V. Tok, S.L. Liow, A. Bongso and S.C. Ng, 1992. *In vitro* decondensation of mammalian sperm and subsequent formation of pronuclei-like structures for micromanipulation. Mol. Reprod. Develop., 33: 338-346.
2) Goto, K., 1997. Current status and future of micromanipulation-assisted fertilization in animals and human. J. Reprod. Dev., 43: 107-119.
3) Wakayama T, *et al.,* 1998. Production of normal offspring from mouse oocytes injected with spermatozoa cryopreserved with or without cryoprotection. Journal of Reproduction and Fertility **112** 11–17 .
4) Zhou, J.B., Y.G. Wu, D. Han, L.Q. Liu, X.W. Tan and N. Liu *et al.,* 2004. Effects of sperm and oocyte quality control on intracytoplasmic sperm injection (ICSI) in goats. Shi Yan Sheng Wu Xue Bao, 37: 367-374.

**Figure 2.11**: Proceeding's poster: 32nd MSAP Annual Conference, 6-9 June 2011, Tawau.

4.2: Nor Farizah, A.H., S.C. Kong, P.J. Kwong, W.E. Wan Khadijah and R.B. Abdulllah. 2010. Effect of repeated laparoscopy ovum pick-up (LOPU) on yield of oocytes recovery on goat. Proc. 7th Asian Reproductive Biotechnology Society. Kuala Lumpur , Malaysia. pp 109.

# EFFECT OF REPEATED LAPAROSCOPIC OOCYTE PICK-UP (LOPU) ON YIELD OF OOCYTES RECOVERY

Nor Farizah Abdul Hamid, Sow Chan Kong, Phek Jin Kwong, Wan Embong Wan Khadijah and Ramli bin Abdullah*

Animal Biotechnology-Embryo Laboratory (ABEL), Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
*Email: ramli@um.edu.my

## INTRODUCTION

LOPU is less invasive surgical for oocytes recovery from antral follicles which were seen as 'pimple-like' protrusion on the ovarian surface. In Malaysia, the source of ovary for obtain oocyte from abattoir is limited. Therefore, LOPU is better alternative to obtain oocytes from does (1,2,and 3). However, it is no certain whether the quantity and quality of oocytes obtained from LOPU is comparable to that of abattoir source.

## OBJECTIVES

a) To determine grades of oocytes obtained from different OR cycle from LOPU.
b) To compare different grades of oocytes retrieved from left and right ovaries.

## MATERIALS AND METHODS

Figure 1: LOPU procedure showing surgical instrument.

## RESULTS

Table 1: Percentage of different grades of oocytes retrieved from different OR cycle

| Grade of oocytes | OR cycle number (n) | | | Total |
|---|---|---|---|---|
| | C1 | C2 | C3 | |
| A | 45.3* (48) | 37.7 (40) | 17.0 (18) | 26.5 (106) |
| B | 44.0* (40) | 33.0 (30) | 23.1 (21) | 22.8 (91) |
| C | 49.7* (77) | 25.8 (40) | 24.5 (38) | 38.8 (155) |
| D | 31.0 (9) | 31.0 (9) | 37.9* (11) | 7.3 (29) |
| E | 5.2 (1) | 36.8 (7) | 57.9* (11) | 4.8 (19) |
| Total | 43.8 (175) | 31.5 (126) | 24.8 (99) | 400 |

Table 2: Percentage of different grades of oocytes retrieved from left and right ovaries

| Grade of oocytes | Side of ovary (n) | |
|---|---|---|
| | Left | Right |
| A | 27.5 (57) | 25.4 (49) |
| B | 24.6 (51) | 20.7 (40) |
| C | 37.1 (77) | 40.4 (78) |
| D | 4.3 (9) | 10.4 (20) |
| E | 6.3 (13) | 3.1 (6) |
| TOTAL | 51.8 (207) | 48.3 (193) |

Figure 2: Different grades of oocyte (a – e) based on COCs layers criteria.

## DISCUSSION

The does were synchronized using CIDR insertion for 14 days after which LOPU was carried out 66 hr of post-PMSG and 60 hr of post-Ovidrel. The results showed that higher percentage of Grades C oocytes were obtained compared to other grades. This is probably due to the oocytes already partially undergone maturation process thereby a few layers of cumulus cells were already loosen. It was noted that Grades A, B and C oocytes were highly obtained from first OR, while high percentages of Grades D and E oocytes were obtained from third OR cycle.

## CONCLUSIONS

LOPU procedure for goat oocyte retrieval could be at least up to three times. However, the developmental competence of oocyte obtained is yet to be determined before it can be recommended for reproduction of in vitro embryos using different reproductive techniques.

## REFERENCES

1) Baldassarre H and Karatzas CN. Advanced assisted reproduction technologies (ART) in goats. 2004. Animal Reproduction Science. 82/83:255-266.
2) Pierson J, Wang B, Neveu N, Sneek L, Cote F, Karatzas CN et al. 2004. Effects of repitation, interval between treatments and seoson on the results from laparoscopic ovum pick-up in goats. Reproductive Fertility Development. 16: 795-799.
3) Mohd Noor Hisham, M. N. 2006. Effect of oestrus synchronisation and superovulation on progestrone and oestradiol level in relation to oocyte recovery in goats. Institute of Biological Science. MSc. Dissertation. The University of Malaya, Kuala Lumpur. pp.1-292.

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**Figure 2.12**: Proceeding's poster: 7th ARBS Annual Conference, 8-12 Nov 2010, Kuala Lumpur.