PRODUCTION OF BOVINE EMBRYOS *IN VITRO* THROUGH *IN VITRO* FERTILISATION (IVF) AND INTRACYTOPLASMIC SPERM INJECTION (ICSI) TECHNIQUES

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ABSTRACT

The objective of this study was to develop an optimal *in vitro* produced embryos (IVP) protocol using two methods of insemination, namely *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) protocols by using different qualities of oocytes in Malaysian cattle. The ovaries were collected from slaughtered cattle from the local abattoir. The oocyte morphology was observed and grouped into three groups: Groups A, B and C, based on cumulus cell compactness. Then the oocytes were subdivided based on the cytoplasm quality, namely homogenous and heterogenous (Experiment 1). The oocytes were matured in humidified 5% CO_2 incubator at 38.5°C at different maturation durations of 20, 24 and 28 hours (Experiment 2). The matured oocytes were fertilised with frozen-thawed sperm before being cultured. The cleavage rate and embryo development were evaluated. The serum was added at every embryo development stage to develop up to blastocyst stage (Experiment 3). In ICSI study, the different oocytes and sperm groups were used (Experiments 4 and 5) to produce ICSI embryos. Finally, comparison was made between IVF- and ICSI-derived embryos (Experiment 6).

The oocyte quality (the presence of cumulus cells and different cytoplasmic group), IVM duration and culture medium supplement were considered among the important factors affecting the maturation, fertilisation and culture competence of bovine embryos during IVMFC procedures to produce IVF-derived embryos. In Experiment 1, the percentage of matured-fertilised oocytes differed between the three groups of oocytes. Homogenous cytoplasm, Grade A oocytes gave the highest 2-cell cleavage rate (78.30%) followed by Grade B (65.92%) and Grade C (24.02%)

compared to heterogenous cytoplasm with 50.90%, 30.48% and 11.66%, respectively. It was consistently shown that homogenous cytoplasm gave higher cleavage rates for all the grades of oocyte. It was also observed that blastocysts were obtained for Grade A – Homogenous (28.55%), Grade A – Heterogenous (2.66%) and Grade B – Homogenous (14.40%) oocytes. In Experiment 2, 24 hours IVM duration gave better results ($P<0.05$) compared to 20- and 28-hours for homogenous cytoplasm. But, there were no significant differences when compared to Group B – Homogenous at same IVM duration. For cleavage rate for 20 hours maturation duration, Grade A - Homogenous gave the highest 2-cell cleavage rate (58.57%), followed by Grade B (38.32%) and Grade C (15.58%). For heterogenous cytoplasm, development values were 45.65%, 39.64% and 21.66%, respectively. It was also observed that higher percent blastocyst was obtained for 24 hours IVM duration from Grade A – Homogenous (28.55%) than Grade B - Homogenous (14.40%) compared to 20 and 28 hours IVM duration. In Experiment 3, the blastocyst development after serum addition at morula stage was 63.06%, including fully expanded and hatch blastocyst formation (47.58 % and 26.34%, respectively). It showed that serum could be beneficial to be added at morula stage, conversely, addition of serum at earlier stages of development might be detrimental to embryonic development as no blastocyst was developed after serum addition at 2-cell and 4-cell stages.

ICSI-derived embryos production in Experiment 4 showed that Grade A COC gave the highest 2-cell cleavage rate (44.53%) followed by Grade B (38.94%) and Grade C (7.85%). It was also observed that morula stage was only obtained in Grade A (16.65%). The cleavage rates obtained from different sperm quality injected into oocytes during ICSI (Experiment 5) showed that the cleavage rates (at 2-cell stage) of

intact-immobilised was the highest (63.42%) compared to other sperm quality. Comparison study of IVF- and ICSI-derived embryos (Experiment 6) showed that IVF embryos gave higher cleavage rate in all cases of embryonic development than the ICSI embryo with values of 66.77% versus 41.74% for 2-cell and 8.94% versus 0.00% for blastocyst stage, respectively.

In conclusions, the results of the present study showed that the quality of oocytes, sperm, IVM duration and culture supplement influenced the maturation, fertilisation and culture of IVF- and ICSI-derived embryos. The morphology of cumulus cells and oocytes cytoplasm influenced the maturation, fertilisation and developmental competence of both IVP embryos. Group A COC gave better results followed by Groups B and C with homogenous cytoplasm was superior then heterogenous cytoplasm. 24-hour IVM duration gave the best maturational rate compared with other IVM duration. The use of immobilised intact sperm produced better results than other groups of sperm during ICSI procedure. Serum addition in culture medium did help in blastocyst formation if added at later stage of embryo development but could be detrimental to early stage embryos.

ABSTRAK

Objektif kajian ini adalah untuk membangunkan protokol penghasilan embrio yang optimum secara *in vitro* (IVP) dengan menggunakan dua kaedah inseminasi, iaitu protokol persenyawaan *in vitro* (IVF) dan suntikan sperma intrasitoplasma (ICSI) yang menggunakan oosit berbeza kualiti pada lembu Malaysia. Ovari dikumpulkan daripada lembu yang disembelih dari rumah sembelihan tempatan. Morfologi oosit telah diperhatikan dan dikelaskan kepada tiga kumpulan: Kumpulan A, B dan C, berdasarkan kepadatan sel kumulus. Kemudian oosit dibahagikan semula berdasarkan kualiti sitoplasma, iaitu homogenus dan heterogenus (Eksperimen 1). Oosit dimatangkan dalam inkubator lembap 5% CO_2 pada suhu 38.5°C pada jangka masa kematangan yang berbeza iaitu pada 20, 24 dan 28 jam (Eksperimen 2). Oosit matang disenyawakan dengan menggunakan sperma sejukbeku-cair sebelum pengkulturaan dilakukan. Kadar pembelahan dan perkembangan embrio dinilai. Serum ditambah pada setiap peringkat perkembangan embrio yang dihasilkan sehingga ke peringkat blastosis (Eksperimen 3). Dalam kajian ICSI, oosit dan sperma dari kumpulan berbeza telah digunakan (Eksperimen 4 dan 5) untuk menghasilkan embrio ICSI. Akhir sekali, perbandingan telah dibuat antara embrio terbitan IVF dan ICSI (Eksperimen 6).

Kualiti oosit (kehadiran sel kumulus dan sitoplasma berbeza kumpulan), tempoh IVM dan penambahan serum dalam medium pengkulturan dianggap sebagai antara faktor penting yang mempengaruhi kecekapan pematangan, persenyawaan dan pengkulturan embrio lembu dalam prosedur IVMFC bagi menghasilkan embrio terbitan-IVF. Dalam Eksperimen 1, peratusan persenyawaan oosit matang adalah berbeza antara tiga kumpulan oosit. Sitoplasma homogenus, dari oosit Gred A

memberi kadar pembelahan peringkat 2-sel yang tertinggi (78.30%) diikuti oleh Gred B (65.92%) dan Gred C (24.02%) berbanding sitoplasma heterogenus dengan 50.90%, 30.48% dan 11.66%, masing-masing. Secara konsisten, ia menunjukkan bahawa sitoplasma homogenus memberikan kadar pembelahan sel yang lebih tinggi bagi semua gred COC. Diperhatikan juga bahawa blastosis diperolehi bagi COC Gred A - Homogenus (28.55%), Gred A - Heterogenus (2.66%) dan Gred B - Homogenus (14.40%). Dalam Eksperimen 2, tempoh IVM 24 jam memberi keputusan yang lebih baik ($P<0.05$) berbanding dengan 20- dan 28-jam untuk sitoplasma homogenus. Tetapi, tidak berbeza dengan ketara berbanding dengan Kumpulan B - Homogenus bagi tempoh IVM yang sama. Kadar pembelahan sel untuk tempoh pematangan 20 jam pula, Gred A - Homogenus memberikan kadar pembelahan 2-sel tertinggi (58.57%), diikuti dengan Gred B (38.32%) dan Gred C (15.58%). Untuk sitoplasma heterogenus, kadar perkembangan adalah 45.65%, 39.64% dan 21.66%, masing-masing. Diperhatikan juga bahawa peratus blastosis yang lebih tinggi diperolehi bagi tempoh 24 jam IVM dari Gred A - Homogenus (28.55%) dari Gred B - Homogenus (14.40%) berbanding bagi tempoh IVM 20 dan 28 jam. Dalam Eksperimen 3, perkembangan blastosis selepas penambahan serum pada peringkat morula adalah 63.06%, termasuk pembentukan blastosis berkembang penuh dan blastosis menetas (47.58% dan 26.34%, masing-masing). Ia menunjukkan bahawa serum boleh memberi manfaat jika ditambah pada peringkat morula, sebaliknya, penambahan serum pada peringkat awal pembangunan mungkin boleh menjejaskan perkembangan embrio seterusnya di mana tiada embrio peringkat blastosis dihasilkan selepas penambahan serum pada peringkat 2-sel dan 4-sel.

Penghasilan embrio terbitan-ICSI dalam Eksperimen 4 menunjukkan bahawa COC Gred A menghasilkan kadar pembelahan 2-sel tertinggi (44.53%) diikuti oleh Gred B (38.94%) dan Gred C (7.85%). Diperhatikan juga bahawa penghasilan embrio peringkat morula hanya diperolehi dalam Gred A (16.65%). Kadar pembelahan yang diperolehi daripada sperma berbeza kualiti yang disuntik ke dalam oosit semasa ICSI (Eksperimen 5) menunjukkan bahawa kadar pembelahan (pada peringkat 2-sel) bagi sperma hidup-imobilisasi adalah yang tertinggi (63.42%) berbanding dengan kualiti sperma yang lain. Kajian perbandingan embrio terbitan-IVF dan ICSI (Eksperimen 6) menunjukkan bahawa embrio IVF memberikan kadar pembelahan yang lebih tinggi dalam semua kes berbanding embrio ICSI dengan nilai 66.77% berbanding 41.74% untuk peringkat 2 sel dan 8.94% berbanding 0.00% bagi peringkat blastosis, masing-masing.

Kesimpulannya, keputusan kajian ini menunjukkan bahawa kualiti oosit, sperma, tempoh IVM dan penambahan serum dalam medium pengkulturan mempengaruhi pematangan, persenyawaan dan pengkulturan embrio hasil terbitan-IVF dan ICSI. Morfologi sel kumulus dan sitoplasma oosit mempengaruhi kecekapan pematangan, persenyawaan dan perkembangan kedua-dua embrio IVP. COC dari Kumpulan A memberikan hasil yang lebih baik diikuti oleh Kumpulan B dan C dengan sitoplasma homogenus adalah lebih baik berbanding sitoplasma heterogenus. Tempoh pematangan 24 jam memberikan kadar pematangan terbaik berbanding tempoh IVM lain. Penggunaan sperma hidup-imobilisasi menghasilkan keputusan yang lebih baik daripada kumpulan sperma lain semasa prosedur ICSI. Penambahan serum dalam medium pengkulturan membantu dalam pembentukan blastosis jika

ditambah pada peringkat akhir perkambangan embrio tetapi boleh menjejaskan embrio jika ditambah pada peringkat peringkat awal perkembangan.

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ABBREVIATIONS

°C	Degree Celcius
1PN	One pronucleus
2PN	Two pronuclei
3PN	Three pronuclei
6-DMAP	6-dimethyl aminopurine
ABEL	Animal Biotechnology-Embryo Laboratory
AI	Artificial insemination
ANOVA	Analysis of variance
ART	Asissted reproductive technique
BO	Brackett-Oliphant
BOEC	Bovine oviduct epithelial cell
BSA	Bovine serum albumin
Ca-I	Calcium ionophore
CC	Cumulus cells
COC/s	Cumulus-oocyte-complex/es
CR1aa	Charles Rosenkrans 1 amino acid
dH_2O	Distilled water
DMSO	Dimethyl sulphoxide
D-PBS	Dulbelcco's phosphate buffer saline
DVS	Department of Veterinary Services
ECS	Oestrus cow serum

EGF	Epidermal growth factor
ET	Embryo transfer
FCS	Foetal calf serum
FSH	Follicle stimulating hormone
g	Gramme
GAG/s	Glycosaminoglycan/s
GVBD	Germinal vesicle breakdown
hCG	Human chorionic gonadotrophin
HCl	Hydrochloric acid
HFl	Hydrofluoric acid
IU	International unit
IBHK	National Animal Biotechnology Institute
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVMFC	*In vitro* maturation, fertilisation and culture
IVP	*In vitro* production
KRB	Kreb-Ringer bicarbonate
KSOM	Potassium Simplex Optimisation Medium
LH	Luteinising hormone
LN_2	Liquid nitrogen

MEM	Minimal Essential Medium
mg/ml	Milligramme per millilitre
MI	Metaphase I
MII	Metaphase II
ml	Milliliter
mM	Milli molar
MOET	Multiple ovulation and embryo transfer
mOSM	MilliOsmole
MPF	Maturation-promoting factor
NaCl	Sodium chloride
NCS	Newborn calf serum
NT	Nuclear transfer
OCS	Oestrus calf serum
PB	Polar body
PHE	Penicillamine, hypotaurine and epinephrine
PN	Pronuclear
PVP	Polyvinyl-pyrrolidone
PZD	Partial zona dissection
RNA	Ribonucleic acid
SE	Standard error
SEM	Standard error of means
SOF	Synthetic oviductal fluid
SPSS	Statistical Package for Social Sciences

SS	Steer serum
SUZI	Sub-zona insemination
T1	Telophase 1
TCM 199	Tissue culture medium 199
vs.	Versus
WS	Washing solution
ZD	Zona drilling
ZP	Zona pellucida
µg/ml	Microgramme per millilitre
µl	Microlitre
µM	Micromolar
MESA	Microsurgical epididymal sperm aspiration
PESA	Percutaneous epididymal sperm aspiration
PLC-X	Phosphoinositide-specific phospholipase C
ROSI	Round spermatid injection
ROSNI	Round spermatid nucleus injection (ROSNI)
TESE	Testicular sperm extraction

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Chapter 1

1.0 INTRODUCTION

Since a few decades ago, assisted reproductive technology (ART) was creating many new opportunities from cardinal research in laboratories to practical applications in the fields of medicine, agriculture and wildlife. In agriculture, the techniques involved include artificial insemination (AI); superovulation; *in vitro* maturation, fertilisation and culture (IVMFC); intracytoplasmic sperm injection (ICSI); cryopreservation; sexing; embryo transfer (ET); nuclear transfer and gene transfer. The ART helps in improvement of livestock in their genetic merit and commercial value, especially to improve quantity, healthiness, meat and carcass quality, milk production and growth rate (Galli *et al*., 2003a) The development of these techniques is timely to meet the ever increasing demand for human food worldwide due to increasing human world population as well as increasing the quality of life of people around the world. Using these techniques, they could be an integral component of livestock breeding programme in an effort to produce genetically superior animals at a rapid rate.

In Malaysia, the ART development is still at an infancy stage where the techniques are only at the research stage in laboratories involving mainly mice, cattle, goats and sheep. In order to make animal agriculture as a business entity, it is imperative that the animal farming practices should include modern and advanced technologies such as ART. The ultimate goal of successful livestock farming is to produce healthy live born offspring for human food security and safety at a rapid rate. This can be achieved through application of embryo technologies such as IVMFC,

ICSI, embryo cryopreservation and embryo transfer (Galli *et al*., 2003a,b). However, realistically the success rate of applying reproductive techniques in farm practices is relatively low. There are many constraints involving internal and external factors affecting the success of applying these techniques. Such factors include quality of oocytes obtained from different sources, medium differences during the IVMFC processes, the skill of the operators, the breeds, the environment, the management and the nutrition.

In cattle, theoretically in one oestrous cycle, an ovary can generate almost hundred follicles that may produce oocytes that can be inseminated to produce embryos. But, in reality, only few oocytes will mature and become a calf. Most of the oocytes are not matured and finally degenerated (Hyttel *et al*., 1997). During maturation process *in vitro,* which is the final phase of oogenesis, oocyte at metaphase II stage will be released from follicle. Oocytes maturation process involves nuclear, meiotic and cytoplasmic events, including changes in the organisation of the plasma membrane as a part of cytoplasmic maturation (Hyttel *et al*., 1997). Nuclear maturation mainly involves chromosomal segregation, whereas cytoplasmic maturation involves organelle reorganisation and storage of mRNAs, proteins and transcription factors that acts in the overall maturation process, fertilisation and early embryogenesis (Krisher and Bavister, 1998; Stojkovic *et al*., 2001; Ferreira *et al*., 2009). With better culture condition, it is believed that those immature oocytes could be matured, fertilised and cultured *in vitro* and could be preserved in oocyte or embryo bank for future use.

Selection and grouping of oocytes according to morphological appearance helped to improve maturation ability (Leibfried-Rutledge and First, 1979; Shioya *et*

al. 1988*;* Nagano *et al.* 1999; Jeong *et al*., 2009). Selection of oocytes are based on visual assessment, follicle cells cumulus-oocytes-complexes (COC) and corona radiata via their thickness and compactness (Nagano *et al.* 1999; Jeong *et al*., 2009). Another way of selection is based on the appearance of oocyte cytoplasm (ooplasm). Oocyte with dysmorphic ooplasm can be identified using stereo microscope. It is proven that, the success of IVP is affected by various factors, such as the culture system, oocyte quality and oocyte or embryo density (Makarevich *et al*., 2002; Jeong *et al*., 2009).

IVF is one of the primary techniques of ART that was used to produce *in vitro* embryos through the process of *in vitro* maturation of oocytes, capacitation of sperm and culture of embryos in appropriate media (Kato and Nagao, 2009). IVF could be useful for analysing fertilisation processes as well as in determining the role of various sperm component in fertilisation and embryo development. To date, there are thousands of offspring have been produced through this technique worldwide in livestock, wildlife and laboratory animals as well as human beings. In spite of this success, there is still room for improvement to make this technique more acceptable, economical and easier to be applied routinely in the research laboratories as well as industry settings.

Following IVF, the ICSI technique has been popularly used to alleviate human infertility particularly in male oligospermia (Gerris *et al*., 1995; Joris *et al.*, 1998). ICSI is a mechanical placement of a sperm directly into the cytoplasm of an oocyte. ICSI can bypass all normal barriers for sperm penetration during fertilisation processes such as cumulus cell-hyaluronic acid matrix; zona pellucida and the ooplasmic membrane; acrosome reaction and capacitation. This technique is still at

research level in both laboratories, wildlife and livestock animals. Apparently, even though there are many similarities in general principals, ICSI protocols are quite different from different species of animals (Gerris *et al*., 1995). Hence, it is evident that refined studies need to be conducted and collected so that this technique can be applied successfully for all species of animals as that of the human.

Sperm treatment during immobilisation plays an important role in ICSI. Sperm treatment before ICSI such as permanent immobilisation, using motile sperm, gentle immobilisation or immotile sperm gives different cleavage results (Goto, 1990; Dozortsev *et al*., 1995a). Injection of oocyte without sperm known as sham injection also can be used as control to differentiate between normal fertilisation and parthenogenesis. Oocyte activation using different techniques such as ethanol treatment and calcium ionophore were believed can induce oocyte cleavage and promote embryo development (Jin-Tae *et al.*, 1998; Abdalla *et al*., 2009; Rho *et al*., 1998a; Emuta and Horiuchi, 2001; Galli *et al*., 2003b; Ock *et al*., 2003; Fujinami *et al*., 2004). In cattle, ICSI has been used in many research centres to produce embryos for further storage and subsequent embryo transfer to produce offspring. However, its application in cattle is still limited due to technical problems and difficulties such as failed to produce physiological rates of embryonic or foetal development in the absence of exogenous activation stimulus before it can be applied economically in cattle industry (Garcia-Rosello *et al.*, 2009).

The ability of matured-fertilised oocytes to be cultured and developed up to blastocyst stage depends on factors such as culture medium and condition; using defined media (mono-culture) such as serum (Rizos *et al*., 2003), conditioned media or co-culture with somatic cells such as oviduct epithelial cell (Habsah, 2006) or

Vero cells (Gomez *et al*., 2008). The use of serum in mono-culture media such as steer serum or BSA at early stage of embryonic development was believed that it would retard embryonic development but can enhance embryo development at later stage up to blastocyst stage (Pinyopummintr and Bavister, 1991; Pinyopummintr and Bavister, 1994). With this in mind, therefore the objectives in this study were:

a) To produce embryos *in vitro* using IVMFC technique from oocytes obtained from slaughterhouse cattle ovaries.
b) To produce embryos *in vitro* using ICSI technique from oocytes obtained from slaughterhouse cattle ovaries.
c) To evaluate the effect of introducing embryos to serum in the culture medium during IVMFC on cleavage rate.
d) To determine the effect of COC and cytoplasmic classifications on cleavage rate.
e) To determine the effect of IVM duration on cleavage rate.
f) To evaluate the effect of sperm treatment during ICSI procedure on cleavage rate.
g) To evaluate the effect of oocyte quality during ICSI procedure on cleavage rate.

Chapter 2

2.0 LITERATURE REVIEW

2.1 DEVELOPMENT OF ASSISTED REPRODUCTIVE TECHNOLOGY (ART) IN CATTLE

The 21st century seems to see a revolution in the application of biotechnological procedures in farm animals. This revolution has been heralded by the production of transgenic mice by Brinster *et al.* (1985), followed rapidly by the production of transgenic cattle, sheep and pigs (Wall *et al.*, 1992), the development of practicable sperm sexing in cattle (Seidel and Garner, 2002) and the dramatic production of sheep, Dolly, cloned from a somatic cells by Campbell *et al*. (1996) with all the possibilities that offers for the production of genetically modified animals. However, the successful development and application of this and related technologies are critically dependent on a whole range of basic reproduction technologies such as *in vitro* maturation of oocytes, *in vitro* fertilisation and *in vitro* culture of embryos. Without significant improvements of these reproductive technologies, application of developments in cloning and the production of transgenic farm animals will remain limited and extremely costly (Galli *et al*., 2003a).

2.2 *IN VITRO* PRODUCTION (IVP) OF EMBRYOS IN CATTLE

In vitro embryo production (IVP) is a well known and accepted method of producing embryos from post-puberty heifers and from adult cattle in the laboratory. Although

each ovary contains hundreds of thousands of oocytes at birth, most are lost through atresia. This process starts even before birth. This tremendous loss of genetic material could be reduced by harvesting oocytes from the ovary and using IVP techniques (Brackett and Zuelke, 1993; Hasler, 1998). The oocytes are collected directly from the ovaries of the donor or from slaughtered cattle and fertilised in the laboratory with selected sperm. Oocytes are grown in the laboratory up to the transferable stage when they are transferred into recipient cattle. This technology is simple and safe. IVP differs from the conventional multiple ovulation and embryo flushing system (MOET). In the IVP system, oocytes were harvested directly from the deceased donor's ovaries and the embryos were produced in the laboratory. Whereas in the MOET system, the embryos were formed and grown inside the donor cow (*in vivo*) and are then flushed out to be transferred into recipients.

Bovine IVP is now a well-established and reasonably efficient procedure. Moreover, ovum pick-up at frequent intervals, in combination with *in vitro* fertilisation (IVF), has proved its worth in improving or increasing the yield of embryos from designated donors. In addition, IVP can be used to salvage irreplaceable genetic material following slaughter for infectious disease control or culling for other reasons (Hasler, 2003). IVF has also been used to produce thousands of embryos needed for scientific research (Gordon and Lu, 1990), including efforts to produce embryonic stem cells. The constituent *in vitro* oocyte maturation (IVM) and *in vitro* embryo culture (IVC) techniques are also integral parts of the procedures for cloning by somatic cell nuclear transfer and generating transgenic cattle which produce valuable pharmaceutical proteins in their milk (Niemann and Kues, 2003). *In vitro* fertilisation by intracytoplasmic sperm injection (ICSI), which is so prominent in

assisted human reproduction, is now feasible in cattle, even with freeze-dried sperm (Keskintepe *et al.*, 2002).

2.2.1 *In Vitro* Maturation (IVM)

Oocytes are arrested at the diplotene stage of the prophase I at birth and resume meiosis at puberty. In *in vivo* condition, resumption of meiosis is initiated by a preovulatory LH surge and occurs in fully grown, meiotically competent oocytes from dominant follicles (Mayes and Sirard, 2001). Figure 2.1 shows a diagram of structures of an ovary. The end-point of *in vivo* oocyte development is the release of the oocytes from the follicles at metaphase II stage, with the potential to support normal embryonic development. The final maturation of oocytes involves nuclear, meiotic and cytoplasmic events, including changes in the organisation of the cytoplasmic membrane as a part of cytoplasmic maturation. Maturation of oocyte is the third and final phase of oogenesis, during which several changes occur in the oocyte, preparing it for ovulation and its imminent interaction with the sperm. Most of the oocytes collected from ovaries of slaughtered cattle are still at immature stage. When oocytes were aspirated or sliced from the ovaries, and cultured under *in vitro* condition, meiosis resumes spontaneously resulting in germinal vesicle breakdown (GVBD), spindle formation and chromosome segregation occurrence and the oocytes progress to the metaphase II (MII) stage after 24 hours of culture (Ward *et al*., 2002).

There are a few factors affecting the maturation process including oocyte morphology and cumulus cells layers (Bols *et al.,* 1996; Bols *et al.,* 1997). The other factors are culture condition such as media used and supplementation in culture media

(Lane *et al.*, 2003; Saadeldin *et al.*, 2011), culture period (Sutton *et al.*, 2003; Kochhar *et al.*, 2003; and temperature (Katska and Smorag, 1985; Morstin and Katska, 1986; Shi *et al.*, 1998).

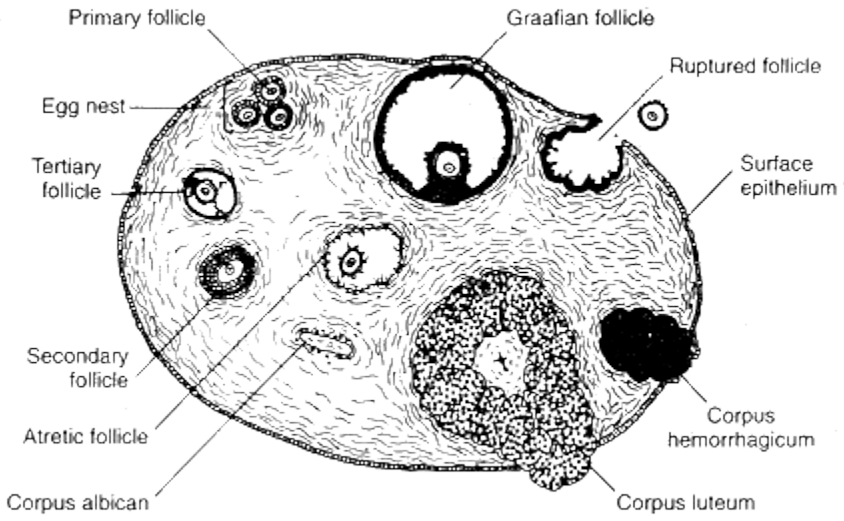

Figure 2.1: Diagram of structures that can be identified in a cross section of an ovary of a reproductively active female. Different maturation stages for follicles and the corpus luteum can be observed. Adapted from Patten (1964).

2.2.1.1 Determination of quality and grading of collected oocytes

Oocyte quality and grading are the most important criteria that affect the results of *in vitro* produced (IVP) embryos. This generally involves assessment of the morphology of the cumulus investment, the cytoplasm and the cumulus cells as a whole. Oocyte classification or grading and selection become an important and essential part of any

IVP study (Varisanga *et al*., 1998; Jeong *et al*., 2009; Satrapa *et al.,* 2011). This is due to the fact that the success of IVP system will affect early embryonic development, establishment and maintenance of pregnancy, foetal development and even adult disease. The other factors are the use of oocyte or embryo for micromanipulation such as for sperm injection (ICSI) using different types of sperm, oocyte and embryo micromanipulation such as splitting and cloning; cryopreservation and embryo transfer. The immature oocytes acquire an ability to resume and complete meiosis, undergo fertilisation process and sustain embryonic development (De Sauza *et al.,* 1998).

2.2.1.1.a *Cumulus cells*

Cumulus cells are cells that surround the oocytes and a sub-population of ovarian granulosa cells with distinct function. The role of cumulus cells is to provide nutriment to the oocytes during its growth, to participate in the zona formation, and following the LH surge, to synthesise the matrix composed of proteins and hyaluronic acid important in oviductal transport or in sperm trapping (Bedford and Kim, 1993). During intrafollicular phase, several layers of cumulus cells tightly surround the oocyte. These cells extend cytoplasmic processes through the zona pellucida in contact with the oolemma. The gap junctions allowed direct cell-to-cell communication (Larsen and Wert, 1988), and somatic cells were able to send small molecules into the oocytes as nourishment. The nutrients are transported into the ooplasm via gap junction between ooplasm and cumulus cells (Tanghe *et al*., 2003). Cumulus cells promoted normal fertilisation with proper pronuclear formation,

control the rate of nuclear maturation and help to maintain penetrability of the oocytes. Cumulus cells also reduce the zona pellucida 'hardening' that occurred during culture (De Fellici and Siracusa, 1982).

Classification of the oocytes varies depending on research objectives. Oocytes were normally graded according to the cumulus cells layers or compactness of the cumulus cells; morphology of the oocytes or ooplasm; homogeneity of the ooplasm and diameter of the ovarian follicles (Leibfried-Rutledge and First, 1979; Shioya *et al.*, 1988; De Loos *et al*., 1989).

2.2.1.1.b *Cytoplasmic quality*

Morphological appearance of cytoplasm has different developmental potential to mature *in vitro*. Oocyte with brown ooplasm had an excellent developmental capacity and oocyte with dark clusters had large numbers of lipid droplets in ooplasm and the greatest developmental capacity after *in vitro* fertilisation (IVF) (Nagano *et al.*, 2006b). Location of lipid droplet is in association with mitochondria and the smooth endoplasmic reticulum and this indicates the utilisation of lipid as an energy substrate for the synthesis of adenosine triphosphate (ATP) by mitochondria. Bovine oocytes use lipids as an oxidative substrate for nuclear and/or cytoplasmic maturation. Thus, the storage of lipid droplets in the ooplasm seems to be important for the production of ATP and maturational ability of oocytes (Cetica *et al*., 2002). Nagano *et al.* (2006a) predicted that the oocytes with a large amount of lipid droplet have high levels of ATP and good developmental capacity; however, oocytes with black ooplasm had less developmental capacity, although they have abundance of lipid droplet in their

ooplasm. Table 2.1 shows the oocyte grading in cattle according to cumulus cells and cytoplasmic quality.

2.2.1.1.c *Follicle size*

There is a general agreement that the origin of an oocyte plays an important role in embryonic development (Yang *et al*., 1998). Some inferences have been made regarding the morphological aspects of the ovaries in relation to the subsequent oocyte developmental competence in an *in vitro* system. Ovarian morphology such as ovary size and follicle size (Varisanga *et al.,* 1998; Calado *et al.,* 2003; Machatkova *et al.,* 2004; Lequarre *et al.,* 2005) can affect embryo competency.

The *in vitro* meiotic and developmental competence of oocytes is related to follicle size, oestrous cycle stage and the level of atresia influenced by other follicles, mainly the dominant follicle (Pavlok *et al*., 1992; Lonergan *et al*., 1994; Machatkova *et al*., 1996; Hagemann, 1999). Oocytes will acquire an intrinsically greater *in vitro* developmental capacity if the follicles reach 7 mm in size. On the other hand, significant differences in oocyte developmental potential during the oestrous cycle, in terms of presence or absence of a dominant follicle, have been observed irrespective of follicle size. Development to blastocyst stage was greater when oocytes were obtained during follicular growth, as compared with follicular dominance (Hagemann, 1999). The presence of a dominant follicle in either one or both ovaries of a pair has a negative effect on the IVF-produced bovine embryos (Varisanga *et al.,* 1998). The small follicle oocyte seems has incomplete cytoplasmic maturation because of mRNA

Table 2.1: Oocytes grading experiments in cattle according to cumulus cells and cytoplasmic quality

Year	Author	Oocyte grading
1979	Leibfried-Rutledge and First	Graded oocytes into 4 categories based on the cumulus cells layers, ooplasm and chromatin: 1) Group 1: >3 complete and compact cumulus cells layers, 2): >3 incomplete or <3 complete and compact cumulus cells layers, 3) Group 3: expended cumulus cells present, cellular investment showed expansion and cumulus cells appearing in scattered clumps in matrix, and 4) Group 4: nude oocytes, cellular investment not present and oocytes were only enclosed by zona pellucida.
1988	Shioya *et al.*	Graded oocytes into 3 categories based on the cumulus cells layers: 1) Class A: with compact and dense cumulus cells layers, 2) Class B: with compact but not dense cumulus cells layers, some were partially naked with compact cumulus cells layers, others those were sub-classed as B' were partially naked oocytes with thin cumulus cells layers or with small remnants of cumulus cells and 3) Class C: consisted of naked oocytes.
1989	De Loos *et al.*	Graded oocytes into 4 categories: 1) Category 1: compact multi-layered cumulus cells investment, homogenous ooplasm, total COC light and transparent, 2) Category 2: compact multi-layered cumulus cells investment, homogenous ooplasm but with a coarse appearance, and a darker zone at the periphery, total COC slightly darker and less transparent, 3) Category 3: less compact cumulus cells investment, ooplasm irregular with dark clusters, total COC darker than categories 1 and 2, and 4) Category 4: expanded cumulus cells investment, cumulus cells scattered, in dark clumps in a jelly matrix, ooplasm irregular with dark clusters, total COC dark and irregular.
1992	Wurth and Kruip	Graded oocytes into 3 categories: 1) A-COC: with clear and compact cumulus cells and translucent ooplasm, 2) B-COC: dark and compact cumulus cells and dark ooplasm and 3) C-COC: dark and

		expanded cumulus cells and dark ooplasm.
1999	Nagano *et al.*	Graded oocytes into 2 categories: 1) Category 1: normal oocytes with homogenous ooplasm and multilayered cumulus cells investment and 2) Category 2: oocyte having heterogenous ooplasm with dark clusters and multilayered cumulus cells investments.
2000	Khurana and Niemann	Graded oocytes into 3 categories: 1) Category 1: oocytes, having a homogenous evenly granulated cytoplasm surrounded by a compact cumulus oophorus with more than three layers, 2) Category 2: Oocytes with fewer than three layers of cumulus cells or those partially denuded but having a homogenous evenly granulated cytoplasm, 3) Category 3: oocytes surrounded by corona radiata cells only and 4) Category 4: denuded oocytes.
2001	De Wit and Kruip	Graded oocytes into 5 categories: 1) COC-A: had a bright, compact cumulus cells investment, 2) COC-B1: had a compact cumulus cells investment, but with darker than COC-A, 3) COC-B2: the colour was comparable, with COC-B1, but the corona radiata appeared to dissociated from the rest of the cumulus cells investment, 4) COC-B3: the cumulus cells was almost black and corona radiata was almost completely dissociated from the rest of the cumulus cells investment and 5) COC-C: was distinguished by their strongly expanded cumulus cells investment with dark spots of degenerated cells.
2001	Mayes and Sirard	Graded oocytes into 3 categories: 1) Class 1: At least five layers of compact cumulus cells and a cytoplasm that was either homogeneous or showed a dark zone around the periphery, 2) Class 2: The second group of COCs showed a slight expansion of the outer layers of cumulus cells and slight granulation of the cytoplasm, 3) Class 3: COCs with atretic or incomplete cumulus.
2002	Bilodeau-Goeseels and Panich	Graded oocytes into 6 categories: 1) Group 1: COC with more than 5 layers, compact and homogeneous ooplasm, 2) Group 2: COC with more than 5 layers, have slight expansion and/or with slight granulation, 3) Group 3: COC with less

		than 5 layers with no or slightly expansion and homogeneous ooplasm, 4) Group 4: COC with less than 5 layers with no or slightly expansion and granulation ooplasm, 5) Group 5: no cumulus variable and 6) Group 6: full expansion with dark clumps and heavy granulation.
2005	Yuan *et al.*	Graded oocytes into 3 categories: 1) Group 1: more than five layers of compact cumulus cells, 2) Group 2: one to five layers of compact cumulus cells and 3) Group 3: expanded cumulus cells.
2006b	Nagano *et al.*	Graded oocytes into 7 categories: 1) Grade 1: brown and homogenous ooplasm, 2) Grade 2: brown and homogenous ooplasm with dark zone around the periphery, 3) Grade 3: brown and heterogenous ooplasm with dark clusters, 4) Grade 4: pale and heterogenous ooplasm, 5) Grade 5: pale and heterogenous ooplasm with dark clusters, 6) Grade 6: black and homogenous ooplasm and 7) Grade 7: variable ooplasmic features and a diameter less than 115 µm.
2009	Jeong *et al.*	Graded oocytes into 3 categories with two or three layers of COCs and homogenously granulated cytoplasm. The cytoplasms were divided into three groups: 1) Group 1: pale colour (PC), 2) Group 2: brown colour (BC) and 3) Group 3: dark colour (DC).

deficiency or protein accumulation (Pavlok *et al*., 1993). Oocytes of larger follicles give a better ability to develop up to blastocyst stage. Blondin and Sirard (1995) reported that follicles that have reached 3 mm in diameter gave better developmental potential. Oocytes from follicles smaller than 3 mm were capable undergoing maturation, and could develop to MII and successfully fertilised by sperm. However, some of the embryos were blocked at either 8-cell or 16-cell stage of development. Oocyte obtained from follicles with 2 to 6 mm in diameter gave the best maturation, fertilisation and cleavage rates results including blastocyst rate (Lonergan *et al*., 1994; Hagemann *et al*., 1999**;** Lequarre *et al.,* 2005). In summary, all researchers agree that follicle size is an important consideration to determine the developmental competence of oocytes and embryos *in vitro*.

2.2.1.2 IVM duration

Immature, partially mature and aged oocytes did not fertilise normally, and consequently resulted in poor embryo development (Yanagimachi, 1994). The rate of oocyte maturation has implications for subsequent development (Dominko and First, 1992). Therefore, proper maturation is a key and essential factor to achieve full developmental competence of embryo development. A number of ultrastructural and molecular changes occurring during oocyte developments are linked to the developmental competence of the gamete (Hytell *et al.,* 1997; Ward *et al.,* 2002). Inappropriate timing of maturation could lead to the formation of abnormal chromatin (Dominko and First, 1997), oocyte aging (Hunter and Greve, 1997) and impaired development (Marston and Chang, 1964). Oocyte age is a determining factor in

activation and reaches a plateau around 30 hours after *in vitro* maturation for bovine oocytes (Ware *et al.,* 1989; Barnes *et al.,* 1993).

During *in vitro* maturation, the extrusion of the first polar body, indicating the attainment of metaphase II, occurs 18-24 hours after the start of maturation (Dominko and First, 1997). Despite the fact that the sperm can penetrate oocytes prior to completion of maturation and the optimum time for *in vitro* fertilisation is at the completion of meiosis. Even though that the oocyte can mature quickly, and able to extrude first polar body by 16 hours after *in vitro* maturation, a further period of culture (normally 8 hours) is necessary before fertilisation process in order to attain full developmental competence (Dominko and First, 1992; Dominko and First, 1997). In general, by 16 hours, approximately 50% of oocytes are at telophase I or in process of polar body extrusion or already reach metaphase II. By 20 hours, more than 80% have reach metaphase II (Lonergan *et al*., 1997; Park *et al.,* 2005). This would suggest that the optimal time for insemination is around 20 hours. It was shown that a duration of 24 hours of IVM gave better blastocyst-reaching result compared with 18 hours IVM (Monaghan *et al*., 1993). Figure 2.2 shows a schematic diagram of the cross-section of a mature mammalian egg, arrested at metaphase II.

2.2.2 *In Vitro* Fertilisation (IVF)

Fertilisation is a complex process of gametes interaction which starts with the specific recognition such as sperm transport through female reproductive tract (in *in vivo* case), sperm capacitation, and sperm binding to the oocyte must occur, followed by the acrosome reaction, penetration of the zona pellucida, binding and fusion of the

sperm with the oocyte plasma membrane, delivery of intact paternal genetic factors into the oocyte and ultimately leads to the fusion of the male and female pronuclei (Lessard *et al*., 2011). Successful in IVF requires appropriate preparation of sperm and oocyte, as well as culture condition that are favourable to the metabolic activity of the male and female gametes (Brackett, 1983). The initial stages of fertilisation depend principally on two structures; that are the acrosome of the sperm and the vitalline coat or zona pellucida of the oocyte. Three major events in sperm-oocyte interaction are i) attachment of the sperm to the zona pellucida; ii) the sperm undergoes the acrosome reaction, as a result of which digestive enzymes are released and the inner acrosomal membrane is exposed; III) the highly fusogenic sperm membrane makes contact with the oocyte plasma membrane and the two membranes fuse together (Brackett, 1983; Lessard *et al*., 2011).

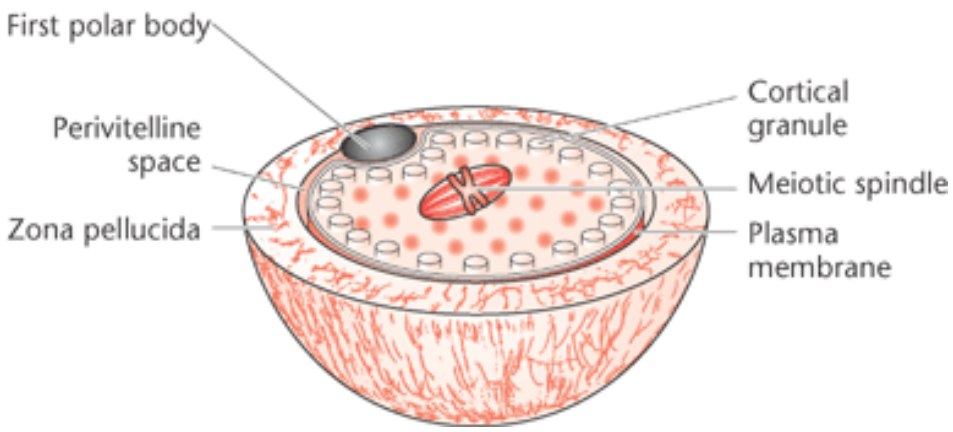

Figure 2.2: Schematic diagram of the cross-section of a mature mammalian egg, arrested at metaphase II, showing the different structures. Note that the meiotic spindle is close to the region from which the first polar body has been extruded. Adapted from Wassarman (1999).

2.2.2.1 Sperm capacitation

Fresh or frozen-thawed sperms are unable to fertilise without undergo a series of preparatory stage known as capacitation (Austin, 1951). Capacitation is associated with removal of adherent seminal plasma protein, reorganisation of plasma membrane lipids and changes in some enzymatic activities such as protein kinase C (Furuya *et al.,* 1993). It involves an influx of extracellular calcium (Singh *et al.*, 1978), increase in cyclic AMP (White and Aitken, 1989) and decrease in intracellular pH (Vredenburgh-Wilberg and Parrish, 1995).

In *in vivo* condition, capacitation occurs while sperm reside in the female reproductive tract for a period of time during gamete transport and required several hours. In *in vitro* condition, the sperm was capacitated by incubation in high ionic strength fertilisation medium (Brackett *et al.*, 1982). Cattle sperm could be induced to undergo capacitation *in vitro* by using heparin (Parrish *et al.*, 1986; Chamberland *et al*., 2001; Breininger *et al*., 2010), caffeine (Niwa and Ohgoda, 1988; Breininger *et al.,* 2010; Li and Funahashi, 2010), glutathione (Slaweta and Laskowska, 1987; Sánchez-Vázquez *et al.,* 2008), a mixture of pennicillamine, hypotaurine and epinephrine (PHE) (Gordon, 1994; Galli *et al*., 2003b; Way and Killian, 2006) and calcium ionophore (Pereira *et al.,* 2000; Januskauskas *et al*., 2000). The biochemical modifications led to a transient change in the pattern of sperm motility. Sperm that have undergone capacitation become hyperactivated and display hyperactivated motility. Capacitation appears to destabilise the sperm membrane to prepare for acrosome reaction (Yanagimachi and Usui, 1974).

Heparin is a glycosaminoglycan (GAG) present in the female genital tract. Heparin and heparin-like GAGs in the oviduct play an important role in capacitation

of cattle sperm. GAG modulates capacitation by binding to the proteins on the sperm membrane (Miller and Hunter, 1986). Heparin-binding protein work by attaching to the sperm surface, enabling heparin-like GAGs in the female tract to induce capacitation (Marks and Ax, 1985; McCauley *et al.*, 1996).

2.2.2.2 Acrosome reaction and zona pellucida penetration

Sperm binding to the zona pellucida is a receptor-ligand interaction with a high degree of species specificity. The carbohydrate groups on the zona pellucida glycoproteins work as sperm receptors. This is a very important part of fertilisation. The sperm then faces the difficult task to penetrate the zona pellucida to get to the oocyte (Vela'squez *et al*., 2007). Figure 2.3 shows schematic diagram of the structure of mammalian sperm and Figure 2.4 shows sperm fusion in the fertilisation process.

Acrosome is a huge modified lysosome that contains with zona-digesting enzymes and located around the anterior part of the sperm head. The acrosome reaction provides the sperm with an enzymatic drill to get through the zona pellucida (Deppe *et al.*, 2008). The same zona pellucida protein that functions as a sperm receptor also stimulates a series of events that lead to the many areas of fusion between the plasma membrane and outer acrosomal membrane. Membrane fusion (actually an exocytosis) and vesiculation which is expose to the acrosomal contents, leading to the leakage of acrosomal enzymes from the sperm head. As the acrosome reaction progress and the sperm passes through the zona pellucida, more plasma membrane and acrosomal contents are lost (Pate *et al*., 2008). During the sperm traverses the zona pellucida, the entire anterior surface of its head, down to the inner

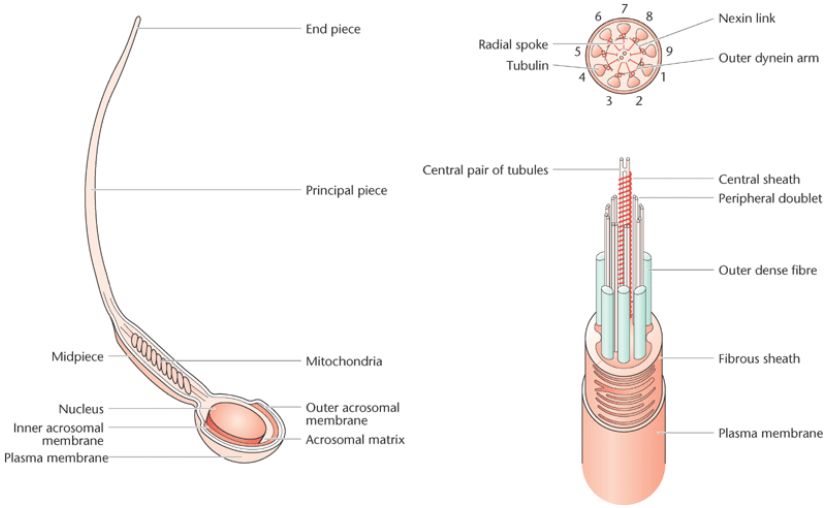

Figure 2.3: Schematic diagram of the structure of mammalian sperm. (Left) Structure showing the head, midpiece, principal piece and end piece of the sperm. The different compartments of the sperm head are shown in cutaway. The helical arrangement of the mitochondria in the midpiece is indicated. Adapted from Wassarman (1999).

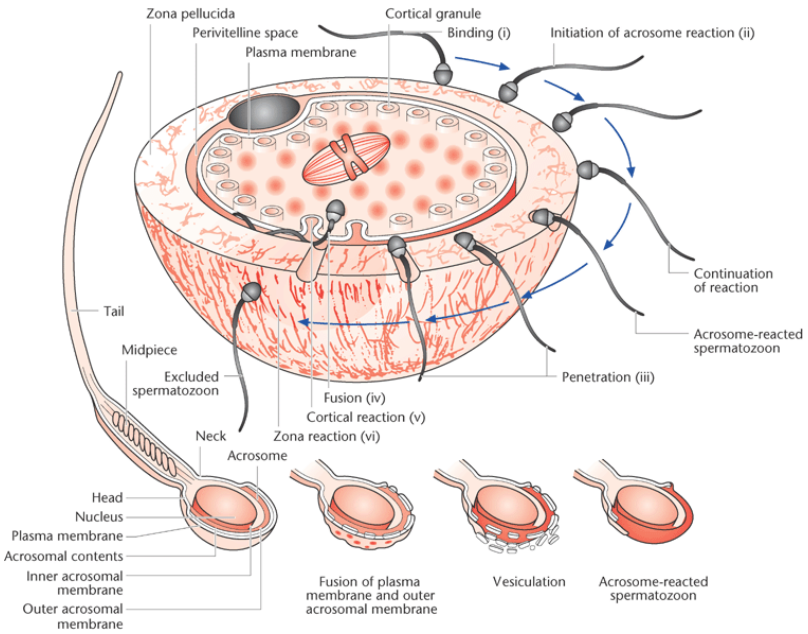

Figure 2.4: Order of events in mammalian fertilization, starting with sperm binding to the outside of the zona pellucida and ending with gamete fusion. Reactions are in sequence (i) to (v), with stages of the reactions labelled. Adapted from Wassarman (1999).

acrosomal membrane, is denuded. Sperm that lose acrosomes before encountering the oocyte are unable to bind to the zona pellucida and fertilise (Tanghe, 2005). The constant propulsive force from the sperm flagellating tail, in combination with acrosomal enzymes, allow the sperm to create a tract through the zona pellucida.

Two factors, motility and zona-digesting enzymes allow the sperm to traverse the zona pellucida. Sperm motility is very important to zona penetration. Once a sperm penetrates the zona pellucida, it binds to and fuses with the plasma membrane of the oocyte. Binding occurs at the posterior (post-acrosomal) region of the sperm head. Sperm glycoprotein called fertilin binds to a protein in the oocyte plasma membrane and induces fusion (Olds-Clarke, 2003; Gaffney *et al.*, 2011).

The absence of acrosomal reaction in post-thawed sperm following prolonged incubation *in vitro* has been reported to be significantly correlated with fertility (Saacke and White, 1972). Acrosome reaction can be induced spontaneously *in vitro* in pre-incubated sperm or by exposure to any of several inducers. The physiological inducers such as cumulus cells (Mattioli *et al.*, 1998) follicular fluid (Suarez *et al*., 1986; Talbot *et al*., 1976; Tesarik *et al*., 1993; Thomas and Meizel, 1988), progesterone (Cheng *et al.*, 1998), zona pellucida proteins (Wassarman, 1990) and glycosaminoglycans (Ax *et al*., 1985; Parrish *et al.*, 1985; Whitfield and Parkinson 1992) or by non-physiological inducer such as calcium ionophore (Whitfield and Parkinson, 1995).

The ability of glycosaminoglycans to bind to bull sperm (Marks and Ax, 1985) and to induce acrosome reaction *in vitro* appear to reflect field fertility to some extent (Ax *et al*., 1985; Lenz *et al*., 1988; Whittield and Parkinson, 1992). Whitfield and Parkinson (1995) were the first to demonstrate a relationship

between the induction of acrosome reaction in frozen-thawed bull sperm by calcium ionophore and fertility after AI.

2.2.2.3 Oocyte activation

Before fertilisation, the oocyte is in inactive state, arrested in metaphase of the second meiotic division (MII). Upon binding of a sperm, the oocyte rapidly undergoes a numbers of metabolic and physical changes that collectively are called oocyte activation. Prominent effects include the rise in the intracellular concentration of calcium, completion of the second meiotic division or called cortical reaction. Cortical reaction is a massive exocytosis of cortical granules seen shortly after sperm-oocyte fusion (Tae *et al.,* 2008; Tanghe, 2005). Cortical granules contain a mixture of enzymes, including several proteases, which diffuse into the zona pellucida following exocytosis from the oocyte. These proteases alter the structure of the zona pellucida, inducing zona reaction. Components of cortical granules interact with the oocyte plasma membrane (Tanghe, 2005).

Zona reaction is an alteration in the structure of the zona pellucida catalysed by proteases from cortical granules. The critical importance of the zona reaction is that it represents the major block to polyspermy that results in induced changes in the zona pellucida (Wang *et al,* 1997; Way and Killian, 2006; Coy and Aviles, 2010). The zona pellucida hardens, the sperm that have not finished traversing the zona pellucida by the time the hardening occurs are stopped in their tracks. Sperm receptors in the zona pellucida are destroyed. Therefore, any sperm that have not yet bound to the zona pellucida will no longer be able to bind (Landim-Alvarenga *et al*., 2002).

Following fusion of the fertilising sperm with the oocyte, the sperm head is incorporated into the oocyte cytoplasm. The nuclear envelope of the sperm disperses, and the chromatin rapidly loosens from its tightly packed state in a process called decondensation (Barroso *et al.,* 2009). Chromatin from the sperm and oocyte are encapsulated in a nuclear membrane, forming pronuclei. Each pronucleus contains a haploid genome. The membranes break down, and the two genomes condense into chromosomes and produce diploid organism (Rho *et al.,* 1998a; Lu and Sidel, 2004).

2.2.3 *In Vitro* Culture (IVC)

The development of more defined embryo culture systems have been one of the key achievements in cattle embryology over the last decade. It is now generally accepted that although it is an efficient system for embryo production, co-culture is difficult to improve upon, due to the different interactions between medium components and the two cell types in culture. Over the past decades, knowledge of the basic cell biology of early embryo development and the in-vivo environment has substantially increased. Initially this led to revised formulations, such as SOFaaBSA or new formulations, such as CR1 (Thompson and Peterson, 2000).

2.2.3.1 Factors affecting oocyte maturation, fertilisation and culture

There are numerous factors affecting the *in vitro* maturation of oocytes obtained from abattoir ovaries. Consequently, these factors would affect the fertilisation, embryo cleavage, pregnancy and parturition rates. Various factors including the quality of

oocyte itself, protein source, somatic cells, culture media, oxygen concentration, number of embryos per culture unit (embryo density) and energy substrates may affect preimplantation embryo development *in vitro* (Bavister, 1995).

2.2.3.1.a *Culture medium*

Preimplantation embryos normally develop within *in vivo* environment of the female reproductive tract. The ability of embryos to develop *in vitro* in a particular medium does not necessarily indicate the beneficial or preferable environment but may instead simply reflect their ability to tolerate artificial conditions (Bavister, 1995).

A number of *in vitro* maturation media in cattle have been developed in laboratories worldwide. These media range from simple to complex media such as Ham's F-10 (Xu *et al.*, 1986), Ham's F-12 (Fukushima and Fukui, 1985), TALP, Menezo B2 and TCM-199 (Edwards, 1965; Katska and Smorag, 1985; Schellander *et al*., 1990). Generally, oocytes are matured in buffered TCM-199, supplemented with pyruvate, hormones (oestradiol, FSH and LH), serum (Goto *et al*., 1988; Fukui, 1990), granulosa cells (Fukui and Ono, 1989) and cumulus cells (Fukui and Sakuma, 1980). All of these supplementations are nescessary to induce full maturation of the nucleus and cytoplasm.

2.2.3.1.b *Serum*

Serum is a protein source that is added to IVM medium. It is considered as a semi-defined protein because it contains unidentified growth factors, hormones and

peptides that may support growth and development of oocytes. It is found that serum provides nutrients to cells in the COC and prevents zona pellucida hardening (Wani, 2002). Most IVM media generally are supplemented with 10 to 20 % heat-inactivated serum. Sera used in IVM medium include foetal bovine serum (FBS), foetal calf serum (FCS), steer serum (SS) and homologous or heterologous oestrus cow serum (OCS).

In culture medium for IVC, serum has a biphasic effect on *in vitro* embryo development (Pinyopummintr and Bavister, 1991). It seems no beneficial effect on embryo development from the 2-cell to the morula stage but serum enhance and increase cell number of blastocyst and stimulate hatching (Pinyopummintr and Bavister, 1991; Pinyopummintr and Bavister, 1994; Wang *et al*., 1997). Generally, when bovine embryos are cultured in media supplemented with serum, they become arrested in development at the 8- to 16-cell stage (Camous *et al.,* 1984; Eyestone and First, 1989). Serum is undefined, or in some cases semi-defined, biological product that most likely provides other physiological active materials such as energy substrates, amino acids and vitamins, all of which may act in concert with growth factors to enhanced embryo development *in vitro*. Adjustment of serum concentrations to 5% during post-cleavage culture and higher concentrations (10-20%) during post-cleavage culture is an effective way to overcome the detrimental effects of serum on early-stage embryos while taking advantage of the embryotrophic properties during later stages (Wang *et al.,* 1997). The use of serum with various somatic cells and growth factors during *in vitro* culture could overcome 8- to 16-cell stage block. Growth factors, for example platelet-derived growth factor (PDGF), have regulatory roles during the fourth cells cycle (Larson *et al.,* 1992a), transforming

growth factors ß$_1$ (TGFß$_1$) and fibroblast growth factor (FGF) are required during fifth cell cycle and blastocyst formation (Larson *et al.*, 1992b).

Embryos cultured in absence of protein exhibit differences in their metabolic activity (Eckert *et al.*, 1998; Krisher *et al.*, 1998), lower developmental capacity (Thompson *et al.*, 1998) and lower cells number (Lee *et al.*, 1998; Lim *et al.*, 1994; Pinyopummintr and Bavister, 1991; Pinyopummintr and Bavister, 1994) compared to embryos cultured in presence of protein. Although the beneficial effects of serum maybe due to its antioxidant properties (Rieger, 1992), serum is not a naturally occurring biological product but a pathological fluid formed by blood clotting, a process that may lead to the chemical changes which have detrimental effects on embryo culture (Maurer, 1992).

However, serum has been found to induce a wide range of abnormalities *in vitro*. Its presence has been associated with the abnormal accumulation of cytoplasmic lipid droplets (Abe *et al.*, 1999) where embryos with larger amount of lipid content are more sensitive and detrimental to micromanipulation procedure such as during embryo cryopreservation (Mohr and Trounson, 1981; Leibo and Loskutoff, 1993) as well as it has been shown to be associated with abnormally large foetuses or offspring known as Large Calf Syndrome (Thompson *et al.*, 1995; Behboodi *et al.*, 1995; Numabe *et al.*, 2000; Walker *et al.*, 1996). This syndrome often occurs from embryos that have been cultured in a few days in synthetic media, which indicates the long-term medium effects carried into gestations and it results in high numbers of neonatal death (Walker *et al.*, 1992; Thompson *et al.*, 1992). In addition, it has also been shown that serum may introduce parthogenic agents into the culture medium (Paloma *et al.*, 2003).

Serum-free media have been introduced, using substitutes such as polyvinyl alcohol (PVA), hyaluronate to replace albumin (Rinehart *et al.,* 1998). Commercially-prepared serum replacements contain fibroblast growth factors (FGF), epidermal growth factors (EGF), insulin, BSA, transferrin, selenium, binding proteins, adhesin factors, vitamins, hormones and mineral trace elements (Paloma *et al.,* 2003).

2.2.4 Intracytoplasmic Sperm Injection (ICSI)

Intracytoplasmic sperm injection (ICSI) is one of the assisted reproductive technique (ART) with the mechanical placement of a sperm directly into the cytoplasm of an oocyte. The normal barriers to sperm penetration including the cumulus cell-hyaluronic acid matrix, the zona pellucida and the ooplasmic membrane are bypassed. Therefore, ICSI overcoming problems associated with sperm penetration of oocyte (Chung, 1999; Suttner *et al*., 2000; Kato and Nagao, 2009).

2.2.4.1 Application of ICSI

An opportunity for investigation of fundamental aspects of fertilisation such as mechanisms of gamete interaction, sperm-induced oocyte activation and first cell cycle control. The advantage of ICSI is that it requires few sperm. In humans, ICSI is applied clinically to overcome several types of male infertility such as oligospermia (Van Steirteghem *et al*., 1993a). In domestic animals, however, the benefit of this technique lies in the use of genetically and/or economically valuable sperm.

Since the first report of ICSI in rodents (Uehara and Yanagimachi, 1976), this technique was successfully used in several species and offspring were obtained in mice (Kimura and Yanagimachi, 1995), rabbit (Hosoi *et al.*, 1988), sheep (Catt *et al.*, 1996; Gomez *et al.*, 1998), non-human primates (Chan *et al.*, 2000), humans (Palermo *et al*., 1992), horse (Cochran *et al.*, 1998). In cattle, ICSI was applied for the injection of both non-sexed sperm (Goto, 1990; Wei and Fukui, 2002) and sexed sperm heads (Hamano *et al.*, 1999).

Westhusin *et al.* (1984) first reported pronuclear formation in sperm-injected bovine oocytes in 1984. Since that report, a number of researchers have tried to improve the ICSI technique in cattle. While offspring have been reported (Goto *et al*., 1990; Hamano *et al.*, 1999), poor *in vitro* development of the microinjected bovine oocytes has commonly been encountered. This problem may be related to the inconsistent level of sperm decondensation and subsequent pronuclear formation observed following ICSI in cattle.

Several assisted fertilisation techniques have emerged and have been rapidly developed where conventional IVF was not possible. Techniques of zona manipulation such as zona drilling (ZD) (Gordon and Talansky, 1986; Depypere *et al.*, 1988; Ahmad *et al.*, 1989), partial zona dissection (PZD) (Odawara and Lopata, 1989) and sub-zonal insemination (SUZI) (Fishel *et al.*, 1994; Parinaud *et al*., 1994). The technique of PZD was developed to increase the probability that a sperm capable of fertilisation comes in contact with the oocyte. Although this method improved conventional IVF results, the improvement was only marginal and relatively large numbers of sperm are still required. This drawback applied less to the subsequent technique of SUZI (Fishel *et al*., 1994). However, for all of these techniques

fertilisation rates remained low, rates of polyspermic fertilisation were increased. Ock *et al.* (2006) found that bovine embryos injected with round spermatid (ROSI) have been reported with extremely low developmental efficiency and did not obtain offspring after the transfer of embryos in cattle (Goto *et al*, 1996). In most species, oocytes in metaphase II stage either fertilised or injected with elongated spermatid can be activated for further development. However, oocytes injected with round spermatid exhibited variations in the effectiveness of activation among different species (Ogura and Yanagimachi, 1993; Ogura *et al.*, 1994; Goto *et al*, 1996).

In human, *in vitro* fertilisation and embryo transfer as treatment for male factor infertility is associated with lower fertilisation and pregnancy rates (Van Steirteghem *et al*., 1993a). ICSI has been used throughout the world as a treatment of last chance for men with extremely severe oligo- and asthenozoospermia but also as a more efficient treatment in less severe cases in which the standard *in vitro* fertilisation could also be envisaged. Due to a strong clinical demand, the human application of ICSI has advanced animal studies significantly (Van Steirteghem *et al*., 1993a; Tesarik, 1996).

2.2.4.2 Activation in ICSI

Intracytoplasmic sperm injection is associated with a slightly different pattern of calcium oscillations in comparison with normal fertilisation (Tesarik, 1998). There are a few activation methods can affect ICSI result. Activation treatment before and after ICSI by using chemical and mechanical activation can activate sperm and oocyte. Fertilisation and cleavage rates were improved by additional oocyte activation

treatment with calcium ionophore A23187 (Goto *et al*., 1990; Chen and Seidel, 1997), ethanol (Hamano *et al.,* 1999; Emuta and Horiuchi, 2001), electric stimulation (Hwang *et al*., 2000) or ionomycin plus 6-dimethylaminopurine (DMAP) (Rho *et al*., 1998a; Chung *et al.,* 1999; Suttner *et al*., 2000; Ock *et al*., 2003). Table 2.2 shows ICSI in cattle.

2.2.4.2.a Oocyte activation treatment

Although live births derived from embryos produced through ICSI have been reported in cattle (Goto *et al.,* 1990; Hamano *et al.,* 1999), the efficiency of ICSI in cattle is far inferior compared to humans (Van Steirteghem *et al.,* 1993a) and mice (Kimura and Yanagimachi, 1995). This is because bovine sperm nucleus is structurally stable due to its richness in protamine disulfide bonds, and this stability is a drag on sperm head decondensation and male pronuclear formation (Rho *et al.,* 1998a).

Oocyte activation is the restoration of metabolic activity in the quiescent oocyte or the process of releasing the oocyte from the second meiotic arrest when the sperm fertilises it (Yanagimachi, 1994). The very early cellular event observed in all activated mammalian oocytes is an intracellular rise in Ca^{2+} concentration. The site of Ca^{2+} release and sequestration is thought to be the endoplasmic reticulum, where inisitol 1,4,5-triphosphate (IP_3) reseptors are present (Kline and Kline, 1992). The first Ca^{2+} transient is followed by a series of shorter Ca^{2+} transients of high amplitude. As fertilisation progresses, the amplitude and frequency of the Ca^{2+} transients decrease, while duration increases until and absolute cessation of Ca^{2+} oscillations

during entry into interphase and PN formation, several hours after sperm entry (Jones *at al*., 1995).

Unlike in human and mouse ICSI, the operation of sperm injection and the presence of a sperm in the ooplasm can not provide enough stimulation to provoke oocyte activation in cattle (Rho *et al.,* 1998b). To solve the problems, attempts have been made to induce sperm decondensation before ICSI and to activate oocyte after ICSI. Compared to human and mouse sperm, bovine ooplasm is opaque and the bull sperm head is large. This leads to extreme difficulty in proper delivery of a sperm into an ooplasm and an increase in the volume of vehicle solution injected into oocytes. As a result, a delivered sperm might not be in the ooplasm, or if it is there, the sperm might be surrounded by enough vehicle solution to prevent it from incorporating with the ooplasm. In that case, no good result can be expected (Wei and Fukui, 2000).
Technical improvement by clarifying the oocyte cytoplasm by polarise the ooplasmic lipid and provide fine visibility for the ICSI operation by centrifugation at 6,000x g for 7 minutes (Wei and Fukui, 2000), 5 minutes Rho *et al*., 1998a; Chung *et al*., 1999). This technique also helpful in minimising the volume of PVP solution injected into oocytes. Cutting the sperm tail and reducing the PVP concentration can significantly benefit in ICSI cattle (Wei and Fukui, 2000). Table 2.2 shows ICSI in cattle.

There are several reports on the birth of healthy offspring in the human and mice after the transfer of embryos produced by ICSI without any additional oocyte activation treatment (Teserik and Mendoza, 2003; Yanagimachi, 1998). However, in cattle, there are a few reports on the birth of calves following ICSI (Goto *et al.,* 1990; Hamano *et al*., 1999; Horiuchi *et al*., 2002; Wei and Fukui, 2002; Galli *et al*., 2003b).

Table 2.2: ICSI in cattle

Author	Result
Wasthusin *et al.,* 1984	Cleavage (4%)
Kameyama *et al,* 1985	Fertilisation (13%)
Keefer *et al*. 1990	Cleavage (38%)
Goto *et al*. 1990	Cleavage (12%), offspring
Heuwieser *et al.* 1992	Fertilisation (39%)
Pavasuthipaisit *et al*. 1994	Cleavage (67%), blastocyst (9%)
Rho *et al*. 1998a	Cleavage (61%), blastocyst (24%), pregnancy (38%)
Horiuchi *et al*. 1999	Cleavage (72%), blastocyst (28%)
Chung *et al*., 1999	Cleavage (62.1%), blastocyst (3.0%)
Katayose *et al.*, 1999	PN (85%), cleavage (72%)
Wei and Fukui, 2002	PN (86.3%), cleavage (71.8%), blastocyst (22.7%), ET (live born)
Horiuchi *et al.*, 2002	Cleavage (72%), blastocyst (20%), offspring (50%)
Keskintepe *et al.*, 2002	PN (74%), cleavage (63.3%), blastocyst (29.6%)
Galli *et al.*, 2003b	PN (88.6%), cleavage (59.7%), blastocyst (6.1%)
	PN (88.9%), cleavage (58.5), blastocyst (7.3%), offspring (9.1%)
Li *et al*., 2004	Cleavage (83%), blastocyst (25%),
Rho *et al.*, 2004	PN (54.8), cleavage (70%), blastocyst (16.3%)
Fujinami *et al.*, 2004	Cleavage (51%), blastocyst (14%)
Oikawa *et al*., 2005	Cleavage (75.6%), blastocyst (39.4%), offspring (47.4%)
Kato and Nagao, 2009	Cleavage (68.7%), blastocyst (20.9%)

In these species, fertilisation and cleavage after sperm injection is very low and *in vitro* development is very limited.

Oocyte activation is characterised by a dramatic rise in intracellular calcium concentration, which in mammals take the form of calcium oscillations (Stricker, 1999; Hafez *et al.*, 2004) driven by an elevation in inositol triphosphate concentrations (Rice *et al*., 2000). The causative agent of these oscillations is proposed to be a recently described phosphoinositide-specific phospholipase C, PLC-X, which is a soluble sperm factor delivered to the oocyte following membrane fusion (Saunders *et al.*, 2007).

Mechanical process of injection can provide an activating stimulus in some species, less than 5% of sham-injected bovine oocytes have been activated (Keefer *et al.*, 1990*;* Rho *et al.*, 1998a,b). Although *in vitro* matured bovine oocytes can be activated by sperm following *in vitro* fertilisation, resulting in production of developmentally competent embryos, these unaged oocytes have proven difficult to activate parthenogenetically (Susko-Parrish *et al*., 1994). When bovine oocytes are exposed to a single chemical (e.g., calcium ionophore) or electrical stimulus, which induce a transient rise in intracellular calcium, the levels of histone kinase briefly decrease but subsequently return to pre-stimulus concentrations (Collas *et al.*, 1993). In fertilised oocytes, however, multiple calcium oscillations are observed, and histone kinase levels remain low until the subsequent cell cycle commences (Liu and Yang, 1999; Sun *et al*., 1994). Treatment of oocytes with multiple calcium stimuli or a single stimulus followed by DMAP, an inhibitor of phosphorylation, or cycloheximide, an inhibitor of protein synthesis, can prevent the rise in histone

kinase and permit pronuclear formation (Susko-Parrish *et al.*, 1994; Soloy *et al*., 1997).

Studies with ethanol (Nagai, 1987) or ionornycin (Ware *et al.*, 1989) indicate that the activation rates are dependent upon the age of oocytes. Nagai (1987) reported that bovine oocyte activation with ethanol approaches peak efficiency around 27 h of maturation *in vitro.* The time course for responsiveness to ethanol activation is similar to the time course reported by Ware *et al*. (1989) for both ionomycin and electrical pulse. However, although activation rates are higher in aged oocytes than in young oocytes, it is generally accepted that aged oocytes are beyond their normal fertilisable life span at the moment of artificial stimulation. Therefore, it is reasonable to expect that such activated oocytes will be less viable.

Although it is understandable that a sham-injected oocyte would require further treatment (e.g., exposure to calcium ionophore and DMAP) in order to be fully activated, it is unclear why the injected bovine sperm cell, itself, cannot stimulate the activation process as it would do during normal fertilisation. Bovine sperms have been shown to be more stably packaged than human, mouse, chinchilla and hamster sperm cells (Perreault *et al*., 1988). Table 2.3 present the result of oocyte activation in ICSI.

Higher blastocyst rates were obtained by a treatment using a combination of ionomycin and DMAP after ICSI compared with other activation treatments, however, no calves were produced after using this activation treatment (Rho *et al*., 1998b). It is important to examine both *in vitro* developments to blastocyst as well as calf production following their transfer, because oocyte activation treatments induce parthenogenetic development (Lie *et al*., 1999) and cause abnormal ploidy of embryos

Table 2.3: Bovine oocytes activation in ICSI

Author	Activation	Result
Katayose *et al.*, 1999	Piezo	PN (85%), cleavage (72%)
Chung *et al*., 1999	Ionomycin Ionomycin + DMAP	Cleavage (27.3%) Cleavage (62.1%), blastocyst (3.0%)
Wei and Fukui, 2002	Piezo	PN (86.3%), cleavage (71.8%), blastocyst (22.7%), ET (live born)
Horiuchi *et al.*, 2002	Ethanol	Cleavage (72%), blastocyst (20%), ET (live born, 5/10, 50%)
Keskintepe *et al.*, 2002	Ionomycin + DMAP	PN (74%), cleavage (63.3%), blastocyst (29.6%)
Galli *et al.*, 2003b	Ionomycin + cyclohexamide No	PN (88.6%), cleavage (59.7%), blastocyst (6.1%) PN (88.9%), cleavage (58.5), blastocyst (7.3%), ET (live born, 1/11, 9.1%)
Li *et al*., 2004	Ionomycin Ionomycin + DMAP	Cleavage (50.7%), blastocyst (5.8%) Cleavage (83%), blastocyst (25%),
Rho *et al.*, 2004	Ionomycin + cycloheximide	PN (54.8), cleavage (70%), blastocyst (16.3%)
Fujinami *et al.*, 2004	Piezo+ ethanol	Cleavage (51%), blastocyst (14%)
Oikawa *et al*., 2005	Ionomycin + DMAP Ethanol	Cleavage (83.9%), blastocyst (40.1%), ET (live born, 1/11, 9.1%) Cleavage (75.6%), blastocyst (39.4%), ET (live born, 9/19, 47.4%)
Kato and Nagao, 2009	PVP	Cleavage (68.7%), blastocyst (20.9%)

(Rho *et al*., 1998a; Ock *et al*., 2003). Table 2.3 present the result of activation in ICSI bovine oocytes.

2.2.4.2.b *Sperm activation treatment*

As ICSI bypasses the normal process of zona penetration and oocyte-sperm fusion, the initial steps involved in destabilising the sperm nucleus may also be bypassed. As recent studies indicate that factors bound to the sperm nuclear matrix are involved in oocyte activation (Perry *et al*., 1999), the pretreatment of bovine sperm may be critical to whether or not activation occurs following sperm injection.

Even though first ICSI calf was born in 1990 (Goto *et al.*, 1990) by using dead sperm, the cleavage rate from ICSI was low, and early embryonic development *in vitro* was limited. With human ICSI, high fertilisation and implantation rates were achieved by use of immobilisation of motile sperm (Dozortsev *et al.,* 1995a,b; Gerris *et al*., 1995; Palermo *et al.,* 1996).

Killed sperm by repeated freeze-thawing were far inferior to immobilised sperm in their ability to participate in embryo development. This is because, immobilised sperm were injected into oocytes almost immediately after the plasma membrane was disrupted, while 'killed' sperm were injected some time (as long 1 hour) after their plasm membrane disruption. Immediate injection of membrane-disrupted sperm is recommended for ICSI in any species (Yanagimachi, 2001).

Immobilisation by scoring sperm tails increases the incidence of successful fertilisation by ICSI (Van de Bergh *et al.,* 1995; Fishel *et al.,* 1995). The success of sperm immobilisation is believed to be due to the local distruption of the plasm

membrane. As the sperm membrane has no wound-healing ability, the entire plasma membrane must disintegrate faster within the ooplasm when it is locally damaged prior to ICSI than when a membrane-intact sperm is injected (Yanagimachi, 1998). The sperm nucleus is a vulnerable organelle. Once the plasma membrane of the sperm is disrupted even locally, the nucleus is exposed to the medium components that are very different from the intracellular environment (Horiuchi *et al*., 2002). Horiuchi *et al.* (1999b) also found that the use of immobilised sperm improved cleavage rates and early embryo development *in vitro* with bovine ICSI compared to killed sperm.

Polyvinylpyrrolidone (PVP) has been used successfully in ICSI to increase viscosity of sperm solution, thus facilitating the handling and immobilisation of sperm. For ICSI, sperm are first suspended in a medium containing PVP and a single sperm is chosen and injected into the oocyte, together with a small amount of medium (Hlinka *et al*., 1998).

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

Cells that routinely are in contact with PVP had increased mucoid secretion as a result of the interaction between this agent. PVP solution affected the acrosomal status of sperm and enhanced pronuclear formation in ICSI. The present of PVP solution in embryo arising from ICSI may effect fetal development in both human and farm animals (Kato and Nagao, 2009).

Studies have indicated that sperm treatment prior to injection can affect the rate of oocyte activation and male pronuclear formation in the rabbit (Keefer, 1989). In the bovine, treatment of sperm with dithiothreitol (DTT) to destabilise disulphide bonds prior to injection, combined with artificial oocyte activation after injection, resulted in improved rates of pronuclear formation and blastocyst development (Rho *et al.*, 1998b). While DTT treatment facilitated sperm processing in the activated oocyte, injection of DTT-treated sperm alone was not sufficient to stimulate oocyte activation. Activation was achieved where injected oocytes were initially activated with 5 pM calcium ionophore, followed by a 3 hours culture period to allow extrusion of the second polar body and incubation in DMAP for 3 hours to complete the activation process (Chung, 1999). Table 2.4 shows result of sperm used during ICSI.

Table 2.4: Result of sperm used during ICSI

Author	Sperm treatment	Result
Horiuchi *et al.*, 2002	dead	PN (65.9%), cleavage (27.5%), blastocyst (0.8%)
	immobilised	PN (70.3%), cleavage (72.2%), blastocyst (20.3%)
Westhusin *et al.*, 1984	dead	PN (25%), cleavage (4%)
Kameyama *et al.*, 1985	dead (freeze, dried)	PN (13%)
Keefer *et al.*, 1990	dead	PN (76 %), cleavage (38%)
Goto *et al.*, 1990	dead	Cleavage (12 %), blastocyst (2 %), ET (live born)
Heuwieser *et al.*, 1992	dead	PN (39 %)
Pavasuthipaisit *et al.*, 1994	live	PN (67 %), cleavage (67 %), blastocyst (9 %)
Iwasaki and Li, 1994	dead	Cleavage (36%), blastocyst (4%)
Catt *et al.*, 1995	live	PN (25%)
Horiuchi *et al.*, 1998	live	PN (78 %), cleavage (74 %), blastocyst (29 %)
Rho *et al.*, 1998a	live	PN (60%), cleavage (61 %), blastocyst (24 %), pregnant (38%)
Horiuchi *et al.*, 1999	live	Cleavage (72%), blastocyst (28%)

Chapter 3

3.0 MATERIALS AND METHODS

3.1 GENERAL INTRODUCTION

The aim of this study was to produce viable *in vitro* bovine embryos derived from *in vitro* fertilisation (IVF), intracytoplasmic sperm injection (ICSI) and embryo cryopreserved methods. The experiments were carried out in Animal Biotechnology-Embryo Laboratory (ABEL), Institute of Biological Sciences (ISB), Faculty of Science, University of Malaya; Nuclear Transfer and Reprogramming Laboratory (NaTuRe) of Institute of Research Management and Consultancy (IPPP), University of Malaya for IVF and ICSI experiment and National Animal Biotechnology Institute (IBHK), Jerantut, Pahang Darul Makmur for embryo transfer (ET) experiment.

Cattle ovaries and frozen semen were obtained from the Department of Veterinary Services (DVS), Ministry of Agriculture and Agro-based Industries, Malaysia. The ovaries were collected from Shah Alam Abattoir of Selangor Darul Ehsan and Senawang Abattoir of Negeri Sembilan Darul Khusus. Frozen semen was obtained from the National Animal Biotechnology Institute (IBHK), Jerantut, Pahang Darul Makmur.

3.2 FACILITIES

The facilities used in this study were EMiL, ABEL and NaTuRe laboratories for oocyte collection, *in vitro* maturation (IVM), *in vitro* fertilisation (IVF),

intracytoplasmic sperm injection (ICSI) and embryo cryopreservation experiment such as embryo vitrification and direct plunging. Shah Alam Abbatoir of Selangor DVS Headquaters and Senawang Abbatoir of Negeri Sembilan DVS for ovary and blood collection. IBHK of DVS at Jerantut, Pahang for embryo transfer experiment and collection of frozen semen supply.

3.3 EXPERIMENTAL ANIMALS

Ovaries of slaughtered cattle of unknown breed, age, health status and origin were used as oocyte source. After slaughtering, while the cattle are still hanging the pelt were removed and the abdominal part (near the mammary area) was incised with very sharp knife and reproductive tract was identified. The left and right ovaries were cut with sharp scissors and stored in the thermo flask containing warm (35-38°C) PBS or normal saline. Only ovaries with good follicles appearance (approximately with 2 mm diameter of each follicle size) were selected and brought to the laboratory (25 km from the laboratory). The frozen semen from IBHK was from Mafriwal and Nellore breeds.

3.4 MATERIALS

All equipment and instruments are listed in Appendix Table 1.1

3.4.1 Equipment and Instruments

The major equipment used in this study were laminar flow chamber, incubator with CO_2 (5%), O_2 (5%), N_2 (90%), inverted microscope with micromanipulator, fluorescent microscope, micropipette puller, micropipette grinder, microforge and Milli-Q water purifier. Full list of equipment and instruments are listed in Appendix Tables 1.1 and 1.2, respectively.

3.4.2 Glassware, Labware and Disposables

All glassware, labware and disposables used in this study are listed in Appendix Tables 1.2

3.4.3 Chemicals

All chemicals, reagents and media were purchased from Sigma, otherwise stated. The chemicals are listed in Appendix Tables 1.3.

3.5 METHODOLOGY

Main experimental methodology was divided into 4 main methods that were *in vitro* maturation, *in vitro* fertilisation, *in vitro* culture and intracytoplasmic sperm injection methods. Sterilisation method as a crucial and routine practice in all embryo laboratories will be described in subsequent segment. The manufacturers for various

chemicals, glassware, disposables, instruments, equipment are not included in the text. However, they are listed in the appropriate appendices.

3.5.1 Sterilisation

Sterilisation is a very imperative step in embryo laboratory to guarantee no contamination occurred and to optimise the outcome of all experiments procedures. Sterilisation steps involve all equipment, instruments, glassware, labware and disposables that were used in this study. Laboratory area and personal hygienic practice is also important before handling experiment in order to avoid contamination to the samples.

3.5.1.1 Sterilisation, maintenance and servicing of equipment and instrument

Floor in IVP laboratory was cleaned and mopped with odourless and clinical grade detergent every month or less depends on usage capacity. All the equipment and instruments were well maintained and serviced. The surface and working bench were cleaned and dust free. Working bench especially in laminar flow chamber was wiped with 70% alcohol. Equipment and instrument were maintained and serviced such as the laminar flow chamber was with well air ventilation and flow and the air filter was changed and serviced every year by-product supplier depending on usage capacity. Milli-Q water purifier' filter was replaced every 3 months or less to ensure best water quality and serviced by-product provider every 6 months. The solution and buffer for pH meter was replaced with fresh solution every 3 months. The most important

equipment to be well maintained and serviced was CO_2 incubator. Before cleaning, the incubator was switched off. The pooled water was cleaned and the water was replaced with Milli-Q water every month. The surface of incubator plate was cleaned with mildly clinical grade detergent (7X) and rinsed thoroughly three times with sterile Milli-Q water. Then, it was wiped with sterile cotton towel until dry and finally the surface was wiped with 70% alcohol and let the alcohol to dry and evaporate out by opening the incubator's door. For better results, for every three months, the incubator was heat sterilised to kill all germs and other contaminants.

3.5.1.2 Sterilisation of glassware, labware and disposables

Glassware such as glass stock bottle, beaker, conical flask, measuring cylinder, volumetric flask and autoclaveable plastic conical tube used in this study were washed thoroughly with non-toxic detergent (7X). By using sponge and nylon brush, the detergent was diluted and the glassware was cleaned thoroughly to remove dirts and impurities. The glass was rinsed thoroughly with tap water until all detergent removed. For better results, it was immersed in 10% hydrochloric acid (HCl) overnight before being soaked for a while and rinsed with reversed osmosis (RO) or distilled water until all acid was fully removed. The stock bottle was loosely capped and covered with a layer of aluminium foil whereas the other glassware with no cap, the opening was covered securely with a layer of aluminium foil. An autoclave tape was stuck on the aluminium foil surface with user initial and date wrote on it. An autoclaveable conical tube was loosely capped and placed in autoclaveable plastic bag before being stuck with autoclave tape. The same method applied to disposable

micropipette tip, microcentrifuge tube, towel and gauze, which were placed in autoclaveable plastic bag, heat sealed, labeled and placed in autoclave machine. The autoclave machine was operated for 20 minutes sterilisation at 121℃. Then, the autoclaved materials were placed in hot dry oven at 60°C for a few days until properly dry.

For unautoclaveable material such as Petri dish, plastic pipette, French straw and plastic conical tube; the materials were placed in plastic bag, heat sealed and were sterilised with ultraviolet light in laminar flow chamber for 15 minutes. A quarter cut of razor blade also can be sterilised with UV light or immersed in 70% alcohol for a few minutes and were let to dry before being used for ovary slicing.

3.5.1.3 Personal hygienic practice in the laboratory

Personnel who involved in embryo work should practice a very hygienic method before handling all embryo material to avoid cross contamination. Personnel should wear a clean lab coat and face mask. The hand was washed with soap properly and rinsed with tap water and then with Dettol or Lysol to kill all germs. The hand was wiped with clean tissue paper and wear gloves. Before handling experiment, personnel should spray the gloves (for non-sterile latex gloves) with 70% alcohol and were let to dry.

3.5.2 Preparation of Stocks and Media

The stocks and media used in this study encompass all experiment media such as for ovary collection, IVM, IVF, ICSI and embryo staining.

3.5.2.1 Buffer

Ovary collecting medium used was phosphate buffered saline (PBS) or normal saline supplemented with penicillin and streptomycin antibiotics. Ten tablets of PBS tablet were added into Milli-Q water (1 L) in Schott stock bottle (1 L). The tablets were let to dissolve in Milli-Q water and added with penicillin (100 IU/ml) and streptomycin (100 µg/ml). In order to dissolve the tablets faster, the magnetic stirrer was used. The stock solution was ready to use and kept in a refrigerator for maximum three months. Normal saline was prepared by adding 9 g of sodium chloride (NaCl) into 1 L of Milli-Q water before being added with antibiotics and kept in a refrigerator.

3.5.2.2 Collecting medium

This medium was also known as working medium. PBS supplemented with antibiotics (pennicillin and streptomycin) was used for ovary and cumulus-oocyte complexes (COCs) collecting and washing. The pH was adjusted between 7.30 to 7.40. This collecting medium was maintained in a water bath at 38°C before use.

3.5.2.3 Oil

Silicone oil could be used directly and does not required sterilisation, but to make sure there were no toxin in the oil, silicone oil must be washed (1X) to remove possible water-soluble contaminants it could be purified by shaking gently the oil with ultrapure water (Milli-Q) or sterile saline solution (0.9% NaCl) by adding in ratio 3:1 (oil to aqueous solution) in a glass bottle. The bottle was covered with aluminium foil to avoid light and the mixtures were let to separate three days at room temperature. The oil layer was carefully collected by using serological pipette then stored in other sterile tissue culture bottle for maximum 3 months in refrigerator (4°C). Before the oil can be used, it should be equilibrated overnight in CO_2 (5%) incubator with a simple culture medium without protein or lipid-soluble component such as TCM-199, BO or mSOF for maturation, fertilisation or culture purposes, respectively. Washed oil was stored in refrigerator at 4°C for maximum 2 weeks. Oil overlays were equilibrated in CO_2 (5%) incubator for minimum 3 hours (or overnight) before introducing media to oocytes or sperm.

3.5.2.4 Maturation medium

Preparation of medium for *in vitro* maturation (IVM) of oocyte was described earlier by Habsah (1997) with slightly modification. Maturation medium used was Tissue Culture Medium 199 (TCM-199). The medium was supplemented with steer serum or commercial foetal bovine serum (FBS) (10%), sodium pyruvate (20 mM) and gentamycin (1.25 µl/ml) solution, follicle stimulating hormone (FSH), luteinising hormone (LH) and oestradiol (1 µg/ml). Nine microdroplets of 100 µl each were

prepared in Falcon culture dish (35 mm) and covered with equilibrated silicone oil. The medium was equilibrated in 5% CO_2 incubator overnight or fresh prepared for minimum of 3 hours before starting maturation process.

3.5.2.4.a *Oestradiol-17β*

Oestrogen stock was prepared by adding oestradiol (1 mg) into absolute ethanol (1 ml). The solution then was aliquot 15 µl in each tube and was kept in refrigerator at 4°C for maximum 3 months. Final concentration in maturation medium was 1 µg/ml.

3.5.2.4.b *FSH*

FSH (70 IU) for stock 1 was prepared from concentration FSH (7,000 IU), Milli-Q water (10 ml) was added into bottle. The stock was kept in freezer for maximum 3 months.

FSH working solution with concentration of 0.02 IU was prepared by adding FSH stock (10 µl) 1 to Milli-Q water (3.5 ml). The solution was aliquot 15 µl each in small tubes and kept for maximum 3 months at freezer (-20°C). FSH working solution (10 µl) was added into IVM medium for maturation purposes.

3.5.2.4.c *Gentamycin*

Gentamycin stock solution was prepared by dissolving gentamycin sulphate (50 mg) in normal saline (1 ml). The solution was filtered with membrane filter (0.2 µm). The

stock was aliquot in sterile microtubes and kept in refrigerator until used maximum for 3 months.

3.5.2.4.d *Sodium pyruvate*

The sodium pyruvate stock (0.2 mM) was prepared by adding pyruvate acid (0.11 g) with of sodium chloride (0.06 g). Milli-Q water was added up to 10 ml into the tubes. The stock was sterilised with Millipore (0.22 μm) filter and kept in refrigerator (4°C) for 2 days. Fresh stock should be prepared each time of experiment.

3.5.2.4.e *Serum*

Serum used in this experiment was steer serum or foetal bovine serum (FBS) that was purchased from commercial company. Steer serum was prepared by taking blood from freshly slaughtered of castrated male in sterile beakers (500 ml). The blood was then immediately poured into conical tubes (15 ml). The blood was let to clot for 30 to 60 minutes. The blood was centrifuged at 3,000 rpm for 20 minutes at room temperature. Carefully, the serum layer was collected from blood layer and placed in conical flask by using sterile Pasteur pipette. The serum was heat inactivated at 56°C for 30 to 45 minutes in waterbath. The serum was let to cool down at room temperature before being centrifuged again at 3,000 rpm for 20 minutes to remove denatured protein. By using Pasteur pipette, the serum was collected carefully to avoid protein pellet. The serum was then filtered with serum filter (0.2 μm), aliquot 1 ml each in tubes and stored in freezer (-30°C) for maximum 6 months.

3.5.3 Fertilisation Medium

Brackett-Oliphant (BO) solution was used for fertilisation. Heparin (1 µl/ml) was added to the BO working solution and was filtered with Millipore (0.22 µm) filter. Solution for oocyte washing and IVF droplets were added with 10 mg/ml bovine serum albumin (BSA). Nine microdroplets of 100 µl each were prepared in Falcon culture dish (35 mm) and covered with equilibrated silicone oil. All the solutions were equilibrated in CO_2 (5%) incubator overnight or minimum for 3 hours. Otherwise stated, all fertilisation medium was using BO medium. The other medium used for fertilisation medium was mSOF working solution. This solution was used as basic medium and was added with BSA, heparin and theophyline before being used as fertilisation medium.

3.5.3.1 Heparin solution

Heparin stock solution was prepared by adding heparin (50 mg) in sterile BO medium (working solution) (2.5 ml) to obtain 100 µM of heparin solution. The solution was aliquot and kept cooled in a freezer (0 °C) until used for maximum 3 months.

3.5.3.2 Calcium ionophore A23187

A solution containing DMSO and absolute ethanol in 3:1 ratio was prepared and calcium ionophore (1 mg) was added into the solution. 20 µl of the solution was added with Milli-Q water (1 ml). The solution was aliquot and kept cooled in a freezer (-20 °C) until used for maximum 6 months.

3.5.3.3 Hepes

Hepes (20 mM) was prepared by adding hepes solution (2 µl) to ICSI injection solution (mSOF) (98 µl). The solution was aliquot and kept in refrigerator for maximum 3 months.

3.5.3.4 Polivinylpyrrolidone (PVP)

PVP (10%) (40,000 IU) was prepared by dissolving PVP in culture medium (mSOF working solution). The solution was aliquot and kept in refrigerator for maximum 2 weeks.

3.5.3.5 Penicillin/streptomycin

Commercial penicillin and streptomycin solutions can be directly used in media preparation. The solutions were aliquot in sterile microtubes and were kept in refrigerator for maximum of 2 weeks.

3.5.3.6 Hyaluronidase

Hyaluronidase stock (80 IU) was prepared by dissolving hyaluronidase (1 mg) in BO working solution (5.88 ml). The stock was aliquot and stored in refrigerator for maximum 2 weeks.

3.5.4 Culture Medium

Culture medium used in this study was synthetic oviductal fluid medium (mSOF). mSOF working solution was prepared and filtered with Millipore membrane filter (0.22 µm) and kept in refrigerator for maximum 3 months. For culture purposes, the working solution was added with BSA (0.003 g/ml).

3.5.5 Preparation of Pipettes and Micropipettes

Pipettes used in this study were glass Pasteur pipette, COC picking pipette, embryo picking pipette, sperm picking pipette for ICSI and ICSI micropipette such as holding pipette and injection needle.

3.5.5.1 Pasteur pipette and glass capillary cleaning and sterilisation

Pasteur pipettes used in this study mainly used during sperm washing prior IVF and ICSI. This pipette also used for cumulus-oocyte complexes (COC) picking during IVM whereas capillaries used for picking embryos and denuded oocytes were haematocrit-capillaries with 75 mm/75 µl, diameter 1.5-1.6 mm. Capillaries used for holding and injection pipettes preparation were borosilicate capillaries with an inner and an outer diameter of 0.69 and 0.97 mm, respectively and length of 10 cm.

All pipettes and capillaries (for haematocrit capillary, the blue mark at one-end was removed by wiping the mark with absolute alcohol) were washed thoroughly with non-toxic detergent (7X) before being rinsed with tap water until no excess detergent left before being washed again 5 times with RO or distilled water. Then the pipettes

were immersed in 10% hydrochloric acid overnight and rinsed 20 times with RO or distilled water to remove the acid perfectly from pipette's inner side. The pipettes were packed individually in autoclaveable biohazard plastic bag and were autoclaved (121°C for 15 minutes) before being dried in oven at 60°C for a few days until dry.

3.5.5.2 Pasteur pipette

Sterile Pasteur pipette (1 ml) mainly used during sperm washing and preparation for IVF and ICSI. The pipette also was used during media preparation. It was drawn with alcohol/spirit flame. When the pipette softens and melt, the both end was pulled to produce a very thin lumen. The cutting point was cut with diamond stone and fire polished to smooth rough edges. The end tip was ready for sperm transferring to ICSI dish.

3.5.5.3 Picking Pasteur pipette

Sterile Pasteur pipette was used to prepare COC picking pipette. This pipette also could be used during denudation process by aspirate in and out the COC. The pipette open end was held by clean and sterile hand and the center part of the pipette was melted by using Bunsen burner. The pipette was rotated to make sure that all round surfaces were evenly heated. When the heated part softens and melt, the pipette was pulled quickly to produce a very fine lumen. The open-end was cut by rubbing the cut point with other pipette lumen (for unsterile pipette breaking, the cut point was cut by using diamond stone cutter) and carefully break at the friction point. The final inner

diameter was about 200 µm. The open size should be fit enough to make sure that during suctioning, the cumulus cells were not accidentally removed. The pipette open-end was fire-polished by introducing it to a very minimal heat by using spirit or alcohol flame until perfectly blunt. The picking pipettes were ready to use.

In order to store the prepared pipette, the pipette fine-end part was placed and locked inside a blue pipette tip (1000 µl) to avoid storing. This simple low cost invention could avoid the fragile end-tip from breaking and contamination during working. The pipettes were packed individually in autoclaveable plastic bag and reautoclaved; then were let to dry in oven (60°C). In order to use the pipette, La Banane, a silicone gas tubing with outer diameter 1 cm was used to control the aspirating and expelling of the COC. The La Banane was prepared by cutting tubing for about 3 inches long. One of the open-end was completely seal with silicone glue. About 1 inch from the seal-side, the tubing was half-tied with cotton thread to facilitate sucking control. The open-end was attached with pulled capillary and ready to use.

3.5.5.4 Oocyte and embryo picking pipette

Haematocrit capillaries were used to prepare pipette for picking denuded oocyte and embryo mainly during culture medium changing and embryo cryopreservation. The haematocrit pipette also could be used for COC picking. The capillaries were heated on a spirit burner and pulled immediately after it softened. The long and thin part of the pulled lumen were cut with diamond cutter (heart-shaped) with diameter of 200 µm and 100 µm each for COC and oocyte/embryo, respectively. The ends of the tips

were fire polished by heating it to the flame for a second. The pipettes were placed in conical tube (15 ml) containing a piece of sterile gauze at the bottom of the tube. The tube was loosely capped; wrapped with aluminium foil; sterilised by using autoclave (121°C, 20 minutes) and dried in oven (60°C).

To hold the pulled-haematocrit pipette, the La Banane was used for picking COC. The open-end was attached with plastic holder (of mouth-piece pipette control) and ready to use.

3.5.6 Preparation of Micropipette

To prepare micropipette for ICSI, three main equipments used were micropuller, microforge and microgrinder. Micropuller used in ABEL laboratory was horizontal programmable pipette puller. To prepare holding and injection pipette, sterile borosilicate glass capillary tubing with inner and outer diameter of 0.69 and 0.97 mm, respectively, and 10 cm length were used.

3.5.6.1 Holding pipette

To prepare holding pipette, the capillary was drawn horizontally with a programmable pipette puller to make a pair of a long fine lumen. Different parameters were used if different types of glass were used. The parameters such as temperature, velocity, time and pulling force influenced the shape, diameter and thickness of the needle. By using a microforge, the pulled needle was fixed to the needle holder. The cutting-point outer diameter was identified by using a micro-scale on the microscope eyepiece. The

needle was placed on the platinum filament that contained a glass-bead; then the heating process was started and the filament with hot glass-bead was brought up to the needle cutting point slowly and carefully touched the needle. The heating was stopped suddenly, the filament cool down and back to normal position. The glass was snapped, produced a straight cut and the excess tip was moved from the glass-bead. The needle opening was faced-up to the glass-bead. The sharp edge was polished by moving the hot glass-bead up to the needle without touching the surface. The process was let to continue until the inner and outer diameters were about 15 µm and 180-200 µm, respectively. At about 1 mm from the opening, the pipette was bent to 35° angle. This technique was done by placing the bent point horizontally to the glass-bead. The hot glass-bead was moved up to the pipette bent-point without touching the pipette surface. The pipette hot point surface was let to melt and moved down until the correct angle achieved. The holding pipette was stored in a close container. The individual pipette was stored in a reused commercial pipette container before being sterilised again by ultra violet light for 15 minutes.

3.5.6.2 Injection pipette

The injection pipette was prepared by cutting the tip of pulled capillary pipette by touching a desire position of the capillary with the hot glass-bead on a heated platinum filament of the microforge. The inner and outer diameters of injection pipette were 8-9 and 10-12 µm, respectively. The end of the pipette was ground with microgrinder at an angle of 45°. The pipette was then washed three times with 10% hydrofluoric acid by using a 20 ml syringe attached to the pipette holder, before being

rinsed 10 times with Milli-Q water. The water in the pipette was blown out by pressing the syringe containing air vigorously. By using microforge, the opening of the needle was turned to the front to make a spike. It was made by touching the end tip to the hot glass-bead surface and pulled back the needle. Finally, the needle was bent to an angle of 35°. The pipette was stored in a closed container before being sterilised again by ultra violet light for 15 minutes.

3.5.7 Preparation of Razor Blade for Ovary Slicing

Stainless steel razor blade that was used to slice ovary was cut to four sections by folding the blade two times. The paper that cover the blade was removed and 4 pieces of a quarter size blades were packed in plastic bag each and the bag was heat sealed. The blades were then sterilised by UV light for 15-30 minutes before placed in a clean container until used. During ovary slicing process, the blade also was immersed in a alcohol and wiped dry with sterile gauze.

3.5.8 *In Vitro* Maturation (IVM) Procedure

The procedures involved in IVM include ovary collection and slicing; oocyte collection and grading and culture of IVM oocytes.

3.5.8.1 Collection of ovaries

The ovaries were collected using collecting medium in a thermo flask containing Dulbecco's Phosphate Buffered Saline (DPBS) or in normal saline (0.9% NaCl) supplemented with antibiotics. The temperature of collecting medium was maintained between 35 to 39°C. The ovaries were removed from reproductive tract by cutting with sterile scissors. The ovaries were washed with warmed (30-35°C) RO or distilled water and then were placed in the thermo flask containing warmed (30-35°C) PBS or normal saline supplemented with penicillin and streptomycin. The ovaries were then transported to the laboratory within 1 hour after slaughter.

3.5.8.2 Oocyte recovery and maturation

In the laboratory, the ovaries were washed 3 times with warmed PBS (the PBS stock were kept warm in water bath at 38°C during recovery) supplemented with antibiotics. Two sterile beakers (250 and 100 ml), and Petri dish (60 mm) were filled with warm (30-35°C) PBS and were placed on a heated stage that was set at 39°C. The ovaries were then placed in the beaker (250 ml). The ovary was held with forceps and the excess part of ovary was cut with sterile scissors. Sterile haemostat was used to hold sterile razor blade. Checkerboard incision was made to the entire surface of the ovary by using the razor blade inside the Petri dish. The sliced ovary was rinsing in the beaker (100 ml). All the process was repeated until all of the ovaries were sliced.

PBS containing cumulus-oocyte complexes (COC) and debris in the Petri dish was poured into the ovary washing beaker. The Petri dish was rinsed with fresh PBS

to make sure that no COC were left in the Petri dish. The debris containing COC in the beaker was let to settle down for 3-5 minutes. The upper part of the PBS was aspirated by using sterile Pasteur pipette until only solution containing debris with COC left. 20 ml of fresh PBS was added to the beaker.

A quarter of PBS containing debris and COC was poured into sterile Petri dish (35 mm). By using sterile micropipette for picking COC, the COC were searched and collected under stereo microscope equipped with heated stage. The COC were placed in the 4-well dish that contained two wells of PBS supplemented with sterile steer serum (10%) and two wells of maturation medium. All the steps for collecting COC were repeated until all of the COC were collected from the collecting beaker. The COC were washed two times in PBS and two times in maturation medium (TCM 199). After washing, the COC were placed in the maturation (TCM-199) droplets that were equilibrated overnight. Then, the COC were let to mature in humidified CO_2 incubator (5%) at 39°C for 24 hours (Figure 3.1 and 3.2)

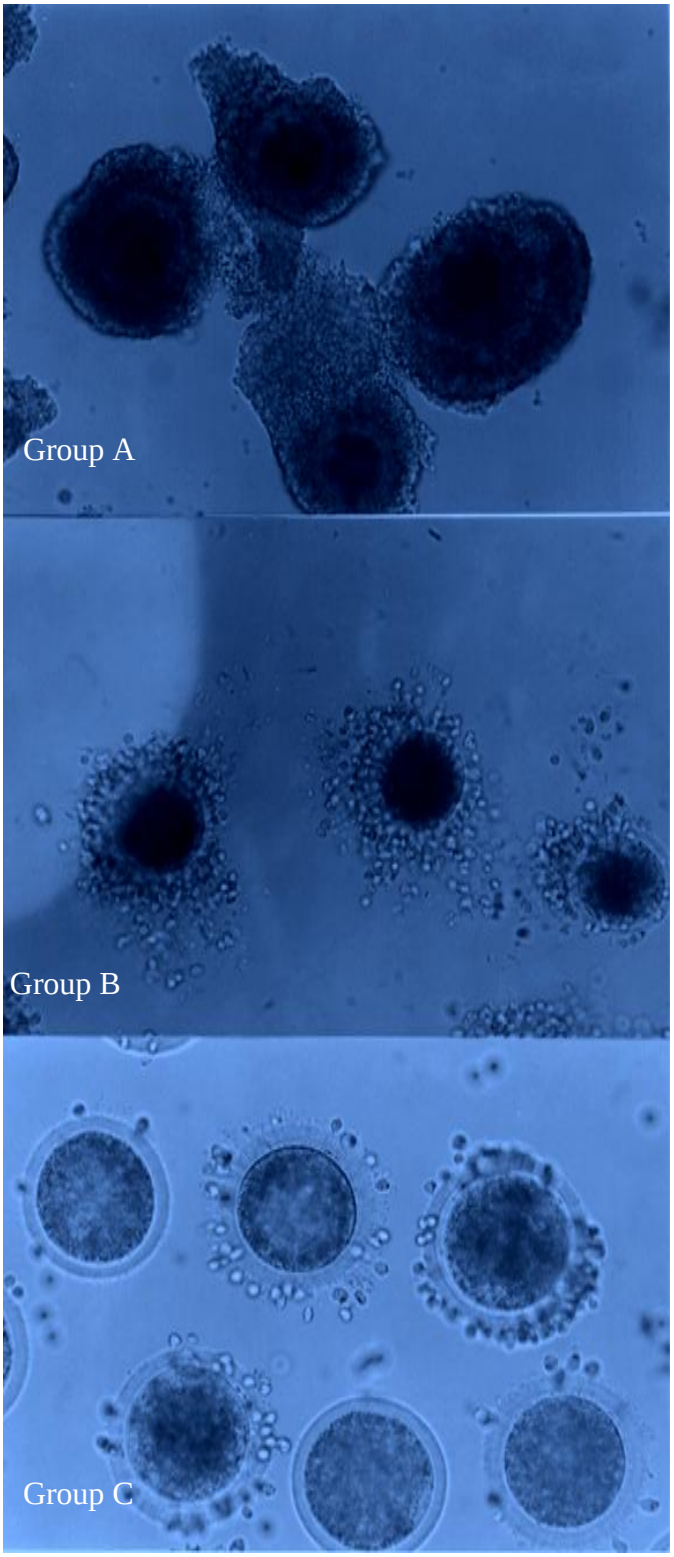

Figure 3.1: Categories of oocytes used for IVMFC. Group A: oocyte with compact and dense cumulus cells with more than three layers; Group B: oocytes with less than 3 layers and some partially naked oocytes; Group C: Naked oocytes.

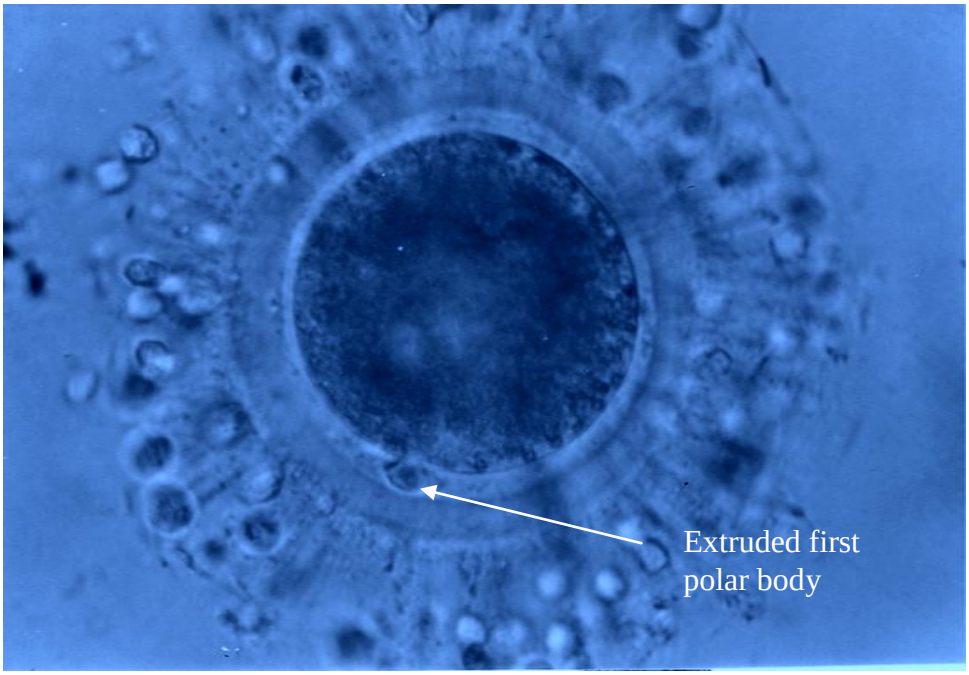

Figure 3.2: Matured oocytes after 24 hours of IVM.

3.5.9 *In Vitro* Fertilisation (IVF) Procedure

For *in vitro* fertilisation, Brackett-Oliphant (BO) medium was prepared earlier. Sperm washing solution, oocyte washing solution and insemination droplets were prepared a day before and equilibrated overnight prior used in CO_2 incubator.

3.5.9.1 Sperm preparation and counting

Two types of sperm preparations were used that were sperm washing and swim-up methods. For both methods, two to five straws (0.25 ml) of frozen Mafriwal semen were thawed in air for 45 seconds, respectively. The straws were then placed in water bath (38°C) for 30 seconds. Then, the straw surfaces were wiped with alcohol (70%). The scissors was wiped with alcohol (70%) and used to cut at the end of the straws. A sperm droplet of each straw was placed on a glass slide. Sperm viability and motility was identified by using inverted or upright objective microscope. Sperm staining using Hoechst stain was also done to identify sperm acrosome-intact image. Only straw with high motility sperm (more than 80%) was used. Every time of new stock and batch of frozen semen arrived to the laboratory, other than using upright microscope to evaluate sperm motility and viability, computerised sperm analyser by IVOS was also used to give a complete and accurate result of sperm parameter such as quality and quantity.

For swim-up method, the semen was sucked with sterile Pasteur pipette and placed at the bottom of the round-bottom tube containing Brackett-Oliphant (BO) medium (2 ml). The tube was slanted (45°) and the sperm was let to swim-up for 1

hour in CO_2 incubator (5%) at 38.5°C. After 1 hour, 1 ml from the upper part of the medium was aspirated from the tubes slowly by using sterile Pasteur pipette and then placed in new sterile conical centrifuge tube. Fresh and warm BO medium (4 ml) was added to the sperm solution before being centrifuged (1000 rpm) for 5 minutes at room temperature (25°C). The supernatant was removed by using Pasteur pipette and the sperm pellet was added with new warm and fresh BO medium before being centrifuged again at 1000 rpm for 5 minutes at room temperature (25°C). The supernatant was removed again and the sperm pallet was added with fresh BO medium (1 ml).

For washing method, the semen was added to fresh BO medium (5 ml) in a conical tube before being centrifuged two times at 1000 rpm for 5 minutes at room temperature. For both methods, sperm concentration was measured by using haemacytometer and sperm concentration was adjusted by adding BO medium until final sperm concentration in IVF droplet was 1 million sperm per ml BO medium.

3.5.9.2 Insemination and culture

Insemination droplets were equilibrated in CO_2 incubator (5%) at 38.5°C overnight. Prior insemination, matured oocytes were washed four times with fertilisation medium before being placed in the fertilisation medium droplets. This method was done to remove excess maturation medium and to loosen the COC. The sperm was introduced to the oocytes by inserted the sperm to the insemination droplets by using micropipettor. The oocytes-sperm mixture was incubated for 18 to 20 hours in 5% CO_2 incubator at 39°C (Figure 3.3). Then, the fertilised oocytes (Figure 3.4) were

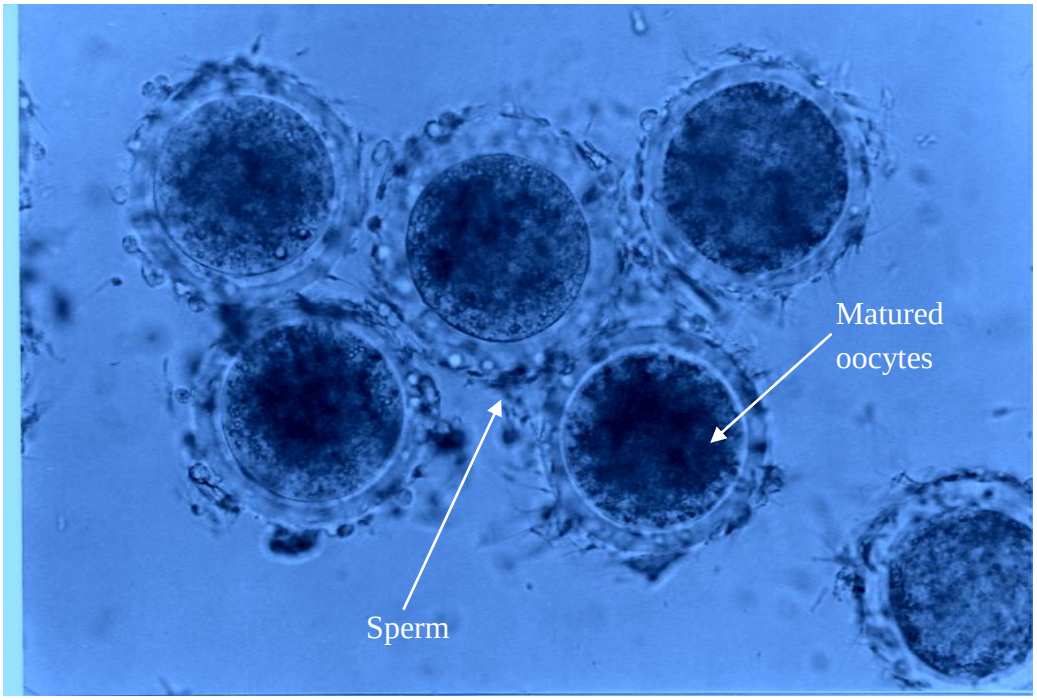

Figure 3.3: Sperm oocyte incubation of Grade C oocytes.

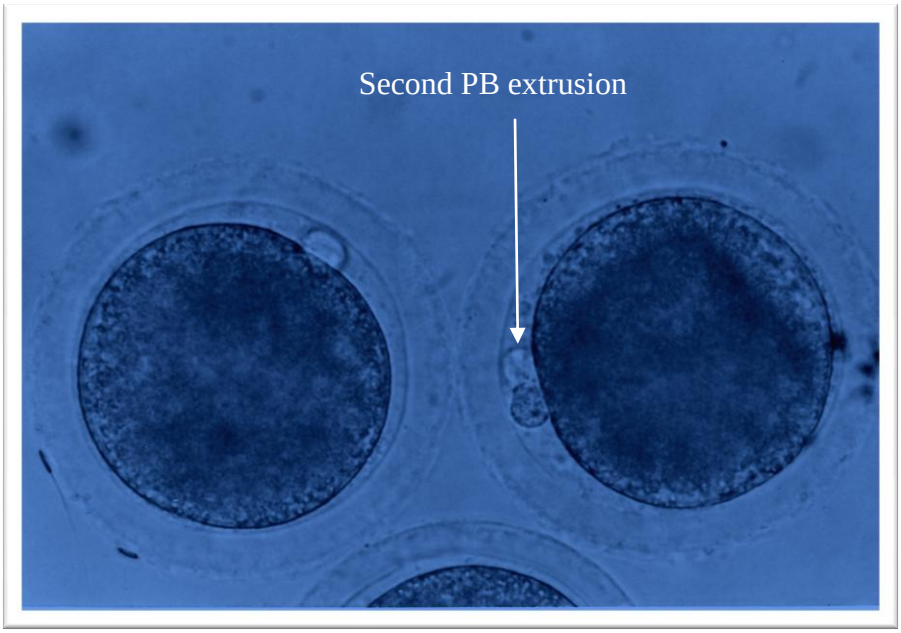

Figure 3.4: Fertilised oocytes after 20 hours of IVF.

washed four times with culture medium to remove excess sperm that attached to the zona pellucida. The oocytes were transferred to culture droplets covered with silicone oil that were prepared and equilibrated earlier. Maximum of 10 oocytes were placed in each droplet (100 µl). After insemination, the zygotes were transferred to culture medium. The numbers of PN and second PB extrusion were accessed. Then, numbers of cleaved embryos were recorded daily until blastocyst stage. Culture medium was changed with fresh ones every 3 days of culture.

3.5.10 Intracytoplasmic Sperm Injection (ICSI) Procedure

ICSI procedures were consist of preparation of holding and injection pipette, preparation of ICSI droplet and alignment. The most important and crucial method was sperm immobilisation and injection before the injected oocyte was activated and cultured for further embryonic development.

3.5.10.1 Micromanipulator and micropipette alignment

Heating stage was warmed to 39°C. All the knob (x-,y-,z-control) and the syringes were adjusted to the center of the scale (re-zero). Holding and injection pipettes were inserted to the pipette holders and tightened well. Under low magnification, started with holding pipette, the pipettes were aligned so that the working tips are parallel to the microscope stage. Then, the position of both pipettes were checked under high magnification to assure the accurate alignment and parallel. A culture dish containing media covered with oil was also prepared. Briefly, the tips of both pipettes were

touched in the oil, then in the medium. So that the ends of the tips were filled by capillary action (a drop of oil behind drop of medium act as a buffer).

3.5.10.2 ICSI dish preparation

ICSI dish was prepared by pipetting droplets (2-5 µl) of HEPES buffered (20 mM) culture medium (mSOF) supplemented with BSA (5%) on the right side of the dish. Then, the droplets were numbered. Then, two larger and elongated (5-10 ul) droplets for sperm were prepared on left side. Elongated (10 µl) PVP (10%) in mSOF droplet was prepared at the centre of the dish and it was smeared for sperm immobilisation. The oocyte droplets were prepared not too close to the edge of dish and to sperm and PVP droplets to avoid mixing. All the microdroplets were covered with equilibrated mineral oil immediately to avoid evaporation. The ICSI dish was equilibrated for at least 15 minutes on the heating stage. At the same time, two culture dishes that contain culture droplets for washing and culturing were prepared and equilibrated at 38.5°C in 5% CO_2 incubator.

3.5.10.3 Sperm preparation

A straw of frozen Mafriwal semen was air thawed for 45 seconds. Then, the straw was plunged in water bath at 38°C for 30 seconds. The straw was emptied and examined under microscope for sperm motility and viability. The sperm was washed in BO medium by centrifuging at 1000 rpm for 5 minutes. The sperm pellet was

diluted with 500 µl of BO medium. By using a fine-pulled Pasteur pipette, about 1 µl sperm was aspirated and placed in elongated sperm droplet in ICSI dish.

3.5.10.4 Cumulus-oocyte complexes (COC) denudation and oocyte preparation

Prior to denudation, three of 100 µl hyaluronidase (80 IU) in culture medium droplets were prepared and covered with silicone oil and warmed on heated stage for minimum 15 minutes. After 24 hours of *in vitro* maturation in CO_2 (5%) incubator, matured COC were collected and placed in a hyaluronidase droplet. The COC were denuded mechanically by aspiration and flush out by using sterile micropipette few times until all the cumulus oophorus and corona radiata were removed from zona pellucida surface.

The oocytes were then washed few times in culture medium and placed in culture droplet. The oocytes were then checked for extrusion of first polar body by slowly rotating the oocyte using micropipette. Matured oocytes were separated from immature ones. The immature oocytes were placed in droplets of maturation medium and were incubated few hours more until first polar body were extruded. The matured oocytes were placed in culture droplets in CO_2 incubator until injection work was ready.

3.5.10.5 Sperm immobilisation

Sperm (10 µl) was placed in sperm droplet in ICSI dish by using sperm-picking Pasteur pipette. At the right edge of the sperm droplet, 1-3 sperms were aspirated into

injection pipette and brought into PVP droplet. The right edge of the inside the needle PVP droplet as focused clearly. The injection pipette was lowered and the sperm inside the pipette was focused. Slowly, the sperm was released into the PVP droplet. The sperm was rotated with needle until it was aligned vertically. The needle was brought up and placed onto the sperm mid-piece; stroked down and across; and the tail was crushed against the bottom of the dish to immobilise the sperm. Selected immobilised sperm was aspirated, tail first, into the injection pipette.

3.5.10.6 Sperm injection and culture

Holding pipette was lowered to the first droplet and was positioned adjacent to the oocyte. Using holding and injection pipette, the oocyte was slowly rotated to locate and positioned the polar body at 6 or 12 o'clock (Figure 3.5). The oocyte was aspirated gently so that the oocyte attached to the pipette.

The injection pipette was moved close to oocyte at 3 o'clock position. The spike was touch to the zona pellucida, and the sperm was brought to the end of the tip (20 um) after which the ooplasm was focused. The pipette was advanced through the zona and cytoplasm until the needle almost touched the opposite side at 9 o'clock position. When membrane breaks, there was a sudden flux of cytoplasm into the pipette. Small amount of cytoplasm was aspirated. The sperm was injected slowly into the oocyte with minimal amount of fluid. Gently, the injection pipette was removed. The oocyte was released and all the process was repeated until all 4 or 5 oocytes have been injected.

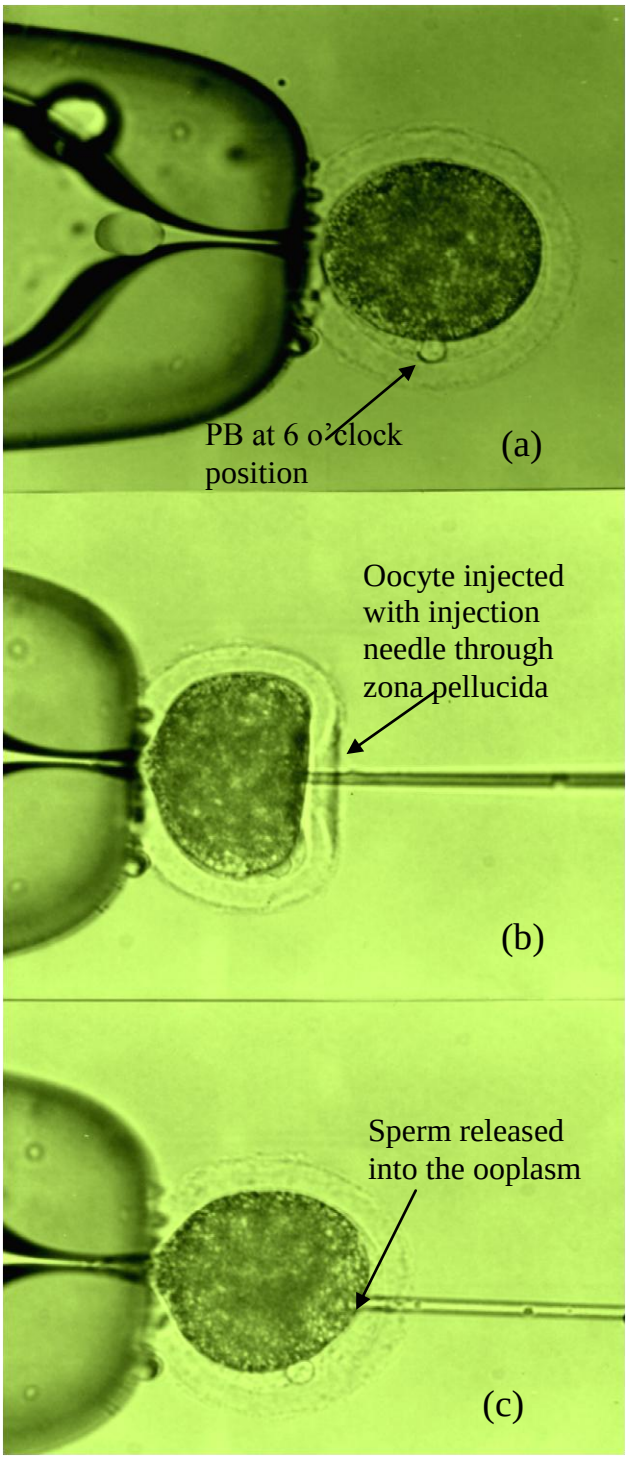

Figure 3.5: ICSI : (a) matured and denuded oocytes was held by using holding pipette at 6 o'clock PB position, (b) matured oocytes injected with injection needle that contain sperm inside the needle and (c) sperm released in the oocyte.

All the oocytes were washed in culture medium four times before transfer to IVC droplet. Maximum 10 oocytes were placed in 100 µl droplet that covered with oil. 18 hours after injection, the number of pronucleus and polar body were accessed. Normal fertilised, cleaved embryos were evaluated after 24 hours of culture. Culture medium was changed with fresh ones every three days of culture.

3.5.11 Embryo and Sperm Staining with Hoechst Stain

Type of staining procedure used was Hoechst stain. Embryos were inserted into acid Tyrode' solution (40°C) until zona pellucida layer was thin. Then, they were washed 5 times with 0.5% TX or PBS. Cover slip that was coated earlier with polylysine was prepared. The embryos were put onto the cover slip and were let to dry to fix them. Later, the cover slip was immersed in paraformaldehyde solution for minimum 2 hours. The cover slip was removed from the solution and was washed again with 0.5% TX for 5 times, before being immersed in 3M TX 1% for 2 hours. After 2 hours of immersion, the cover slip was washed again with 0.5% TX for 5 times.

Hoechst stain was mixed with auto vortex mixer for 1 minute. A volume of 50 µl of the stain was dropped onto the embryos, and the cover slip was placed in the Petri dished before being covered with Petri dish cover and placed in the humidified incubator at 37°C for ½ hour. Then, it was washed 5 times with 0.5% TX. Excess water was removed by wiping with dry tissue. Glycerol (Glycerol:water, 1:4; Dabco:diluted gliserol, 1:2) was dropped onto a clean slide. The cover slip with embryo was touched to the glycerol-Dabco droplet where the embryo side was facing

to the droplet. Excess water was removed. The end of the cover slip was sealed with nail polish before being covered with aluminium foil.

Sperm staining was done after sperm washing and capacitating. Prior to staining, sperm suspension was diluted by transferring 10 µl of sperm into 100 µl BO medium. Of the reconstituted sperm, 10 µl was then transferred onto a pre-cleaned cover slip and allowed to dry on a slide warmer at 37°C. The cover slip was then immersed into 3 ml of 2% paraformaldehyde for two hours. The cover slip with fixed sperm was then washed in 0.1 M phosphate buffer solution (PBS) containing 0.5% Triton X-100 inside a 6-well culture dish five times, five minutes each. Excess solution was dabbed with tissue papers. The sperm was then permeabilised in 3 ml of 1% Triton X-100/PBS for 1 hour. The acrosomes were directly stained with 12.5 µg/ml FITC-PSA or 10 µg/ml TRITC-LCA. A 50 µl of either stains was added onto the sperm containing cover slip and covered with a Petri dish to prevent dehydration and incubated inside a humidified incubator for one hour.

Following the final washing step, the nuclear materials of the sperm was stained with bis Benzimide Hoechst 33258 for 30 minutes, followed by series of washing and finally mounted onto a drop of 1:4 glycerol and water that contained 1% antifade agent 1,4-diazabicyclo-[2,2,2]octane on a glass slide. At each time the sperm was stained in duplicates. The coverslips were sealed with nail polish, labeled and wrapped with aluminium foil.

They were stored at 4ºC inside a refrigerator. However, for high quality images, immediate observation is recommended under the fluorescent microscope. Approximately, a total of 200 sperms were counted at random in at least five different fields per slide. The percentage of the acrosome intact sperms were determined and

averaged by counting those emitted either red (TRITC-LCA) of green (FITC-PSA) fluorescent over the total number of sperm detected from the blue (Hoechst) Fluorescent.

3.6 EXPERIMENTAL DESIGN

The main objective of this study was to produce *in vitro* bovine embryos through IVMFC and ICSI techniques. Six experiments were designed as described below. Figure 3.6 showed the flow chart of experimental design.

3.6.1 Effect of Oocyte Quality on IVF Performance (Experiment 1)

The objective was to produce embryos from slaughter cattle oocytes where the cumulus cells and oocyte cytoplasmic were graded and matured and underwent fertilisation and culture protocol.

The morphological characteristics of the oocytes were examined under a stereomicroscope and characterised according to Shioya *et al*. (1988) and Nagano *et al*.(2006b) with slightly modification. The COCs were divided into three groups based on number of cumulus cell layer namely Group A with a compact and dense with more than three layers of cumulus cells, Group B with one to two layers of cumulus cells or half covered cumulus cells and Group C with naked oocytes. Then, the COCs were regrouped into two groups accoding to the homogenousity of cytoplasm. Homogenous group cytoplasm are brown with dark zone around the periphery whereas heterogenous group characterised by pale with dark cluster.

All the COCs were matured *in vitro* in humidified CO_2 incubator (5%) at 38.5°C for 24 hours and fertilised in BO medium. Embryonic development at 2-cell stage were assessed after 24 hours of insemination and were recorded and used as successful rate for different COCs and cytoplasm qualities of fertilising capability. Embryo development was observed daily until blastocyst stage under inverted microscope at high magnification (200X).

The cleavage rate was recorded as percentage calculated from the number of embryos used for the respective treatments. Data were analysed by analysis of variance (ANOVA), followed by Duncan's multiple range test (D-MRT).

3.6.2 Effect of IVM Durations on Fertilisation Rate after IVF with Different Oocyte Grades (Experiment 2)

The objectives were to produce embryos and evaluate the effect of different IVM durations and oocytes grades. The oocytes were matured at different IVM durations at 20, 24 and 28 hours in humidified CO_2 incubator (5%) at 38.5°C before underwent IVF and IVC. Embryo development at 2-cell stage were accessed and used as successful rate for different fertilising capability. Embryo development was observed daily until blastocyst stage under inverted microscope at high magnification (200X).

The cleavage rate was recorded as percentage calculated from the number of embryos used for the respective treatments. Data were analysed by analysis of variance (ANOVA), followed by Duncan's multiple range test (D-MRT).

3.6.3 Effect Serum Addition on Blastocyst Production after IVF (Experiment 3)

The purpose of this experiment was to evaluate the effect of serum addition on blastocyst production after IVF. The oocytes were matured 24 hours and fertilised in BO medium. The oocytes were cultured and every embryo development stages were added with serum (10%). Embryo development were evaluated and recorded daily under inverted microscope at high magnification (200X).

The cleavage rate was recorded as percentage calculated from the number of embryos used for the respective treatments. Data were analysed by analysis of variance (ANOVA).

3.6.4 Effect of COC Quality on Fertilisation and Cleavage Rates after ICSI (Experiment 4)

The objective of this study was to evaluate the effect of COC quality on fertilisation and cleavage rates after ICSI. The COCs of Group A, B and C were matured 24 hours and injected with intact-immobilised of frozen-thawed sperm. All the oocytes underwent oocytes activation with ethanol (5%) after injection. Presumptive zygotes were subsequently cultured up to Day 9 after injection. Embryo development were evaluated and recorded daily under inverted microscope at high magnification (200X). Developmental differences between embryos produced with different oocytes groups were evaluated.

The cleavage rate was recorded as percentage calculated from the number of embryos used for the respective treatments. Data were analysed by analysis of variance (ANOVA), followed by Duncan's multiple range test (D-MRT).

3.6.5 Effect of Sperm Quality on Fertilisation and Cleavage Rates after ICSI (Experiment 5)

The objective of this study was to determine the effect of sperm quality on fertilisation and cleavage rates after ICSI. The studies were grouped into four namely dead-immobilised sperm, intact sperm, intact-immobilised and sham injection. The COC were matured 24 hours and injected with those of frozen-thawed sperm. All the oocytes underwent oocytes activation with ethanol (5%) after injection. Presumptive zygotes were subsequently cultured up to Day 9 after injection. Embryo development were evaluated and recorded daily under inverted microscope at high magnification (200X). Developmental differences between embryos produced with different oocytes groups were evaluated. At day 10, the embryos were stained with Hoechst stain.

The cleavage rate was recorded as percentage calculated from the number of embryos used for the respective treatments. Data were analysed by analysis of variance (ANOVA), followed by Duncan's multiple range test (D-MRT).

3.6.6 Comparison between IVF and ICSI on Fertilisation and Cleavage Rates (Experiment 6)

The objective of this study was to compare fertilisation and cleavage rate of embryo produced by IVF and ICSI methods. The COC were matured 24 hours and injected with frozen-thawed sperm. For IVF, the oocytes were inseminated with frozen-thawed sperm for 18 hours before being cultured in CO_2 incubator. As for ICSI, the oocytes were injected with intact-immobilised sperm by using frozen-thawed sperm. The oocytes were activated with ethanol (5%) after injection. Presumptive zygotes were subsequently cultured up to Day 9 after injection. Embryo development were

evaluated and recorded daily under inverted microscope at high magnification (200X). Developmental differences between embryos produced with different oocytes groups were evaluated.

The cleavage rate was recorded as percentage calculated from the number of embryos used for the respective treatments. Data were analysed by analysis of variance (ANOVA).

3.7 STATISTICAL ANALYSIS

Calculation of percentages was described in the respective experiment designs. The effects of oocyte quality on IVF performance (Experiment 1); oocyte quality and IVM durations on the rates of cleavage (Experiment 2); serum on the rates of cleavage (Experiment 3); oocyte quality on ICSI performance (Experiment 4); sperm and ooxyte quality on ICSI performance (Experiment 5) as well as comparison on cleavage rates after IVF and ICSI, were analysed by analysis of variance (ANOVA) and the cleavage rate was recorded as percentage calculated from the number of embryos used for the respective treatments. Data were analysed by analysis of variance (ANOVA) and Duncan's multiple range test (D-MRT) using the SPSS statistical software package version 17. A probability of $P<0.05$ was considered significant for all statistical tests. Values are presented as mean±SEM.

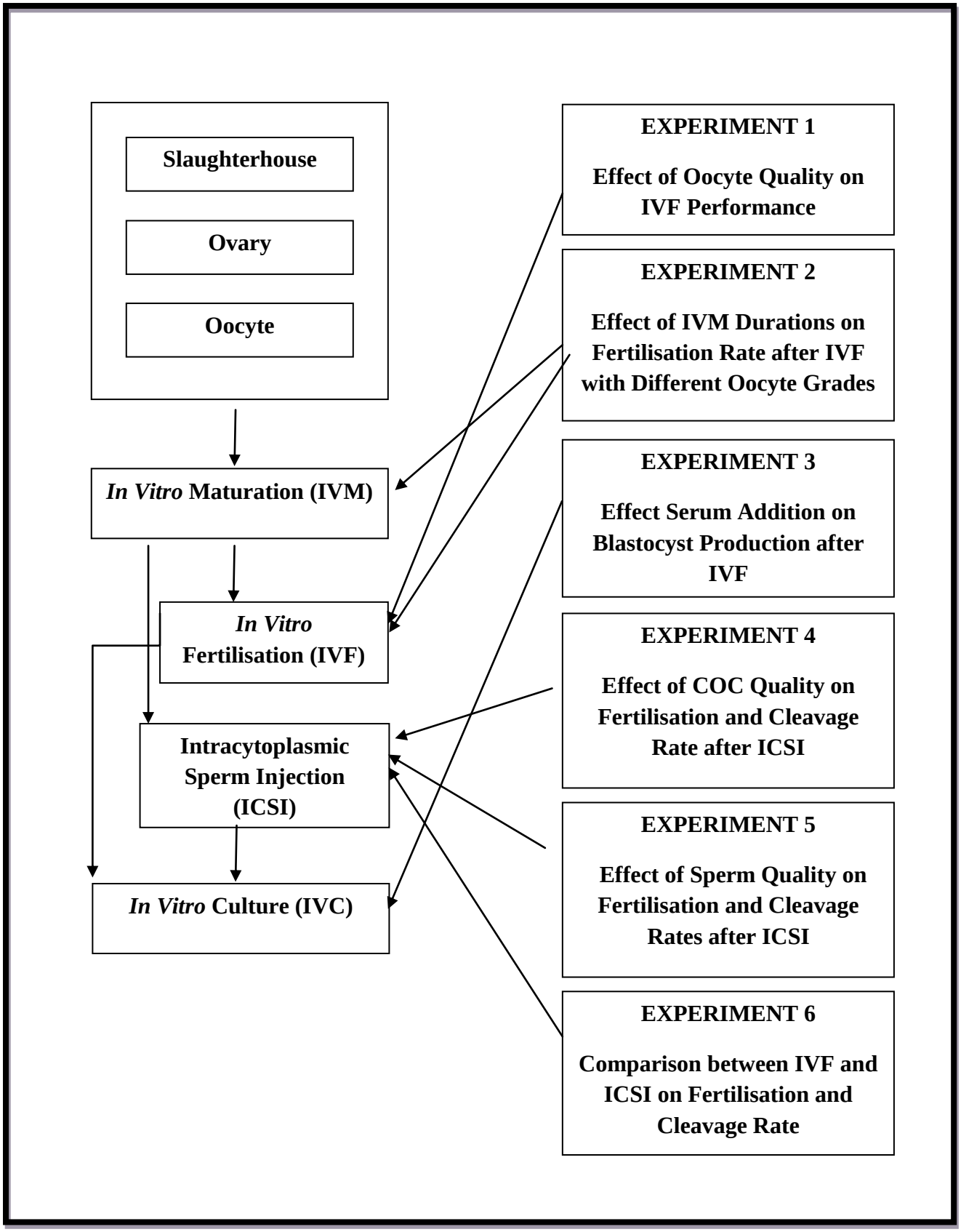

Figure 3.6: Flow chart showing experimental design involved cattle oocytes harvested from abattoir.

Chapter 4

4.0 RESULTS

4.1 EFFECT OF OOCYTE QUALITY ON IVF PERFORMANCE (EXPERIMENT 1)

The cleavage rates obtained after IVF for different grades of COC and cytoplasm are presented in Table 4.1. In all treatments, there was a general trend of decreasing cleavage rates from 2-cell stage to blastocyst stage. For homogenous cytoplasm, Grade A gave the highest 2-cell cleavage rate (78.30%) followed by Grade B (65.92%) and Grade C (24.02%). Similar trend was also observed for heterogenous cytoplasm with the values of 50.9%, 30.48% and 11.66%, respectively. It was consistently shown that homogenous cytoplasm gave higher cleavage rates for all the graded of oocyte COC. It was also observed that blastocysts were obtained for Grade A – Homogenous (28.55%), Grade A – Heterogenous (2.66%) and Grade B – Homogenous (14.40%) oocytes. No blastocyst was obtained for other categories of oocyte quality. Figure 4.1 shows graphical presentation of cleavage rates of embryos obtained from IVF using different oocytes grades (Appendix 3.0)

Table 4.1: Cleavage rate after IVF in different oocyte grades

COC grade	Cytoplasmic grade	Number of oocytes (n)	% embryo development with mean ± standard error (n)					
			2-cell	4-cell	8-cell	Morula	Compact Morula	Blastocyst
A	Homogenous	120	78.30± 1.75^{d} (94)	69.70± 4.57^{e} (84)	60.54± 3.90^{d} (72)	46.97± 5.64^{c} (56)	36.56± 3.59^{d} (44)	28.55± 2.02^{b} (34)
	Heterogenous	126	50.90± 5.02^{c} (64)	38.57± 4.90^{c} (48)	33.75± 3.17^{c} (42)	20.11± 4.75^{b} (24)	9.48± 1.10^{b} (12)	2.66± 1.63^{a} (4)
B	Homogenous	130	65.92± 6.79^{cd} (84)	53.97± 8.10^{d} (68)	44.89± 7.06^{c} (56)	24.81± 2.78^{b} (32)	20.61± 2.85^{c} (26)	14.40± 2.21^{b} (16)
	Heterogenous	112	30.48± 4.02^{b} (34)	19.85± 4.12^{b} (22)	14.64± 5.87^{b} (16)	5.33± 3.43^{a} (6)	1.66± 1.66^{a} (2)	0.00± 0.00^{a} (0)
C	Homogenous	62	24.02± 5.41^{ab} (14)	12.68± 3.41^{ab} (8)	9.35± 4.01^{ab} (6)	2.50± 2.50^{a} (2)	0.00± 0.00^{a} (0)	0.00± 0.00^{a} (0)
	Heterogenous	32	11.66± 7.26^{a} (4)	0.00± 0.00^{a} (0)	0.00± 0.00^{a} (0)	0.00± 0.00^{a} (0)	0.00± 0.00^{a} (0)	0.00± 0.00^{a} (0)

^{a,b,c,d,e} Means within column with different superscripts were significantly different at P<0.05.

4.2 EFFECT OF IVM DURATIONS ON FERTILISATION RATE AFTER IVF WITH DIFFERENT OOCYTE GRADES (EXPERIMENT 2)

The cleavage rates obtained after IVF for different IVM durations are presented in Table 4.2. In all IVM duration treatments, regardless grade of oocytes (COC and cytoplasm), there was a general trend of decreasing cleavage rates from 2-cell stage to blastocyst stage. For 24 hours maturation duration of Grade A - Homogenous cytoplasm, gave the highest 2-cell cleavage rate (78.30%) followed by Grade B (65.92%) and Grade C (24.02%). Similar trend was also observed for heterogenous cytoplasm with the values of 50.9%, 30.48% and 11.66%, respectively. These results showed that the 24 hours IVM duration gave better results ($P<0.05$) compared to 20- and 28-hours for homogenous cytoplasm.

Cleavage rate for 20 hours maturation duration, Grade A - Homogenous gave the highest 2-cell cleavage rate (58.57%), followed by Grade B (38.32%) and Grade C (15.58%). For heterogenous cytoplasm, development values were 45.65%, 39.64% and 21.66%, respectively. Result for 28 hours maturation duration, Grade A - Homogenous gave the highest 2-cell cleavage rate (58.88%), Grade B (49.16%) and Grade C (14.00%). For heterogenous cytoplasm, the values were 30.77%, 28.82% and 4.00%, respectively. There were no significant differences of 20 hours and 28 hours IVM durations of Group A - Homogenous oocyte. Heterogenous oocyte with 20- and 24 hours IVM durations which gave higher cleavage rate ($P<0.05$) compared to 28 hours IVM durations of Groups A and B oocytes.

In Group C oocyte, very low embryonic developments was observed for both homogenous and heterogenous cytoplasm oocyte groups in all IVM durations. The

embryos cleaved only up to early stages of development (2- to 8-cell stage), and all heterogenous oocytes were only developed up to 2-cell stage.

It was also observed that higher percent blastocyst was obtained for 24 hours IVM duration from Grade A – Homogenous (28.55%) than Grade B - Homogenous (14.40%). For 20 hours IVM duration, the blastocyst was obtained in Group A – Homogenous (3.48%) and Group B – Homogenous (2.00%). In 28 hours IVM duration, comparable results (2.5%) of blastocyst rates were obtained in both Groups A and B – Homogenous oocytes.

As for heterogenous cytoplasm oocytes in Group A, very low blastocyst rates were obtained with the values of 2.66% and 1.53% for 24 and 20 hours IVM duration, respectively. No blastocyst was obtained for other categories of oocyte quality. Figure 4.2 shows graphical presentation of cleavage rates obtained after IVF for different IVM durations. Figure 4.2 and 4.3 (Appendix 3.0) showed the mean percentage of embryonic development after IVF for different IVM durations of different COC group with homogenous and heterogenous cytoplasm, respectively. Figure 4.4 to 4.6 showed embryonic development after IVF.

Table 4.2: Effect of IVM duration on fertilisation rate after IVF

COC grade	Cyto-plasmic grade	IVM duration (h)	N	% embryo development with mean ± standard error (n)					
				2-cell	4-cell	8-cell	Morula	Compact morula	Blastocyst
A	Homo-genous	20	120	58.57±3.33^{hi} (70)	45.08±5.50^{fg} (54)	42.08±7.03^{gh} (50)	23.64±3.36^{f} (28)	14.30±6.39^{cd} (18)	3.48±2.13^{a} (4)
		24	120	78.30±1.75^{j} (94)	69.70±4.57^{h} (84)	60.54±3.90^{i} (72)	46.97±5.64^{g} (56)	36.56±3.59^{e} (44)	28.55±2.02^{c} (34)
		28	88	58.88±5.71^{hi} (52)	38.55±4.35^{ef} (34)	29.83±4.74^{efg} (26)	17.88±2.23^{def} (16)	9.44±2.38^{bc} (8)	2.5±2.5^{a} (2)
	Hetero-genous	20	128	45.65±1.61^{fgh} (58)	35.42±4.96^{ef} (44)	19.96±4.02^{cde} (26)	9.35±1.14^{abcd} (12)	4.20±1.72^{ab} (6)	1.53±1.53^{a} (2)
		24	126	50.90±5.02^{ghi} (64)	38.57±4.90^{ef} (48)	33.75±3.17^{fgh} (41)	20.11±4.75^{ef} (24)	9.48±1.10^{bc} (12)	2.66±1.63^{a} (4)
		28	98	30.77±2.68^{def} (30)	28.77±3.45^{cde} (28)	11.13±4.95^{abc} (12)	7.80±3.54^{abc} (6)	5.32±3.60^{ab} (4)	0.00±0.00^{a} (0)
B	Homo-genous	20	114	38.32±2.90^{efg} (44)	28.22±5.51^{cde} (32)	20.88±6.23^{cdef} (24)	12.73±5.19^{cde} (14)	4.00±4.00^{ab} (4)	2.00±2.00^{a} (2)
		24	130	65.92±6.79^{ij} (84)	53.97±8.10^{g} (68)	44.89±7.06^{h} (56)	24.81±2.78^{f} (32)	20.61±2.85^{d} (26)	14.40±2.21^{b} (16)

(continued)

(continued)

		28	72	49.16±5.16^{gh} (36)	33.45±3.16^{def} (24)	25.23±3.13^{def} (18)	11.54±2.96^{bcde} (8)	5.35±3.29^{ab} (4)	2.50±2.50^{a} (2)
	Hetero-genous	20	90	39.64±5.47^{efg} (36)	18.20±3.11^{bc} (32)	11.66±3.52^{abc} (20)	4.72±2.89^{abc} (8)	0.00±0.00^{a} (0)	0.00±0.0 (0)
		24	112	30.48±4.02^{def} (34)	19.85±4.12^{bc} (22)	14.64±5.87^{bcd} (16)	5.33±3.43^{abc} (6)	1.66±1.66^{ab} (2)	0.00±0.00^{a} (0)
		28	84	28.82±1.82^{cde} (24)	21.74±4.32^{bcd} (18)	10.41±2.85^{abc} (8)	7.55±3.28^{abc} (6)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)
C	Homo-genous	20	54	15.85±4.31^{abcd} (8)	13.00±5.38^{ab} (6)	5.00±5.00^{ab} (2)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)
		24	62	24.02±5.41^{bcde} (14)	12.68±3.41^{ab} (8)	9.35±4.01^{abc} (6)	2.50±2.50^{ab} (2)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)
		28	40	14.00±5.78^{abc} (6)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)
	Hetero-genous	20	32	21.66±9.71^{bcd} (6)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)
		24	32	11.66±7.26^{ab} (4)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)
		28	30	4.00±4.00^{a} (2)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)	0.00±0.00^{a} (0)

^{a,b,c,d,e,f,g,h,I} Means within column with different superscripts were significantly different at P<0.05.

n: number of oocytes.

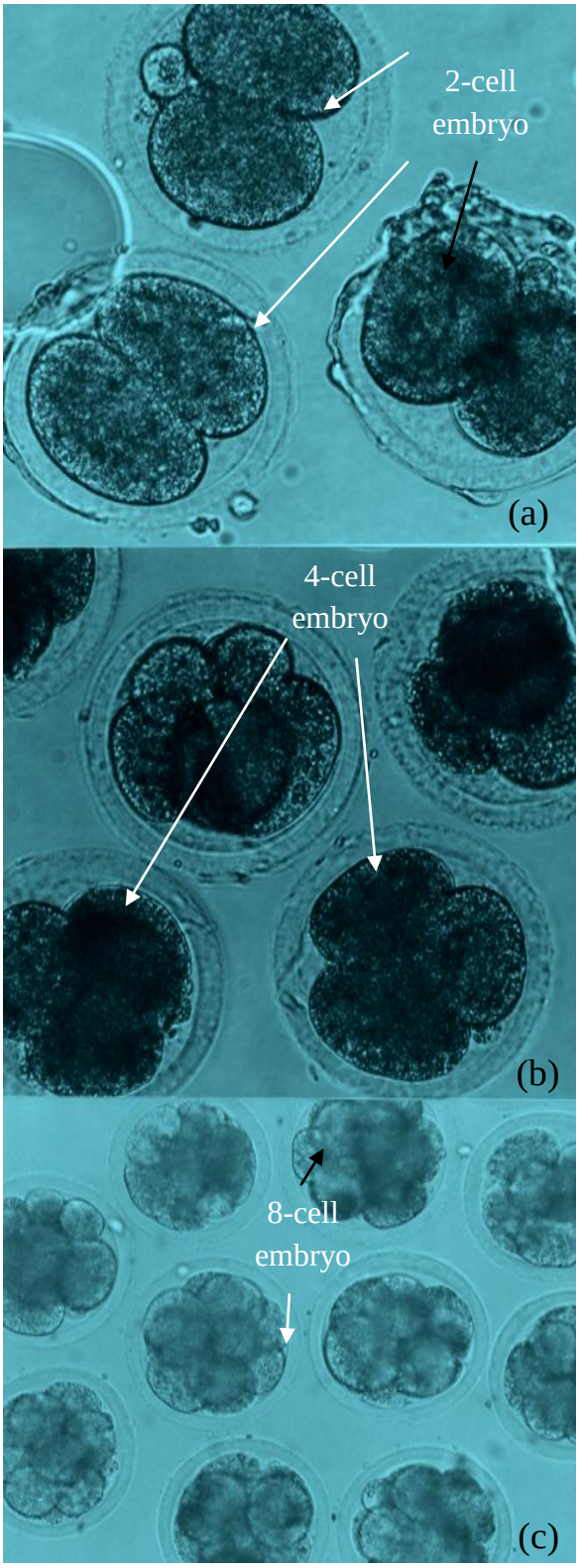

Figure 4.4: Cattle embryo derived from IVM and IVF oocytes after 24 hours of maturation. (a) embryos at 2-cell stage, (b) embryos at 4-cell stage and (c) embryos at 8-cell stage.

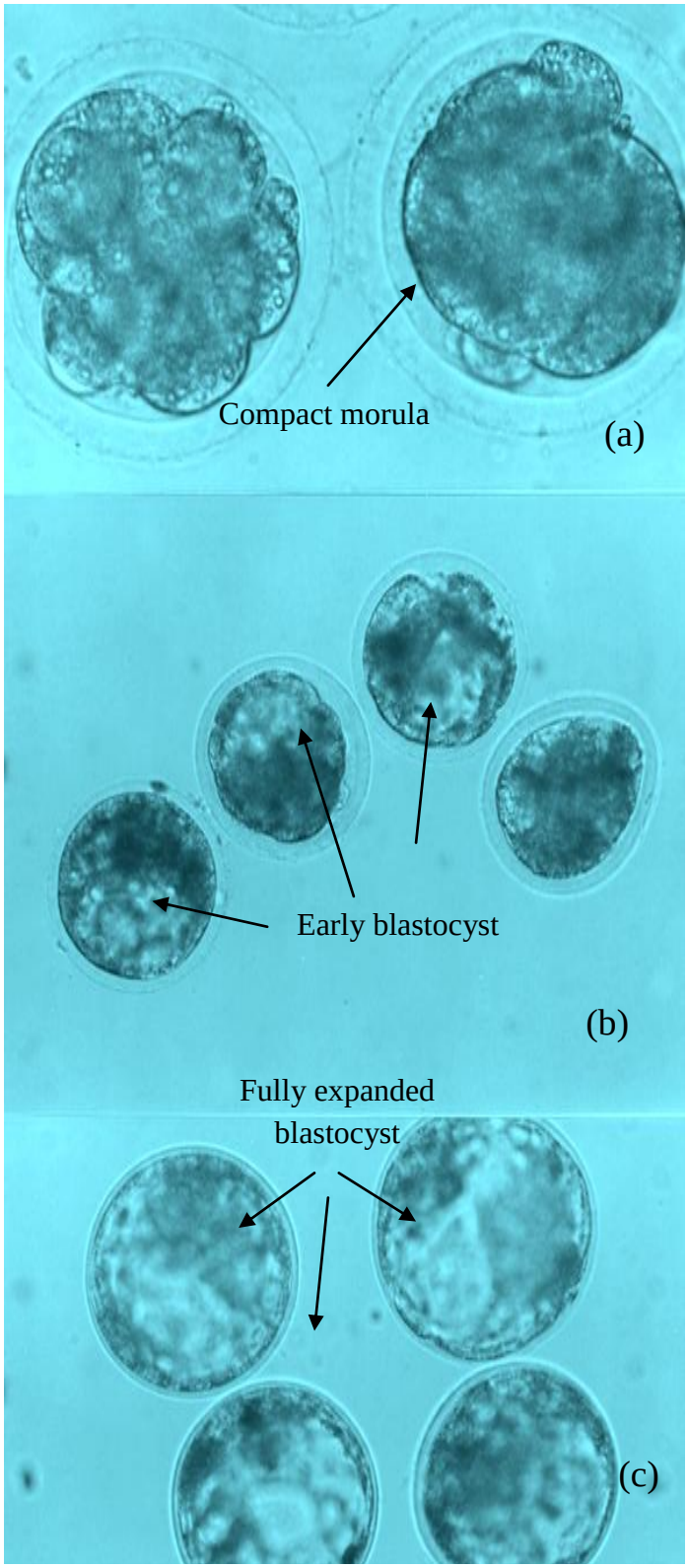

Figure 4.5: Cattle embryo derived from IVM and IVF oocytes after 24 hours of maturation. (a) embryos at morula/compact morula stage, (b) embryos at early blastocyst stage and (c) embryos at fully expanded blastocyst stage.

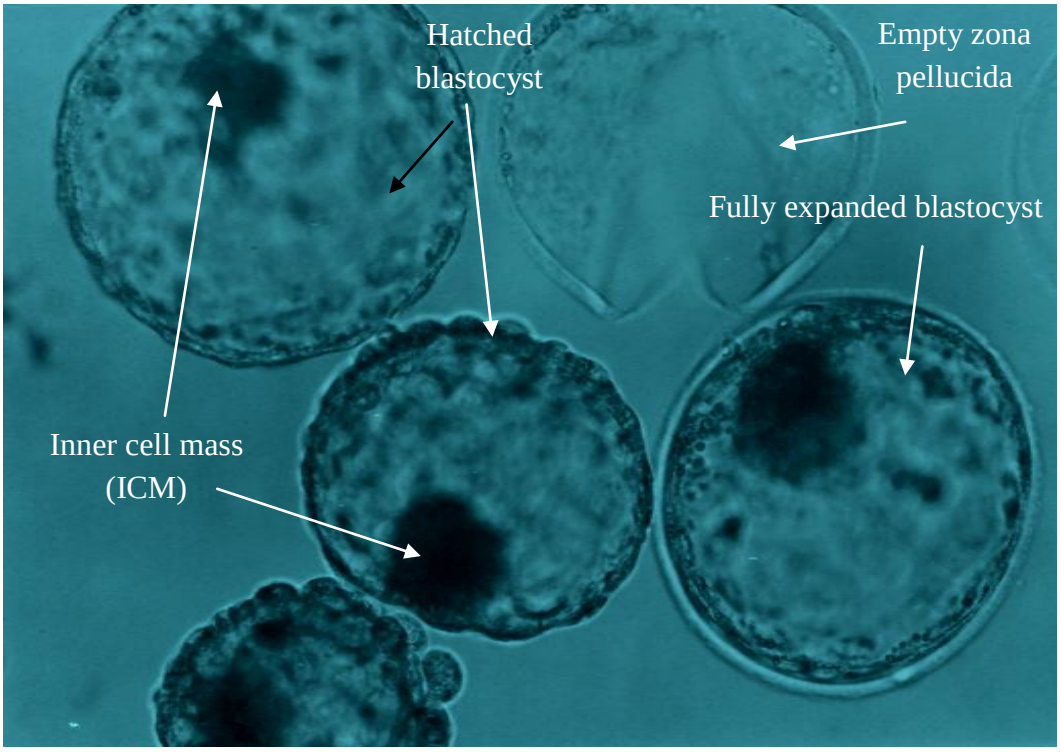

Figure 4.6: Cattle embryo at fully expanded and hatched blastocyst derived from IVM and IVF oocytes after 24 hours of maturation.

4.3 EFFECT SERUM ADDITION ON BLASTOCYST PRODUCTION AFTER IVF (EXPERIMENT 3)

The effect of serum addition on blastocyst production is presented in Table 4.3. In all treatments, there was a general trend of decreasing cleavage rates from 4-cell to blastocyst stage. From the results above, very low early blastocyst developed after serum supplement at 8-cell stage (7.85%). No blastocyst was developed after serum addition at 2-cell and 4-cell stages.

The blastocyst development after serum addition at morula stage was 63.06%, including fully expanded and hatch blastocyst formation (47.58% and 26.34%, respectively). Embryonic developments were observed at fully expanded blastocyst (73.33%) and hatched blastocyst (54.99%) after serum addition at early blastocyst. Compared to control (no serum addition), embryonic development was only up to fully expanded blastocyst (2.50%). Figure 4.7 (Appendix 3.0) showed the gradients of cleavage rate when serum was added at different stages of embryonic development. From the figure, it shows that serum could be beneficial to be added at morula stage, conversely, addition of serum at earlier stages of development might be detrimental to embryonic development. Figure 4.8 showed embryonic development after serum addition.

Table 4.3: Effect of serum addition on blastocyst production after IVF

Embryo stage of serum addition	n	% embryo development with mean ± standard error (n)						
		2-cell	4-cell	8-cell	Morula	Early blastocyst	Fully expanded blastocyst	Hatched blastocyst
2-cell	66	-	14.35±4.21 (10)	6.50±4.15 (4)	0.00±0.00 (0)	0.00±0.00 (0)	0.00±0.00 (0)	0.00±0.00 (0)
4-cell	62	-	-	19.66±6.51 (12)	6.50±4.15 (4)	0.00±0.00 (0)	0.00±0.00 (0)	0.00±0.00 (0)
8-cell	78	-	-	-	18.41±6.40 (14)	7.85±3.22 (6)	0.00±0.00 (0)	0.00±0.00 (0)
Morula	130	-	-	-	-	63.06±3.05 (82)	47.58±2.23 (62)	26.34±2.84 (34)
Early blastocyst	30	-	-	-	-	-	73.33±11.30 (22)	54.99±8.16 (16)
Control	70	62.33±7.08 (44)	39.08±4.92 (38)	39.08±4.92 (28)	24.95±4.25 (18)	10.55±4.09 (8)	2.50±2.50 (2)	0.00±0.00 (0)

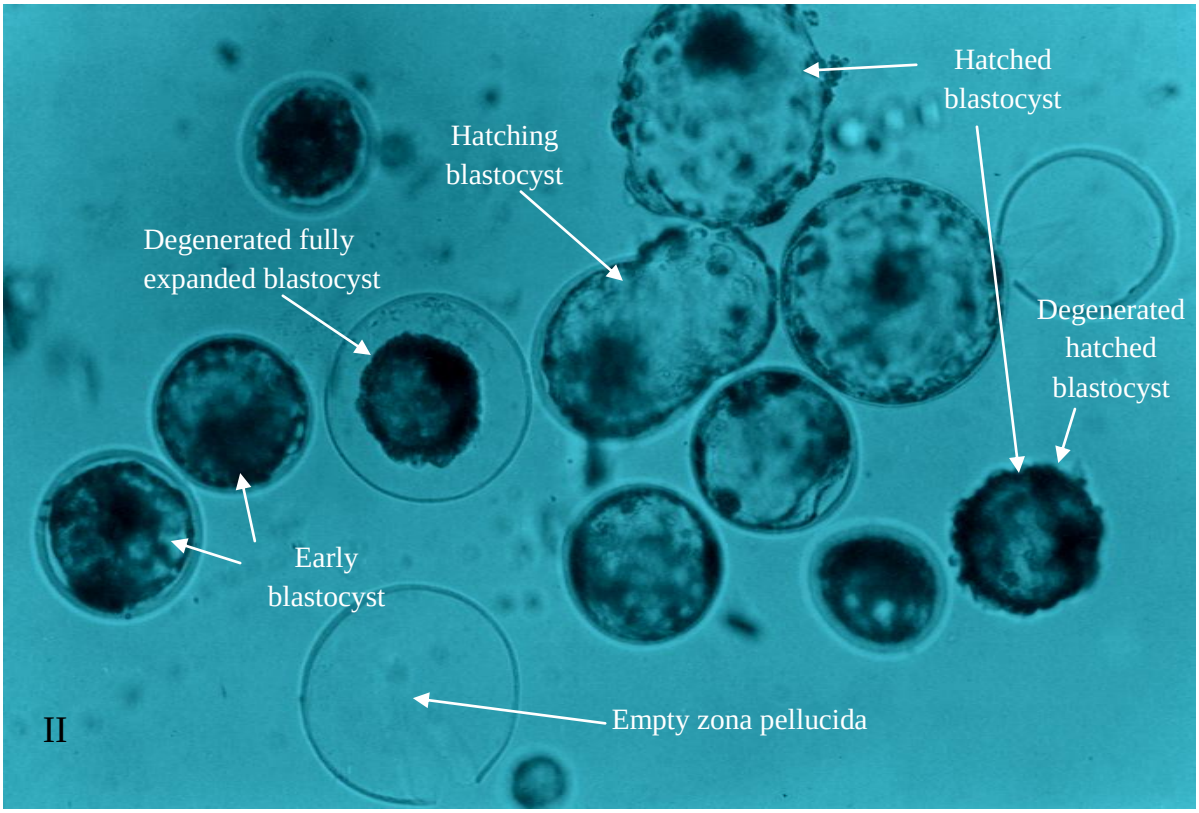

Figure 4.8: Cattle embryo at different embryonic development after serum addition.

4.4 EFFECT OF COC QUALITY ON FERTILISATION AND CLEAVAGE RATES AFTER ICSI (EXPERIMENT 4)

The cleavage rates obtained after ICSI for different grades of COC with homogenous cytoplasm are presented in Table 4.4 and Figure 4.9 (Appendix 3.0). In all treatments, there was a general trend of decreasing cleavage rates from 2-cell stage to morula stage. Grade A COC gave the highest 2-cell cleavage rate (44.53%) followed by Grade B (38.94%) and Grade C (7.85%). It was also observed that morula stage was only obtained in Grade A (16.65%). No further development up to blastocyst stage was observed in all COC grades. There were no significant different in embryo development at 2-cell, and different at 4-cell up to morula stage when compared within Grade A and B COC. In Group B COC, the embryos only developed up to 8-cell, and in Group C COC, the embryos only developed up to 2-cell stage.

Table 4.4: Effect of COC quality on fertilisation and cleavage rates after ICSI

COC group	n	% embryo development with mean ± standard error (n)			
		2-cell	**4-cell**	**8-cell**	**morula**
A	88	44.53±6.05[b] (38)	42.53±7.42[c] (36)	33.51±9.61[b] (28)	16.65±3.73[b] (14)
B	90	38.94±8.11[b] (34)	22.72±4.31[b] (20)	4.22±2.59[a] (4)	0.00±0.00[a] (0)
C	58	7.85±5.10[a] (4)	0.00±0.00[a] (0)	0.00±0.00[a] (0)	0.00±0.00[a] (0)

[a,b,c] Means within column with different superscripts were significantly different at $P<0.05$.

4.5. EFFECT OF SPERM QUALITY ON FERTILISATION AND CLEAVAGE RATES AFTER ICSI (EXPERIMENT 5)

The cleavage rates obtained from different sperm quality injected into oocytes during ICSI are presented in Table 4.5 and Figure 4.10 (Appendix 3.0). The cleavage rates (at 2-cell stage) in dead-immobilised, intact, intact-immobilised and sham injection of Group A oocytes were 28.33%, 24.58%, 63.42% and 39.99%, respectively. Correspondingly in Group B, the cleavage rates were 33.66%, 39.33%, 43.23% and 46.66%; and in Group C, the cleavage rates were 30.66%, 29.71%, 23.83% and 33.33%, respectively. In all cases, no embryos were developed to blastocyst stage. At morula stage, the cleavage rates for intact-immobilised sperm of Groups A and B oocytes were 33.71% and 6.50%, respectively.

Table 4.5: Effect of sperm quality on fertilisation and cleavage rate after ICSI

Sperm treatment	COC group	n	% embryo development with mean ± standard error (n)			
			2-cell	4-cell	8-cell	Morula
Dead and immobilised	A	50	28.33± 4.94^{ab} (14)	19.33± 6.35^{ab} (10)	0.00± 0.00^{a} (0)	0.00± 0.00^{a} (0)
	B	42	33.66± 5.33^{abc} (14)	18.00± 7.84^{ac} (8)	0.00± 0.00^{a} (0)	0.00± 0.00^{a} (0)
	C	40	30.66± 5.28^{abc} (12)	11.66± 7.26^{ab} (4)	0.00± 0.00^{a} (0)	0.00± 0.00^{a} (0)
Intact	A	40	24.58± 3.14^{a} (10)	14.16± 4.78^{ab} (6)	5.00± 5.00^{a} (2)	0.00± 0.00^{a} (0)
	B	52	39.33± 7.02^{abc} (20)	27.99± 6.20^{b} (14)	17.33± 9.15^{b} (4)	4.00± 4.00^{a} (2)
	C	48	29.71± 5.25^{abc} (14)	7.85± 5.10^{ab} (4)	2.85± 2.85^{a} (2)	0.00± 0.00^{a} (0)
Intact and immobilised	A	60	63.42± 5.64^{d} (38)	57.23± 4.71^{c} (34)	37.04± 3.71^{c} (22)	33.71± 5.57^{b} (20)
	B	88	43.23± 3.16^{bc} (38)	27.15± 1.53^{ab} (24)	18.07± 2.43^{b} (16)	6.50± 2.69^{a} (6)
	C	80	23.83± 3.92^{a} (18)	10.41± 2.85^{ab} (8)	0.00± 0.00^{a} (0)	0.00± 0.00^{a} (0)
Sham	A	30	39.99± 6.66^{abc} (12)	13.33± 8.16^{ab} (4)	0.00± 0.00^{a} (0)	0.00± 0.00^{a} (0)
	B	30	46.66± 8.16^{c} (14)	19.99± 8.16^{ab} (6)	0.00± 0.00^{a} (0)	0.00± 0.00^{a} (0)
	C	30	33.33± 0.00^{abc} (10)	6.66± 6.66^{a} (2)	0.00± 0.00^{a} (0)	0.00± 0.00^{a} (0)

^{a,b,c,d} Means within column with different superscripts were significantly different at $P<0.05$.

4.6 COMPARISON BETWEEN IVF AND ICSI ON FERTILISATION AND CLEAVAGE RATES (EXPERIMENT 6)

The cleavage rates obtained after IVF and ICSI are shown in Table 4.6 and Figure 4.11. In all treatments, there was a general trend of decreasing cleavage rates from 2-cell stage to blastocyst stage. IVF embryos gave higher cleavage rate in all cases of embryonic development then the ICSI embryos. The values for IVF versus ICSI are as follows: (2-cell: 66.77% vs. 41.74%, $P<0.05$; 4-cell: 41.48% vs. 32.63%, $P>0.05$; 8-cell: 31.01% vs. 18.87%, $P>0.05$; morula: 19.16% vs. 8.32%, $P<0.05$; compact morula: 13.96% vs. 0.00%, $P<0.05$; and blastocyst: 8.94% vs. 0.00%, $P<0.05$, respectively).

Table 4.6: Comparison between IVF and ICSI on fertilisation and cleavage rate

Treatment	n	% embryo development with mean ± standard error (n)					
		2-cell	**4-cell**	**8-cell**	**Morula**	**Compact morula**	**Blastocyst**
IVF	224	66.77±3.90[a] (150)	41.48±4.85[a] (90)	31.01±5.53[a] (66)	19.16±4.08[a] (42)	13.96±3.34[a] (30)	8.94±3.18[a] (19)
ICSI	89	41.74±4.86[b] (36)	32.63±5.22[a] (28)	18.87±6.77[a] (16)	8.32±3.28[b] (7)	0.00±0.00[b] (0)	0.00±0.00[b] (0)

[a,b] Means within column with different superscripts were significantly different at $P<0.05$.

Chapter 5

5.0 DISCUSSION

5.1 PRODUCTION OF EMBRYOS THROUGH ASSISTED FERTILISATION

This section provides discussion on IVF and ICSI. In the IVF study, it was found that maturation and fertilisation rates were better in oocytes with group A COC followed by Group B and C. The homogenous cytoplasm was superior in affecting maturation and fertilisation than heterogenous cytoplasm. The Group A of homogenous cytoplasm was superior in producing cleavage and blastocyst rate after matured *in vitro* for 24 hours with serum supplementation during *in vitro* culture at morula stage. In ICSI, the oocyte quality did affect embryo development even though with not highly significant between Groups A and B. The use of intact-immobilised sperm gave best result in producing embryo compare to other sperm types.

5.1.1 Effect of Oocyte Quality on IVF Performance (Experiment 1)

The results of the present experiment demonstrated that the cumulus-oocyte complexes (COC) and cytoplasm qualities influenced the fertilisation rate. Morphological oocyte quality based on compactness and numbers of cumulus cell had significant positive effects on the rates of *in vitro* maturation and subsequent fertilisation rate. Group A homogeneous gave highest cleavage rate compared to group B and C with 78.3%, 65.9% and 24.0%, respectively. This result was in

agreement with other researchers (Shioya *et al*., 1988; Younis *et al.*, 1989b; Younis and Brackett, 1991; Zhang *et al.*, 1995; Chian *et al*., 1996; Fatehi *et al.*, 2002). Khurana and Niemann (2000) categorised the oocytes into 4 groups: Category I COC with a homogenous evenly granulated cytoplasm possessing at least 3 layers of compact cumulus-cells; Category II COC had less than three layers of cumulus-cells or were partially denuded but also possessed a homogenous evenly granulated cytoplasm; Category III with corona enclosed oocytes and Category IV with nude oocytes. They found that a greater proportion of Category I and II compared to Category III or IV oocytes completed with these respective results: IVM: 84.5%, 85.0%, 71.0% and 32.7%; IVF: 67.1%, 63.7%, 44.6% and 16.3%; cleavage: 61.4%, 52%, 26% and 12.1%; development to the morula and blastocyst: 33.9%, 13.1%, 0% and 0%. By using a highly selected population of good quality oocytes, it is possible to increase the efficiency in terms of proportion of oocytes progressing to morula and blastocyst. In contrast to these results, Kim *et al.* (1996) found that there was no significant difference in maturation rate (MII) among denuded oocytes (88.9%), oocytes surrounded only a layer of corona cells (82.4%) and cumulus intact oocytes (88.9%).

Nagano *et al.* (1999b) reported that there were no significant differences in embryonic development between homogeneous and heterogeneous ooplasm (with dark clusters) after *in vitro* fertilisation (IVF) with cleavage results of 81.9% and 91.6%, respectively. Heterogeneous oocytes showed a similar or higher normal fertilisation rate due to their lower incidence of polyspermy as compared to homogeneous oocytes. However, the developmental capacity of normal fertilised oocytes did not differ between homogeneous and heterogeneous oocytes.

Nagano *et al.* (2006a,b) suggested that the morphological appearance of bovine oocytes was closely related to their ATP levels and cytoplasmic morphology provides an indication for the selection of oocytes with a high maturational and developmental ability. The oocytes used lipids as an oxidative substrate for nuclear and/or cytoplasmic maturation. Oocytes having brown ooplasm with a dark region at the periphery or dark clusters in their ooplasm produced relatively high levels of ATP at the GV and MII stages, resulting in good maturational and subsequent developmental ability. The oocytes with pale ooplasm (few lipid droplets) had low levels of ATP at the GV and MII stages, leading to poor maturational and developmental ability. In contrast, oocytes with black ooplasm (large amount of lipid droplets) had unusually high levels of ATP at the GV stage, leading to low maturational and subsequent developmental ability. Jeong *et al.* (2009) found that cytoplasmic colour was a useful selection parameter for abattoir-derived oocytes for IVP and had an inverse relationship with the lipid content of the cytoplasm. The cleavage and blastocyst rate was highest with dark colour (81.5% and 18.9%), followed by brown colour (67.7% and 15.5%) and pale colour (50.4% and 9.8%), respectively.

Various factors such as quality of oocytes, oxygen tension, embryo density, quality of sperm and culture conditions may influence the rate of preimplantation embryonic development. The IVF procedures in the present experiment were carried out on oocytes with intact and loosen cumulus cells.

In almost all species a certain proportion of cumulus cells are still present at the site of fertilisation (Motta *et al*., 1995; Yanagimachi, 1994). *In vitro* fertilisation in

the presence of cumulus cells increased the fertilisation rate in cattle (Chian *et al.*, 1996; Fatehi *et al.*, 2002; Tanghe *et al.*, 2003; Dey *et al.*, 2012), mouse (Fraser, 1985; Itagaki and Toyoda, 1991; Siddiquey and Cohen, 1982), hamster (Bavister, 1982), pig (Kikuchi *et al*., 1993) and buffalo (Nandi *et al.*, 1998).

Study of Tanghe *et al.* (2003) concluded that direct gap junctional communication between the oocyte and corona cells is needed to optimally support fertilisation. The cumulus cells are involved in the interaction between the male and female gametes, play a role as sperm trap by guiding sperm towards the oocytes (Hunter, 1988; Schroeder and Eppig, 1984). Sperm upon interaction with the cumulus oophorus encounters a higher redox state stimulating sperm penetration. Cumulus oophorus facilitates the fertilising ability of penetrated oocytes predominantly by secreting a complex mixture of secretions and metabolic product around the oocytes capacitation (Cox *et al*., 1993; Crozet, 1984), acrosome reaction (Chian *et al.*, 1995; Fukui, 1990), maintaining sperm motility and viability (Fukui, 1990; Ijaz *et al.*, 1994), preventing zona pellucida from hardening (Downs *et al.*, 1986; Katska *et al*., 1989, Mattioli *et al*., 1988) and enhancing sperm penetration and *in vitro* fertilisation (Cox *et al.*, 1993; Fukui, 1990; Magier *et al*., 1990; Legendre and Steward, 1993).

In cattle, the presence of cumulus cells surrounding the oocytes are not necessary for successful IVF (Behalova and Greve, 1993; Cox, 1991; Ball *et al*., 1983). It has been shown that almost equal rates of sperm penetration during IVF of cumulus-free and cumulus-enclosed oocytes. Study by Fatehi *et al.* (2002) demonstrated that the present of cumulus cells have a positive influence on the *in vitro* fertilisation. The cleavage rate significantly decreases when cumulus cells are

removed prior to *in vitro* fertilisation. These results were in agreement with previous reports showing that the presence of cumulus cells facilitated fertilisation of cattle oocytes leading to higher penetration rate (Cox, 1993; Chian *et al*., 1995; Chian *et al.*, 1996), higher cleavage rate (Younis and Brackett, 1991; Zhang *et al.,* 1995; Fukui, 1990; Liu *et al.*, 1995) and a higher yield of blastocyst (Zhang *et al.,* 1995; Liu *et al.,* 1995; Kim *et al.,* 1996). In other studies, (Behalova and Greve, 1993; Cox, 1991; Ball *et al.,* 1983) found that removal of cumulus cells did not affect the penetration rate. However, male and female pronucleus formation was impaired (Ball *et al.,* 1983) and higher incidence of polyspermy was observed (Bahelova and Greve, 1993).

Dey *et al.* (2012) revealed that co-culturing COCs and denuded oocytes leads to a synergistic effect on the *in vitro* embryo development potential and embryo quality of both COCs and denuded oocytes. Co-culturing denuded oocytes during IVM increases COC embryo development potential and the total number of blastomeres produced. The capacity for denuded oocytes to reach the blastocyst stage was improved by maturation with intact COC. This synergistic effect can be attributed to increased nuclear and cytoplasmic maturation, prevention zona hardening, and correction of the fertilisation pattern and increased the *GPX1* expression in the oocytes. Denudation at the beginning of IVM decreases the percentage of cattle oocytes that reach metaphase II, fertilisation and embryo development potential. Cox *et al.* (1993) showed that the positive effect of the cumulus cells on fertilisation only when the cells are in contact with the zona pellucida. However, the presence of loose cumulus cells during the IVF of denuded oocytes partially reverses the effect of denudation before fertilisation. The harmful effect of denudation on nuclear and cytoplasmic maturation, zona hardening, *GPX1* expression in the presumed matured

oocytes, the pattern of fertilisation and embryonic quality and developmental efficiency can be improved to the level of normal *in vitro* matured COC.

It is well established that the culture condition employed for IVM could significantly influence IVF rates and subsequently embryonic development. Several culture systems allow for the *in vitro* development of bovine embryos up to the blastocyst stage: cell-free systems (defined simple or complex media, semi-defined media with addition of BSA and/or serum, conditioned media) or co-culture with various somatic cell types (oviduct epithelial cells, granulosa or cumulus cells, Buffalo rat liver cells (BRL) (Bavister, 1995; Donnay *et al*., 1997; Habsah, 2006).

Study of Donnay *et al.* (1997) found that an improvement in the yield of blastocysts developing from singly cultured embryos was observed when co-culture with somatic cells was begun at the 9-16 cell stage or earlier. However, the favourable effect of co-culture on embryo growth, as reflected by the cell number of blastocysts cultured singly, could start between the 5-8 cell and the 9-16 cell stages. Co-culture does not improve the yield or the number of cells of blastocysts cultured in groups, which suggests that the beneficial effects of co-culture with somatic cells, and cooperation between embryos are not additive, at least for the studied parameters. They also found that the highest rate and best quality of blastocysts were achieved when the embryos were cultured with cumulus cells in mSOF supplemented with 10% FCS at 48 hours post-insemination.

Somatic cells act through physical contact of the cells and embryos and the cells secrete mitogenetic factors (Bavister, 1995). He proposed that the cells may secrete beneficial factors and/or remove detrimental factors such as one of the cells could be to lower the oxygen tension in the immediate vicinity of the embryo. The co-

culture with BOEC exerted a pronounced beneficial effect on development of *in vitro* fertilised cattle oocytes through the 16-cell block (Kim and Park, 1990). Habsah (2006) observed that higher numbers of 2-cell stage until the morula stage were observed in Groups A and B oocytes in co-culture system compared to mono-cultured system. In Group B', however, co-culture system produced better results compared to mono-culture systems in all developmental stages. Thus, the beneficial factors secreted by oviductal epithelial cells could help one another in the embryonic development especially in Group B' oocytes.

A cooperative effect between bovine embryos was demonstrated by Donnay *at al*. (1997) in cell-free culture system: mSOF supplemented with 10% FCS 48 hours post-insemination did not allow for the development of bovine embryos from the zygote to the blastocyst stage when cultured singly (0% blastocyst) or in groups of 3 or 6 (6% blastocysts), although 23% of embryos cultured per group of 20 under the same conditions developed into blastocysts. Nevertheless, no effect of embryo number was observed on the rate of early cleavages (5- to 8-cell stage). It was also demonstrated that embryo development or quality is enhanced by increasing the embryo : medium volume ratio (Paria and Dey, 1990; Lane and Gardner, 1992; Ferry *et al.,* 1994; Blondin and Sirard, 1995; Salehuddin *et al.,* 1995; Khurana and Niemann, 2000). This effect was generally attributed to the secretion by the preimplantation embryo of mitogenic factor(s) acting in an autocrine-paracrine fashion (Gandolfi, 1994) when they were allowed to develop close to each other and the inferior development of individually cultured embryos was improved by the addition of growth factors. Increasing the number of embryos per unit volume the concentration of inhibitory components in the medium is reduced on a per-embryo

basis or the concentration maintains some essential intracellular substances, such as amino acids, at a functional level (Bavister, 1995). This result contrasted with Lee and Fukui (1995) who observed no difference in blastocyst rates between bovine embryos cultured singly or in groups in SOF medium with FCS. Study of Khurana and Niemann (2000) found that development of IVM/IVF zygotes derived from the Category I (best quality) oocyte was not affected by the changes in the number of embryos per culture unit. However, the rate of development of one-cell embryos originating from Category II (good quality) oocytes significantly increased when cultured in groups of 40 (22.7% blastocyst) instead of 20 (13.6% blastocyst) embryos per 0.5 ml medium. Study conducted by Nagao *et al.* (1998) concluded that the number of embryos in a drop during *in vitro* culture seriously effect blastocyst development. They cultured different numbers of embryos (1, 5, 10, 25, 50, 100, 250) in a 50 µl drop of medium the blastocyst development of 25, 50 and 100 embryos in a drop (41-47%) were higher than the other groups.

5.1.2 Effect of IVM Duration on Fertilisation Rate after IVF with Different Oocyte Grades (Experiment 2)

The main findings from this study were: (1) IVM duration influenced the fertilisation rate with optimal duration of 24 hours, (2) morphological oocyte quality based on compactness and numbers of cumulus cell had significant positive effect on the rate of IVM and subsequent fertilisation rate with oocytes of Group A showed highest cleavage rate. These results were in agreement with that of Monaghan *et al.* (1993)

and Ward *et al.* (2002) who reported that 24 hours IVM duration was superior to 18 hours in terms of proportion of oocytes reaching the blastocyst stage.

The relatively low level of efficiency achieved using *in vitro* embryo production, manifested by the frequent failure of up to 60% of immature oocytes to reach the blastocyst stage, was almost certainly related to the quality of the oocytes at the beginning of maturation (Ward *et al.,* 2002). During IVM, the extrusion of the first polar body indicating the attainment of metaphase II occurred 18-24 hours after the initiation start of maturation (Gasparrini *et al.,* 2008). In general, by 16 hours, approximately 50% of oocytes were at telophase I in the process of polar body extrusions or had already reached metaphase II, and by 20 hours, more than 80% had reached metaphase II. Monaghan *et al*. (1993) reported that 24 hours was superior to 18 hours in terms of the proportion of oocytes reaching the blastocyst stage. Maturation for 16 and 32 hours resulted in significantly lower blastocyst yields than 24 hours with 20.4, 24.8 and 39.3%, respectively. There were no significant differences of blastocyst yields for 20 and 28 hours of maturation period with 26.4 and 26.5%, respectively (Ward *et al.*, 2002). The result was consistent with that of present study and other studies (Prokofiev *et al*., 1992; Long *et al.,* 1994; Gliedt *et al.,* 1996; Lonergan *et al.,* 1997).

However, Park *et al.* (2005) found that extrusion of the first polar body was highest between 14 and 16 hours of IVM and optimal embryo development at blastocyst stage and pregnancy rate was achieved by reducing the duration of IVM to 18 hours. This was in agreement with studies of Dominko and First (1992, 1997) and Semple *et al*. (1993). The rate at which the oocyte undergoes maturation has implications for subsequent development. In some studies, oocytes that developed fast

in vitro and extrude the polar body early (16 hours) during IVM are more likely to develop to the blastocyst stage (Dominko and First, 1992, 1997). The quality of this rapidly developing blastocyst was similar to those produced *in vivo* (Lonergan *et al.*, 1999). In addition, prolongation of metaphase II arrest before insemination led to gradual loss of successful fertilisation and embryo development (First *et al*., 1988) and an inappropriate time point of insemination could also lead to impaired development (Marston *et al.,* 1964).

In this present study, different oocyte qualities resulted in different fertilisation rates, and the optimal time for all maturation was 24 hours compared to 20 and 28 hours of maturation duration. Habsah (2006) demonstrated that maturation rate (based on MII stage) was significantly different ($P<0.05$) and indicated that the characteristics and quality of COCs influenced the resumption of meiotic process in bovine oocytes. The results of the present study confirmed the earlier finding of Leibfried and First (1979), Dalhausen *et al.* (1981) and Khurana and Niemann (2000). Under local setting, bovine oocytes of Group A of 24 hours of maturation gave highest maturation rate and cleavage rate compared to other COC groups.

The presence of cumulus cells was the most important factor for successful in *in vitro* maturation, fertilisation and culture. Cumulus cells surrounding immature oocytes, played a central role in developmental competence of oocytes by inducing both nuclear and cytoplasmic maturation and also act as a 'go-between' between oocytes and the follicular of culture environment (Sirard *et al*., 1989). In this present study, cleavage rate of Group B-homogenous of 24 hours oocytes was 65.92%, but for Group B-heterogenous oocytes, the cleavage rate was 30.48%. Kim and Park (1990),

Khurana and Niemann (2000) and Habsah (2006) showed oocytes of Group B' completed their first meiotic division with the values of 85, 71 and 59%, respectively. These results showed that Group B oocytes had the ability to mature *in vitro* due to the presence of cumulus cells projections embedded in the zona pellucida (Hytell *et al.*, 1986).

In this present study, all oocyte categories were treated with oestradiol-17β to the maturation medium. It was reported that addition of 1μg/ml oestradiol-17β enhanced maturation rate (Fukui and Ono, 1989; Habsah, 2006). Habsah (2006) reported that the addition of oestradiol 17-β and steer serum to the maturation medium promoted cytoplasmic maturation and consequently embryonic development in poorer quality groups of oocytes. However, Beker *et al.* (2002) reported that addition of 1μg/ml oestradiol to TCM199 significantly decreased the percentage of Metaphase II (MII) compared to control (56.3 and 74.0% respectively), and increased the percentage of nuclear aberrations compared to control (13.3% and 2.1% respectively). The negative effect of oestradiol on nuclear maturation was stronger when denuded oocytes were matured; 25.1 and 60.0% of the oocytes reached MII stage for the oestradiol and control groups, respectively.

These conflicting findings may be accounted for the presence of many factors affecting embryo development *in vitro* including different bulls used in different experiment and oocyte quality (Dominko and First, 1992; Sumantri *et al.*, 1997) transport time and temperature from the abattoir to the laboratory, follicle size, developmental stage of oocytes, oocyte diameter, composition of medium, hormone and serum used in the medium. The overall *in vitro* development of oocytes to the

blastocyst stage has remained between 10% and 30%, whereas oocytes from *in vivo* matured and *in vitro* fertilised and culture reach blastocyst varied from 50% and 80% (Dominko *et al.,* 1997; Rizos *et al.,* 2002). When cumulus-enclosed or denuded oocytes isolated from ovarian follicles and cultured *in vitro*, they resumed meiosis spontaneously and underwent GVBD (Edwards, 1965). During *in vivo* maturation, GVBD occurred between 4 and 8 hours after the LH peak (Kruip *et al*., 1983). The results of Sirard *et al.* (1989) delineated the timing of nuclear events in the bovine oocyte during IVM. The nuclear membrane underwent breakdown after 6.5 hours of culture. A polar body was observed 19 hours post-LH surge compared to 18 hours nuclear maturation *in vitro* proceeds at a similar rate to that *in vivo* if removal from the follicle is compared to time with the LH surge.

Other important factor that effect embryo development of IVM derived oocytes was sperm factor such as sperm-oocyte co-incubation duration. In this present study and other study (Long *et al.,* 1993), the gamete co-incubation duration was 18-20 hours. It has been reported that the duration of sperm-oocyte incubation affect bovine embryo development. Sumantri *et al.* (1997) demonstrated that sperm from sires of different breeds required different periods of co-incubation to achieve maximum fertility. Sperm from individual bulls had been reported to differ in their abilities to fertilise matured oocytes *in vitro* and in the development to the pre-implantation stage (Shi *et al.,* 1990, 1991). Similar findings were also reported in rams (Fukui *et al.,* 1988), sheep, goat and buffalo (Gasparrini *et al.,* 2008). It has been suggested that the optimal time for sperm-oocyte incubation for achieving maximum fertilisation rate after IVM-IVF was 24 hours (Rehman *et al*., 1994), however, study by Chian *et al.* (1996) showed that a significantly higher incidence of polyspermy

occurred if sperm-oocytes incubated for 24 hours compared to 8 hours. Sumantri *et al.* (1997) indicated that the rate of blastocyst formation decreases with increased of sperm-oocyte incubation time for each different bull. But, this finding was in contrast with Long *et al*. (1993) and Rehman *et al.* (1994).

5.1.3 Effect Serum Addition on Blastocyst Production after IVF (Experiment 3)

Results obtained from this present study showed that serum supplementation enhanced embryo development especially when added at later stages of embryonic development. Serum addition at morula and early blastocyst stage produced blastocyst, including fully expanded and hatched blastocyst.

Serum contains beneficial substances for embryonic development such as growth factors and chelators of heavy metals. The omission of serum in the co-culture system resulted only in blastocyst formation and no hatched and hatching blastocyst. Zona hardening has been reported to occur when serum or cumulus cells were excluded (Mochizuki *et al*. 1991). Habsah (2006) observed the mono-culture system resulted in slightly higher blastocyst rate compared to the co-culture system, and suggesting that omission of serum in co-culture system lowered the blastocyst formation. Serum in culture medium has been reported to promote hatching by providing a pool of plasminogen which in cattle embryos was converted to plasmin that proteolytically degraded the zona pellucida and facilitates hatching (Kaaekuahiwi and Menino, 1990).

Many investigators have reported the improvement in embryonic development with serum-supplemented media (Pinyopummintr and Bavister, 1991; Takagi *et al.*,

1991; Yoshioka *et al.,* 1997; Khurana and Niemann, 2000). The result obtained in the present study was in agreement with Lonergan *et al.* (1999), where they found that BSA and FCS significantly improved embryonic development when added to SOF which resulted in superior embryo quality. Additional FCS resulted in significant accelerated embryonic development such as more blastocyst development (on Day 6) and higher overall percentage of blastocyst with higher total cell numbers and hatching rates. Van Langendonckt *et al.* (1997) reported that the addition of FCS to mSOF at 42 hours post-insemination accelerated the development of bovine embryos between the 9- to 16-cell and morula stages and that the first blastocyst was observed 0.9 day earlier in mSOF supplemented with FCS. Yoshioka *et al.* (1997) reported that the development of morula to blastocyst was 1 day earlier in SOF plus FCS than in SOF plus BSA, even when the FCS supplementation was at 120 hours post-insemination. They suggested that FCS initiates earlier blastulation with fewer cells in the morula than when using BSA. Wang *et al.* (1997) reported that the development to the blastocyst stage and hatching rate were better in FCS-supplemented media than in media supplemented with BSA. The proportion of embryos that ceased development at the morula stage was greater in BSA-supplemented media.

Jung and Willard (2009) found that supplementation with FBS did not statistically improve the rate of blastocyst formation, although embryos were derived from culture medium with FBS supplementation exhibited a numerical increase in apparent developmental competence with 36.6% and without serum with 24.3% blastocyst formation. Pinyopummintr and Bavister (1991) found that serum supplementation exhibited a biphasic effect, with inhibition at the first cleavage and stimulation of morula compaction and blastocyst formation. However, blastocyst

development in TCM-199 supplemented with 10% BCS (29.7%) or with BCS + oviduct cell-conditioned medium (21.6%) was significantly greater than in non-supplemented HECM (hamster embryo culture medium), (9.7%) or TCM-199 (13.8%). Fukui and Ono (1989) reported that even the effectiveness of serum supplementation in the culture medium varies by different components of culture conditions used *in vitro*.

It has been reported that some batches of serum decreased the blastocyst rate and cell number and increase apoptotic cells on a particular culture system (Van Langendonckt *et al.*, 1997). Caro and Trounson (1984) did not find any improvement of the embryonic development with the serum-supplemented medium versus non-supplemented medium in the mouse. This was in an agreement with Abe and Hoshi (2003) who evaluated the quality of bovine embryos developed from IVM and IVF oocytes cultured in either serum-free or serum-containing media. Their result showed that bovine embryos cultured in serum-supplemented medium contain numerous cytoplasmic lipid droplets and immature mitochondria compared to those cultured in serum-free medium. The accumulation of cytoplasmic lipids in embryos developed in serum-containing medium may be a result of incorporation of lipoproteins from the serum and may result in impaired function of mitochondria. The survival and hatching rates of embryos produced in serum-free media after post-thaw culture were superior to those of embryos produced in the serum-containing medium, suggesting that the abnormal accumulation of cytoplasmic lipids in embryos may have a negative effect on the sensitivity of embryos to chilling and freezing.

Serum is a pathological fluid formed by blood clotting, which may induce chemical alterations with possible harmful effects for embryo culture. Serum and

serum albumin are two commonly used protein sources in media for the *in vitro* culture of mammalian embryos. Albumin not only serves as a low-affinity, high-capacity reservoir for certain beneficial components (e.g. steroids, vitamins, fatty acids and cholesterol), but also scavenges ions and small molecules (Maurer, 1992). Serum contains a variety of known and unknown substances which may stimulate or inhibit embryo development *in vitro* (Maurer, 1992; Bavister, 1995). Studies have shown that serum supplementation during the early cleavage stages of culture can inhibit the first cleavage but enhances the formation of morulae and blastocyst when serum is added during the later stage of embryo development (Pinyopummintr and Bavister, 1991; Lim *et al.*, 1994).

5.1.4 Effect of COC Quality on Fertilisation and Cleavage Rate after ICSI (Experiment 4)

In this present study, no significant difference in embryo development was observed at 2-cell, however, there were differences from 4-cell up to morula stage when compared between Grade A and B COC. Grade A COC gave the highest 2-cell cleavage rate (44.53%) followed by Grade B (38.94%) and Grade C (7.85%). However, morula stage embryos were only obtained in Grade A COC with a value of 16.65%. Oocytes with visible extruded polar body were selected to be used for ICSI injection in this study. However, the quality and morphological appearance of the polar body were not specified. In human, study of Martini *et al.* (1997) and Fancsovits *et al.* (2006) found that lower rates of fertilisation, cleavage and formation of good quality embryos resulting from oocytes with an enlarged first PB and suggested that

oocytes with enlarged first polar body should not be used for ICSI procedures. The presence of an enlarged first PB may be associated with disproportional segregation of the mitochondria and proteins needed for the occurrence of normal fertilisation and embryo development preimplantation (Navarro *et al.*, 2009).

The low survival and fertilisation rates obtained in the present study was believed to be attributed to many factors, such as wrong selection of morphologically matured oocytes, the poor 'wound-healing' of the oolemma and size of the injection needle. Furthermore, it was admitted that the author had limited technical skill and experience in handling and preparing the micropipettes as well as conducting injection. Nagano *et al.* (2006a) found that the low developmental rates of bovine *in vitro* matured oocytes may be due to degeneration, whereby bovine is a mono-ovulator, therefore most of the oocytes for *in vitro* embryo production are destined to degenerate. It was reported that oocytes with a multi-layered cumulus showed higher nuclear maturation, fertilisation and developmental rates than denuded and oocytes with corona radiata alone (Xu *et al.*, 1986; Shioya *et al.*, 1988). Nagano *et al.* (1999) suggested that denuded oocytes and oocytes with corona radiata alone showed less developmental capacity and that the appearance of the ooplasm reflected the *in vitro* developmental capacity of the oocytes. Byskov (1978) and Hytell *et al.* (1986) found that the nuclear morphology and arrangement of the organelles in the degenerating oocytes are similar to those in maturing oocytes and the phenomenon was called as pseudomaturation. Oocytes from small follicles (2-8 mm) with undulating nuclear membrane and oocyte that had organelle-free area in their peripheral region, which generally appeared 12-18 hours after maturational culture may enter the pseudomaturation phase (Hytell *et al.*, 1986; Nagano *et al.*, 2006a).

In present study, the preliminary work was done by using commercial injection pipette designed for human. The size of the sperm head is dependent on each individual bull. The overall length of bovine sperm is approximately 68 to 74 μm, with the head is about 8-10 μm. The widest portion of the head is about 4 to 5 μm (Salisbury *et al.,* 1978). Human sperm has the head size of 5 μm by 3 μm and a tail 50 μm long (Smith *et al*., 2009). Therefore, the commercial injection pipette used (with outer diameter 6-7 μm) was slightly smaller compared to bovine sperm head size. There was an extremely difficult to suck the larger bovine sperm head and caused sperm head sticking to the opening of injection pipette. The high pressure used to suck the sperm caused sperm head damage, and excessive PVP sucked together with the sperm. To overcome the problem, in this present study, home-made injection needle was used. Due to lack of experience, the injection needle was prepared with respective size of inner and outer diameter of 10 and 12 μm. The inner and outer diameter and the wall thickness have significant influence on the characteristics of the needle obtained that consequently affecting the ICSI performance. The pore size of the injection pipette in bovine ICSI is larger than that used from human. Therefore, the breakage of oocyte plasma membrane (oolemma) by aspiration results in remarkable damage and the oolemma of bovine oocytes are elastics like those of mice and it was difficult to puncture them by conventional ICSI (Horiuchi *et al*., 1999a). In this present study, the conventional ICSI was used with semi-automatic micromanipulator. The research by Horiuchi *et al.* (2002) found that ICSI using a piezo-electric actuator could produce live bovine offspring efficiently due to an affective sperm injection into oocytes using that method. Since the oolemma is very flexible and 'tough', the conventional pipette cannot readily break the membrane. It

did not break even if the tip of a sharply pointed injection pipette reached the centre of the oocyte. It was highly probable that the oolemma was not broken and therefore the sperm was not injected into oocytes. The oolemma may be broken when the pipette tip was pushed to the almost opposite side of the oocyte, but even in such cases the oolemma of many oocytes may not be broken. When the pipette was inserted to the centre of the oocytes and the ooplasm was then aspirated into the pipette, the oolemma may be broken, the sperm could be injected but many or most of the oocytes degenerate later (Keskintape and Brackett, 2000; Horiuchi *et al.*, 2002). Katayose *et al.* (1999) showed that in comparison with conventional ICSI, the rate of male pronuclear formation yield after ICSI using piezo-micromanipulator was significantly higher (81 versus 21%). They produced 50% of ten recipients receiving blastocyst produced by piezo-ICSI with immobilised sperm were pregnant at day 60-80 of gestation period and five normal and healthy calves were delivered.

The difficulty in puncturing bovine oocyes oolemma, the observation and identification of sperm injected into cytoplasm are difficult because bovine oocyte are opaque and the detail of ICSI process are difficult to observed due to bovine oocytes contain vacuoles and lipid droplets in the cytoplasm which prevent clear visualisation. Therefore, the successful puncture of oolemma insufficiently accurate (Horiuchi *et al.* (1999a). In this present study, no centrifugation method was done to facilitate sperm injection. Tatham *et al.* (1996) used centrifuged oocyte before ICSI. Centrifugation permits the removal of excess lipid for visualisation and does not affect subsequent development and facilitated the visualisation during sperm injection. Centrifugation enhances the visibility of nuclear elements, such as germinal vesicles (GV), metaphase spindles and pronuclei, The conformation of a sperm injected into ooplasm

must be effective in order to increase fertilisation rate per injected oocytes (Horiuchi *et al.*, 1999a). The centrifugation of bovine, porcine and murine zygotes does not affect subsequent development (Wall *et al.*, 1985; Nakamura *et al.*, 1986; Wall and Hawk, 1988) and facilitates the visualisation and microinjection of DNA into rabbit, sheep, pig and mouse zygotes (Hammer *et al.*, 1985; Brinster *et al.*, 1985).

5.1.5 Effect of Sperm Quality on Fertilisation and Cleavage Rates after ICSI (Experiment 5)

The present study indicated that sperm quality affects the fertilisation and cleavage rates after ICSI in bovine. Intact-immobilised sperm showed the highest cleavage rate compared to other sperm type ($P<0.05$). This finding was in agreement with Horiuchi and Numabe (1999) and Wei and Fukui (1999). Horiuchi and Numabe (1999) found that motile sperm immobilised by a tail scoring injected into bovine oocytes, followed by the treatment of artificial oocyte activation (7% ethanol for 5 minutes) increased the development rate to more than two cell stage in bovine oocytes with a second polar body after ICSI (74 versus 33%). The blastocyst rate was increased by treatment with 7% ethanol for 5 minutes (29 versus 14%), and thought that the low cleavage rate of ICSI bovine oocytes without additional activation stimuli is caused by insufficient stimulation of oocyte activation for embryo development, not resumption of meiosis and extrusion of the second polar body.

Wei and Fukui (1999) also investigated the effects sperm type (dead, immotile or motile) and sperm pretreatment such as mechanical (tail cutting or tail-scoring) on male pronuclear formation after intracytoplasmic sperm injection (ICSI) in cattle. The

effects of sperm type was examined found that no significant difference on the male pronuclear formation rate when the three types of sperm were injected into oocytes. However, tail-scored sperm achieved a higher male pronuclear rate than that of non-mechanically treated (38.2 versus 13.2%). Immobilisation of a sperm by tail-scoring before ICSI can improve the formation of the male pronucleus.

During ICSI, in several species, the injection procedure itself is apparently sufficient to activate the oocytes, as the sperm nucleus can undergo decondensation and formation of a pronucleus when injected in to the oocytes. In contrast, ICSI with the bovine sperm rarely leads to its decondensation and male pronucleus formation during subsequent culture *in vitro* (Catt *et al*., 1995; Parreault *et al*., 1988; Keefer *et al.,* 1989; Westhusin *et al.,* 1984). Permeabilisation of the sperm membrane by physical means may have a role to play in facilitating decondensation and pronucleus formation after ICSI. In order to improve pronucleus formation following ICSI of bovine oocytes, sperm have often been pre-treated by various methods, including artificial removal of the acrosome and tail by sonication (Kuretake *et al.,* 1996), immobilising sperm and damaging the sperm membrane by freezing and thawing before injection (Goto *et al.,* 1990; Keefer *et al*., 1990) and crushing the sperm tail with the micropipette used for injection have been reported to improve results (Lacham-Kaplan and Trounson, 1994). In this present study, immobilisation was applied by resting injection needle on the tail of a motile sperm which it then presses against the floor of the dish while the injection needle is moved outwards. When performed correctly the sperm can be seen to rotate during the maneuvers and the tail will become kinked. The same method applied by other researcher (Dorzortsev *at al.*, 1995b; Wei and Fukui, 1999; Boediono, 2001; Garcia-Rosello *et al.*, 2009). The

success of sperm immobilisation is believed to be due to local disruption of the plasma membrane. As the sperm plasma membrane has no wound-healing ability, the entire plasma membrane must disintegrate faster within the ooplasm when it is locally damaged prior to ICSI than when a membrane-intact sperm is injected. These treatments focused on to improved male nuclear formation, enhance fertilisation and cleavage rate (Horiuchi and Numabe, 1999; Boediono, 2001; Horiuchi *et al*., 2002) and make the sperm-borne oocyte-activating factor more easily available to the cytoplasm of the oocyte (Garcia-Rosello *et al.*, 2009). In contrast to human sperm that needs plasma membrane damage prior to ICSI for sperm nucleus decondensation, in bovine immobilisation of a sperm by tail-scoring before ICSI is sufficient to improve the rate of male pronucleus formation but not to promote pre-implantation development at the same rate as IVF. The procedures that are usually used for sperm capacitation before ICSI are not sufficient in comparison with complex process of capacitation during IVF, where sperm also capacitates passing through several natural barriers such as cumulus cells and zona pellucida (Galli *et al*., 2003b).

In this present study, natural and newly dead sperm was used to be injected into oocytes. The sperm was tail-scored and injected into oocytes. There was no significance different between oocyte grades observed. In early reports on bovine ICSI, many researchers (Westhusin *et al.,* 1984; Younis *et al*., 1989a; Keefer *et al*., 1990) selected dead sperm for practical use or for basic information on the fertilisability of sperm nuclei and their results showed that sperm nuclei transform into male pronuclei; however, the fertilisation rates were very low. Goto *et al.* (1990) used capacitated bull sperm killed by repeated freezing and thawing without cryoprotectant and successfully produced embryos and the first ICSI calves were

born. This result showed that bovine embryos produced by ICSI with a dead (killed) sperm could developed into live calves. Sperm nuclei could transformed into male pronuclei even if sperm did not survive, however fertilisation and cleavage rate were very low (Younis *et al.*, 1989a; Keefer *et al*., 1990; Heuwieser *et al.*, 1992). Horiuchi *et al.* (2002) found that the sperm "killed" by repeated freeze-thawing were far inferior to "immobilised" sperm in their ability to participate in embryo development with blastocyst formation were 2% and 70%, respectively. This is because immobilised sperm were injected into oocytes almost immediately after the plasma membrane was disrupted, while killed sperm were injected some time (as long 1 hour) after their plasma membrane disruption. For other species, Hoshi *et al*. (1994) and Katayose *et al*. (1992) found that freeze-dried hamster and human sperm nuclei remain able to develop male pronuclei even after 12 month of storage at 4°C after injected into human and hamster oocytes. Other studies done by injecting freeze-dried sperm into same species were in rabbit (Hoshi *et al*., 1994), cattle (Keskintape *et al.*, 2002) and pig (Kwon *et al.*, 2004) oocytes were produced embryo at blastocyst stage. Live offspring produced were reported in mouse (Ward *et al*., 2003) rabbit (Liu *et al.*, 2004) and rat (Hirabayashi *et al.*, 2005) sperm.

The other method of sperm treatment used recently other than freeze-thawed sperm was heat-dried sperm. Lee and Niwa (2006) injected bovine sperm that were dried at 50°C for 8 hours and stored 4°C for 1 month and resulted 15% blastocyst stage embryos. They concluded that bovine sperm dried by heating and used for ICSI could induced oocyte activation and subsequent embryo development. Other studies done using heat-dried sperm were by Yanagida *et al.* (1991) whose isolated sperm nuclei from hamster mouse and human and heated 90°C for 30 minutes and injected

into rabbit oocytes and produced pronuclei. Mouse sperm heated 56°C for 30 minutes were shown to support full embryonic development was done by Cozzi *et al*. (2001) and produced embryo at 8-cell stage in rabbit (Hoshi *et al*., 1992).

In this present study, sham injected oocyte was used as a control method for ICSI. During ICSI in human, rabbit or mouse oocytes, puncture the oolemma and aspiration of cytoplasm is sufficient for oocyte activation (Catt *et al*., 1996). In contrast, bovine oocytes are not activated only by mechanical treatment with the injection pipette or by the injected sperm. Bovine oocytes require additional activation by chemicals or other stimuli (Rho *et al.*, 1998a,b) which is prerequisite for decondensation of the sperm head and formation of the male pronucleus.

In this present study, in all sperm treatments, oocyte activation was performed by treated injected oocytes with 7% ethanol for 5 minutes. It is well known that successful of bovine ICSI also depends on proper activation of the oocytes (Keefer *et al.*, 1990; Lacham-Kaplan and Trounson, 1994; Younis *et al.*, 1989a). Mechanical stimulation by the injection pipette alone can only occasionally bring this about in cattle; in more than 95% of cases of stimulus is insufficient (Keefer *et al.*, 1990; Rho *et al*., 1998a). Activation can be induced by a variety of stimuli, including exposure to calcium ionophore (Ware *et al.*, 1989), ethanol, (Nagai, 1987; Minamihashi *et al.*, 1993), electric current (Ware *et al.*, 1989; Powell and Barnes, 1992), cycloheximide and 6-dimethylaminopurine (DMAP) (Fulka *et al.*, 1991). Activation of oocytes with additional stimuli during the ICSI procedure increases free cytosolic calcium which causes the destruction of the cytostatic factor (CSF), degradation of the maturation promoting factor (MPF), reinitiation of meiosis, extrusion of cortical granules and

transformation of sperm and oocyte nuclei into male and female pronuclei, respectively, which is turn leads to cleavage (Stojkovic *et al*., 1999). Each step in ICSI procedure (such as puncture of the oocyte with a pipette, injection techniques, the injection of Ca^{2+}-containing medium and the sperm itself may potentially contribute to oocyte activation and subsequent cleavage (Dosortsev *et al.,* 1995a,b*;* Dosortsev *et al.,* 1998). Tesarik *et al.,* 1993 reported that oocyte activation is started after a considerable lag period following sperm injection and activation is probably caused by a soluble factor released from the exogenous sperm cell. When a motile sperm immobilised by tail-scoring was injected into oocytes, the treatment of artificial oocyte activation (7% ethanol for 5 minutes) increased the development rate to more than two-cell stage in bovine oocytes with a second polar body after ICSI (74 versus 33%), the blastocyst rate was increased by treatment with 7% ethanol for 5 minutes (29 versus 14%). Therefore, the low cleavage rate of ICSI bovine oocytes without additional activation stimuli is caused by insufficient stimulation of oocytes activation for embryo development, not resumption of meiosis and extrusion of second polar body (Horiuchi and Numabe, 1999).

In contrast to the need of chemical activation after ICSI, it is well documented that ICSI without chemical activation was enough to activate oocytes in hamster (Hoshi *et al*., 1992), rabbit (Keefer, 1989) and human (Van Steirteghem *et al.,* 1993a). It was reported that only the presence of a sperm was necessary for oocyte activation (Dale *et al*., 1985; Ainul, 2010). Lacham-Kaplan and Trounson (1995) obtained cleavage rate (38%) after conventional ICSI in mouse without prior injection of $Ca2^{+}$. It was demonstrated that injection of $Ca2^{+}$ other than present in the medium was not required to achieve high fertilisation rates in human (Van Steirteghem *et al.,* 1993b).

5.1.6 Comparison between IVF and ICSI on Fertilisation and Cleavage Rate (Experiment 6)

IVF and ICSI are considered among the most important methods employed to produce IVP embryos for embryo transfer, cryopreservation and manipulation. In this experiment, comparison was made between IVF and ICSI performance by using *in vitro* matured oocytes from slaughtered cattle and of frozen-thawed sperm. IVF gave higher cleavage rate than ICSI. There were no ICSI embryos developed after morula stage. IVF embryos were developed up to blastocyst stage (8.94%). Therefore, IVF was assumed to be effective than ICSI in generating embryos at this present study setting. However, the efficiency of ICSI and IVF cannot be confirmed based on the results of the present study as the survival and fertilisation rate of ICSI in this study was very low.

The direct comparison between IVF and ICSI in producing embryos by using cryopreserved sperm has confirmed that ICSI has many advantages compared with IVF. ICSI requires only a single sperm to fertilise oocytes, while IVF needs hundreds of thousands of motile sperm in the fertilisation medium even for a few oocytes. A failure in cryopreservation may result in very low number of motile sperm which is too low for IVF; a lot of sperm in the sample are lost. Study of Ward *et al*. (2002) found that a concentration of 0.125×10^6 to 0.5×10^6 sperm/ml provided enough sperm to optimise blastocyst development and was superior in terms of cleavage rate to all concentrations below 0.25×10^6 sperm/ml. However, the problem with low sperm count can be solved by employing ICSI technique. Theoretically, with the ICSI techniques, thousands of calf can be produced from the cryopreserved sperm of a single male, provided the sperm are stored in different straw. In human, the used of

single sperm in ICSI has helped and was widely used to treat male infertility, such as obstructive (azoospermia) where approximately 6% of infertile men have blockages in the genital tract that prevent the passage of sperm between the testes to the penis and resulted in absence of sperm in the semen; men had problem with sperm antibodies, dilated veins in the scrotum (varicoceles) were often present in men (20-40%). Men with subfertility problem, almost 65% men investigated for infertility have sperm present in the semen, but lower numbers than normal, namely oligozoospermia (35%); or adequate numbers, but with reduced motility (asthenozoospermia), abnormal morphology (teratozoospermia) or a combination of both (25%). A few (5%) have normal semen tests (normozoospermia) but there are other abnormalities which impair the fertilising ability of the sperm. At present, very little is known about the mechanisms by which sperm production and function are reduced either in men with an obvious cause such as previous undescended testes or inflammation of the testes, or in men who have no apparent cause for the problem. There are also men in general community with poor sperm test results who have no major problems producing pregnancies (World Health Organisation, 1992; Irwine, 1998; Barratt and John, 1998).

COC played an important role during *in vitro* maturation and fertilisation. The importance of cumulus cells for the maturation and acquisition of developmental competence in oocytes has been established. Removal of cumulus cells from the oocytes prior IVM adversely affects the maturation, fertilisation and embryo development (Zhang *et al.,* 1995; Leibfried-Rutledge *et al.,* 1989; Kim *et al*., 1996; Fatehi *et al.,* 2002). In the present study, during IVM and IVF, the COC were let intact. After IVM, IVF was carried out without removing cumulus cells, involving

oocytes with expanded cumulus cell were inseminated. As for ICSI, after IVM procedure, the oocytes were fully denuded to remove cumulus cell and corona radiata. Cumulus cells influenced in fertilisation by attracting, trapping and selecting sperm (Chian *et al.,* 1996); facilitating sperm capacitation, acrosome reaction and penetration (Cox *et al.,* 1993; Chian *et al*., 1995) as well as preventing precocious hardening of the zona pellucida (Katska *et al.,* 1989). Usually, denuding oocytes was done to perform invasive procedure for embryo sexing and micromanipulation techniques such as nuclear transfer and ICSI, to study sperm-oocyte interaction and to evaluate morphological aspects of oocyte quality, such as the identification of polar bodies, ooplasm granulation and vacuolisation and extent of perivitelline space (Gordon, 1994; Tanghe, 2003). Tanghe (2003) concluded that cumulus oophorus facilitates fertilising ability of penetrating oocytes predominantly by secreting a complex mixture of both cumulus secretions and metabolic products around the oocyte. The importance of gap junctional communication between oocyte and corona cells, and of sperm trapping by the cumulus oophorus during IVF was essential factors to optimally support the fertilisation process. Dey *et al.* (2012) found that co-culturing denuded oocytes with cumulus cells during IVM increased COC embryo developmental potential and total number of blastomeres produced. Capacity of denuded oocytes to reach blastocyst stage, together with the actual number of blastomeres, was improved by maturation with intact COC. These synergistic effects was probably attributed to increase nuclear and cytoplasmic maturation, preventing of zona pellucida hardening, correction of fertilisation pattern and increased glutathione peroxidase 1 (*GPXI*) expression in the oocytes.

Fertilisation is a complex process of molecular events involving mature haploid male and female gametes and their mutual recognition and fusion to establish the genotype of new individuals. Fully grown, matured oocytes and normally differentiated haploid sperm were the prerequisite for the success of the fertilisation process (Kupker *et al.*, 1998). In the present study, the normal frozen sperm were used for IVF and ICSI treatment. During IVF, morphological normal sperm was a very important factor that affected fertilisation and cleavage outcome. Ampullary sperm was highly selected population with a great facility to penetrate oocytes. To acquire fertilisation ability, sperm underwent modification, involving capacitation, hyperactivation, acrosome reaction and finally penetration of zona pellucida (Cox *et al.*, 1993; Chian *et al*., 1995; Mortimer, 1997). Therefore, sperm morphology defects such as sperm with nuclear or head defects (teratoid, double, macrocephalic, microcephalic, rolled/crested head, pyriform, tapered), acrosomal or tail defects (knobbed acrosome, stump tail, pesudodroplet, cork screw, fractured flagellum, distal droplet, bent midpiece) were avoided during IVF (Walters *et al.,* 2005; Nothling and Irons, 2008). As for ICSI, all the events were normally associated with sperm entering into the ooplasm were bypassed and modified after ICSI. ICSI was applied in cytoplasmic injection techniques, such as the injection of morphologically abnormal sperm, spermatids and spermatocytes (Hewitson *et al*., 2000). The ability of obtaining fertilisation with injection of sperm head had simplified ICSI. In mice, numerous reports had been published on the normal fertilisation achieved after injection of oocytes with sperm heads which were isolated by various techniques, such as piezo pulses (Kimura *et al.,* 1998), sonication (Kuretake *et al.,* 1996), mechanically detached using injection needle (Dosortsev *et al*., 1997) or by the freeze-thawed

procedure (Moreira *et al*., 2005). However, to carry on the exogenous DNA, round spermatid had been suggested to be more effective than sperm, because of the nuclear proteins of spermatids are formed with histones and removal of disulfide bonds led to the complex compaction of the DNA (Yamazaki *et al.,* 1998). In cattle, an attempt to produce embryo by using round spermatid was still low (Ock *et al.,* 2006). However, Goto *et al.* (1996) successfully produced blastocyst after injecting round spermatid. They reported that development of the embryos reconstructed with round spermatid still remains low. Two methods were reported for producing embryos reconstructed with round spermatid. These include intracytoplasmic injection of round spermatid into ooplasm (ROSI) (Ogura and Yanagimachi, 1993; Goto *et al.,* 1996) and electric fusion after round spermatid insertion into the perivitelline space of the oocytes (Ogura *et al*., 1994). Both methods had resulted in obtaining offspring in mice (Ogura *et al*., 1994, 1996), rabbits (Sofikitis *et al.*, 1994), and humans (Tesarik *et al*., 1995). However, ROSI had a relatively low efficiency in embryo development and pregnancy after transfer into surrogate recipients and offspring were obtained by ROSI in cattle (Goto *et al*., 1996).

In the present study, normal IVM without cryopreservation oocytes were used. The use of cryopreserved oocytes to be fertilised by IVF or ICSI techniques had been studied to compare both techniques. Mavrides and Morroll (2005) showed the cleavage rate in controls (non-cryopreserved IVM oocytes with standard IVF) was much higher than ICSI. When cryopreserved oocytes were used, the rates were lower in each group. The cleavage rates were similar between study groups in ICSI but very different when IVF was used. This was believed to be due to the effect of cryopreservation on zona pellucida hardening (Hinsch *et al.,* 1994; Matson *et al.,*

1997). Hinsch *et al.* (1994) found that during IVF, the number of sperm binding to the zona was not altered, but there was a reduced zona penetration in oocytes exposed to standard IVF sperm solution. Compared to IVF, ICSI bypassing the effect the zona pellucida changes after cryopreservation and improved fertilisation rates. Studies in other species including humans, insemination with ICSI following cryopreservation yields a much better fertilisation rate compared to standard IVF giving 46% versus 13.5% in humans and 16.5% versus 7.9% in mice. The reason for this was mainly attributed to zona hardening and reduction of ZP3 receptors in the zona, which were responsible for sperm binding (Kazem *et al.,* 1995). Other than oocytes, sensitivity of bovine embryos to cryopreservation was also affected by method of embryo production. The IVF-derived zygotes have lower cryotolerance than *in vivo* derived counterparts (Massip *et al.,* 1995). The faster-developing IVF-derived bovine blastocysts (harvested 7 days after insemination) were cryopreserved better than slower-developing ones (harvested 8 days after insemination) (Han *et al.,* 1994; Saha *et al.,* 1996; Dinnyes *et al.,* 1999; Gomez *et al.,* 2008). Among blastocysts which developed on the same day, embryos reaching more advanced stage or larger diameter were more likely to survive cryopreservation (Han *et al.,* 1994; Dinnyes *et al.,* 1999; Park *et al.,* 1999; George *et al.,* 2008). Abdalla *et al.* (2010) found that ICSI-derived expanded blastocysts (≥200 μm in diameter) were slightly sensitive to vitrification than IVF-derived. They recommended that *vitro* culture of ICSI-derived embryos (until fully expanded blastocyst) should be prolonged prior to cryopreservation.

5.2 GENERAL DISCUSSION

In the present study, production and manipulation of bovine embryos by using various assisted reproductive techniques (ART) were carried out. Numerous factors affecting the outcomes of IVF and ICSI performances were discussed in previous section. This section will discuss general aspects relating to this research.

5.2.1 *In Vitro* Production of IVF Embryos

It is well known that the following factors affect the *in vitro* fertilisation performance such as oocyte quality, *in vitro* maturation duration, concentration and quality of sperm, *in vitro* culture condition and co-culture. Numerous studies have been conducted to optimise the *in vitro* environmental conditions for the IVMFC procedure. In the present study, the factors affecting IVF-derived embryos were focused on oocyte quality, IVM duration and serum addition in culture medium. The results of the present study demonstrated that the COC and ooplasm qualities during maturation influenced the cleavage rate after IVF. The characteristics and quality of cumulus cells are the main factors involved in determining the ability to attain proper oocyte maturation and subsequent developmental ability after fertilisation. Morphological oocyte quality based on compactness, number of cumulus cell layers and ooplasm quality had significant positive effects on the rates of IVM and IVF. The oocytes of these different groups were shown to be able to mature and fertilise *in vitro*. Oocytes of Group A (compact oocytes and dense with more than 5 layers of cumulus cells) showed the highest cleavage rate followed by Group B (oocytes with less than 5 layers and some with partially naked) and Group C (naked oocytes). The

differences in ooplasmic quality also affected maturation and fertilisation rate after IVF. In the present study, the ooplasm was divided into 2 groups, namely homogenous (brown-coloured ooplasm) and heterogenous (light-coloured ooplasm).

The homogenous oocytes of different group of COC gave higher cleavage rate compared to heterogenous oocytes. This result suggested that the appearance of the ooplasm reflected the *in vitro* developmental capacity of the oocyte. The brown ooplasm indicates an accumulation of lipids and good developmental potential, while a light-coloured ooplasm had low density of organelles and poor developmental potential. The maturation duration was also affected cleavage rate after fertilisation. In the present study, the 24 hours maturation duration gave the highest cleavage rate after fertilisation followed by 20 hours and 28 hours in homogenous ooplasm compared to heterogenous ooplasm with Group A gave highest cleavage rate followed by Groups B and C oocytes. In order to enhance blastocyst formation after culture, the effect of serum addition in culture medium was studied with addition of 10% steer serum at different embryonic developmental stages. Serum addition at morula and later stage starts showed improvement in blastocyst and hatching rate compared to early embryonic developmental stages (at 2-, 4- and 8-cell stages) and control. It was believed that serum promote embryo hatching by providing a pool of plasminogen which in cattle embryos was converted to plasmin that proteolytically degraded the zona pellucida and facilitates hatching (Kaaekuahiwi and Menino, 1990) and produced superior quality embryos (Lonergan *et al.*,1999).

In this study, the significant factors which were identified that contribute to the low cleavage rate and development into blastocyst *in vitro* in IVF embryos were the lower quality and quantity of oocytes collected from the local abattoir. This was

reflected in the low cleavage rates obtained. The ovaries were collected from unknown source of slaughtered cattle, that is with unknown age, breed, health status and reproductive background. Furthermore, the number of slaughtered cattle was quite limited which produced low quality ovary. The unknown reproductive cycle during slaughtering resulted in unknown stages of oestrous cycle. Most of the cattle were slaughtered at old age. Therefore, small and depletion in the number of follicles in the ovary were obtained with low quality oocytes produced. Another critical issue was the competition with other research institutions for the limited number of ovaries available at the slaughter house. Normally, these limited ovaries were shared among the researchers involved. Due to lack of oocytes sources, all grades of oocytes were used in this study. The used of all grades of oocytes quality may reduce percentage of oocyte cleavage and less ovary resulted low number of replication in experiment.

In summary, from the results of this study indicated that cattle oocytes harvested from the local abattoir were able to develop via IVF in our culture system, regardless of the quality of oocytes. It was clearly shown that lower quality oocytes would develop but into lesser number of viable embryos.

5.2.2 *In Vitro* Production of ICSI Embryos

In general, factors known to affect the outcome of ICSI experiment such as sperm and oocyte quality, sperm treatment and oocyte activation before and after ICSI, injection procedure and microtools preparation such as injection and holding pipette; and the use of piezo injection pipette. Numerous studies have been conducted to optimise the

in vitro environment conditions for the ICSI procedure. In the present study, the factors affecting ICSI-derived embryos were focused on sperm quality and treatment; and cumulus-oocyte quality. The results of the present study demonstrated that the ICSI embryos can be produced with different sperm and oocyte quality. However, the ICSI experiment in the present study did not achieve much significant result. However, it did provide author the knowledge and technical aspects as a learning curve in terms of micropipette preparation and injection techniques involved which could be applied later in micromanipulation studies.

In this present study, conventional ICSI was performed using frozen-thawed sperm with intact-immobilised, dead-immobilised and intact sperm. This study was conducted with Group A COC of intact-immobilised sperm which showed the highest cleavage rate after ICSI, followed by sham, dead-immobilised and finally intact sperm. Due to embryo development after sham injection and only progress up to 4-cell stage, it was believed and proven after embryo staining that partinogenetically embryo could be produced with this sham injection technique. This was believed to be due to injection itself, hyaluronidase exposure during oocyte denudation and oocyte activation with 5% ethanol after ICSI. Similar trend of embryo development was observed in dead-immobilised sperm. The embryo only developed until 4-cell stage. Even though Goto *et al.* (1990) produced ICSI calves by using dead sperm, other researchers (Younis *et al.*, 1989a; Keefer *et al*., 1990; Heuwieser *et al.*, 1992) found that the fertilisation and cleavage rate of this type of sperm were very low. When compared to immobilised sperm to dead sperm, the blastocysts were 70% and 2%, respectively (Horiuchi *et al.*, 2002).

The quality of oocytes affected fertilisation and embryonic development after ICSI. Even though ICSI bypassed all oocyte barrier factor and only oocytes with extruded polar body were injected, the low quality oocytes of Grade C only developed up to 2-cell stage. The Grade A oocyte developed until morula stage followed by Grade B oocytes at 8-cell stage. This was believed to be due to role of cumulus and mural granulosa cells together with the oocyte form a gap-junction-mediated syncytium, which is essential for oocyte growth to proceed. The granulosa cells supply the oocytes with nutrients and connect to the external world, and by using a highly selected population of good quality oocytes, it is possible to increase the efficiency in terms of proportion of oocytes progressing to morula and blastocyst (Van Soom *et al*., 2002). The other factor was the timing of the appearance of the first polar body in combination with timing of fertilisation influenced the developmental competence of the zygotes (Dominko and First, 1997).

Comparison study of IVF versus ICSI, it was found that cleavage rate and embryo development in IVF embryos were higher than ICSI. The difference in embryo obtained in this study was believed to be due to lack in author experience in micropipettes preparation especially injection needle and sperm injection procedure itself. There were problems encountered during the making of micropipettes. At the earlier part, borosilicate capillaries were used with an inner and outer diameter of 0.6 and 0.97 mm, respectively and a length of 10 cm. The size of inner diameter was 8-10 μm with 45° bevel and sharp spike at the tip. However, the injection procedure was found to be extremely difficult as the pipette was observed to have difficulty to penetrate the though and elastic zona pellucida and oolemma, where in most cases, the pipette was slightly bent (as a result of extreme puncture). This was believed due

to spike was not sharp enough. From personal observation, the wall thickness of the injection pipette was slightly thick, the lumen diameter of injection pipette was lesser and sometimes was just fit enough to suck sperm with the head size is about 8-10 μm. Therefore, bigger needle opening was prepared and resulted more damage to the oocyte and it was observed that most of the oocytes lysed after injection. As alternative, a thin walled glass capillary with inner diameter (0.78 mm) and outer diameter (1 mm) (Clark Glass, England) was used. This seemed slightly improved the injection procedure where thinner yet harder injection needle was prepared. Other regular problem encountered in this present study was attachment of the sperm on the spike and outer part of injection needle during sperm release in oolemma. Therefore, it required rotating and sometimes a coarse movement in order to release sperm into ooplasm. This might have damaged the oocytes and thus poor cleavage rate.

5.3 RESEARCH CONSTRAINTS

There were a few constraints indicated in this research such as lower quality and quantity of oocytes collected from the local abattoir was reflected in the low cleavage rates obtained. The ovaries were collected from unknown source of slaughtered cattle that is with unknown age, breed, health status and reproductive background. The number of slaughtered cattle was quite limited which produced low quality ovary. Most of the cattle were slaughtered at old age. Therefore, small and depletion in the number of follicles in the ovary were obtained with low quality oocytes produced. The unknown reproductive cycle during slaughtering resulted in unknown stages of

oestrous cycle and also competition with other research institutions for the limited number of ovaries available at the slaughter house.

Due to lack of oocytes sources, all grades of oocytes were used in this study. The used of all grades of oocytes quality may reduce percentage of oocyte cleavage and less ovary resulted low number of replication in experiment. The difference in embryo obtained was believed to be due to lack in author experience in micropipettes preparation especially injection needle, sperm immobilisation and injection procedure itself. Difficulty in injection procedure such as though and elastic zona pellucida and oolemma, identification and observation of sperm during injection of sperm due to opaque cytoplasm and attachment of the sperm on the spike and outer part of injection needle during sperm release.

5.4 FUTURE DIRECTION

The results obtained from the present study provide additional knowledge to the existing information regarding production of bovine embryos via IVF and ICSI. It is suggested that the naked oocytes and heterogenous or dysmorphic ooplasm should be discarded during morphology assessment by using stereomicroscope. This is because, in order to produce high quality embryo with the best embryonic development, especially for embryo transfer and cryopreservation, the low quality oocyte seems not developed further and longer time used to select and group the oocyte will reduce the oocyte quality and maturation rate; except for further study to optimise and salvage low quality oocyte. Further research should be conducted to produce cryopreseved bovine embryos for embryo transfer. Due to poor embryo development after thawing

after cryopreservation done, the embryo transfer procedure cannot be done by using frozen embryos. In this study, an attempt had been carried out to transfer fresh embryos to IBHK, Jerantut, Pahang that was about 200 km from UM with 5 times replication, by using portable incubator. Unfortunately, no pregnancy was observed. Therefore, cryopreservation method should be optimised in order to facilitate the embryo transfer.

Chapter 6

6.0 CONCLUSIONS

In summary, many factors have been reported to affect the IVMFC of bovine oocytes *in vitro*. The main factors affecting the success IVM procedure and subsequently embryo development after fertilisation are the oocyte quality and IVM duration. The results of this study indicated that the quality of oocytes based on cumulus investment and ooplasmic morphology influenced the success rate of IVMFC. The compact and dense cumulus cells layers around the oocytes and dark and homogenous ooplasm were advantageous during IVMFC.

Problems encountered with oocyte aging and immature oocytes during IVM were clarified with IVM duration study under local setting. The use of serum addition in culture medium during IVC helped improved embryonic development of later stage embryos to produce blastocyst. In ICSI, the use of high quality oocytes improve fertilisation and cleavage rate after injection. Other than oocytes, sperm types used during ICSI influenced the outcome of ICSI-derived embryos. In this study, comparison between IVF- and ICSI-derived embryos showed that, IVF gives better cleavage rate and embryo development compared to ICSI. However, it is not fair enough to compare the embryo outcome because ICSI need to have professional and technical skill in handling microtools preparation and injection procedure itself. With refined optimisation, the use IVF and ICSI techniques to produce bovine embryos can be routinely applied to be used in cryopreservation, embryo transfer and micromanipulation of embryos and oocytes.

This study successfully produced bovine embryos after IVF and ICSI. From specific finding of the research it can be concluded that:

a) Oocyte morphology based on cumulus cell and cytoplasmic classifications affect cleavage rate and embryonic development after fertilisation.
b) *In vitro* maturation duration at 24 hours gave better maturation rate by producing high cleavage rate after fertilisation under local setting.
c) In order to optimise embryonic development, serum addition was shown to be able to stimulate early expansion of blastocyst by accelerating blastulation process and improved blastocyst yield after introducing at later stage of embryonic development.
d) ICSI could produce embryos by using self-prepared microtools and conventional injection procedure. However, embryonic development following ICSI was low.
e) The use of intact-immobilised sperm to produced embryos with aid of oocyte activation after injection is important factor in bovine ICSI compared to other sperm treatment.
f) The IVF is a better technique than ICSI in generating embryos by using frozen-thawed sperm.

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APPENDICES

APPENDIX 1.0: LIST OF MATERIALS

Appendix Table 1.1: List of Equipment and Instruments

Equipment/Instrument	Model No.	Manufacturer
Autoclave machine	HA-300MII	Hirayama Hiclave, Japan
Bunsen burner	EN-521	Portagaz, Malaysia
Centrifuge	4490321	Medilite ThermoIEC, USA
CO_2 incubator	Binder 7001-0030	GmbH, Tuttlingen, USA
Digital balance	AL204	Mettler, Toledo, Switzerland
Digital camera (Evolution MP for Fluorescent microscope	-	MediaCybernatics, Toronto, Canada
Digital camera (X-cam-α for ICSI microscope	-	MicroLambda, Kuala Lumpur, Malaysia (supplier)
Dissecting microscope	SZH10	Olympus, Japan
Fluorescent microscope	Axiovert 135M	Carl-Zeiss, Inc., Germany
Haemacytometer	L42471	Neubauer, Germany
Hot plate magnetic stirrer	34533	Snijders b.v., Tillburg, Holland
Impulse sealer	KF-300H	Khind, Taiwan
Inverted microscope (embryo observation)	CK2	Olympus, Japan
Inverted microscope fitted with micromanipulators (for ICSI)	IX71	Olympus Optical Co., Ltd, Tokyo, Japan
La Banane (hand-controlled pipette for Handling oocyte and embryo)	-	Self-assembled

Laminar flow chamber	HLF-120	Gelman Sciences, Australia
Liquid nitrogen tank	SC2/1V	MVE, USA
Microforge	-	Technical Product International, USA
Microgrinder	EG-4	Narishige Co. Ltd., Tokyo, Japan
Micropipette dispenser (2-20-, 5-50-, 10-100-, 200-1000-µl)	-	Appendorf, Humburg, Germany
Micropipette puller	P-97	Sutter Instrument Co., USA
Microprocessor pH meter	HI-122 / HI8417	Hanna Instruments, Singapore
Narishige hydraulic micromanipulators	ON3-99D	Narishige Co. Ltd., Tokyo, Japan
Optical lamp	500-I	Intrax, Switzerland
Oven	40050-IP20	Memmert GmbH, Germany
Refrigerator and freezer	GR-M31MPD	Toshiba, Japan
Spirit burner (150 ml)	-	Shanghai Machinery, China
Stage warmer (Thermoplate) for ICSI or inverted microscope	HATS-U55R30	Tokai Hit, Japan
Stage warmer for dissecting microscope	C0102D	Linkam Scientific, UK
Stereomicroscope	SZH10	Olympus Optical, Japan
Surgical set	-	Aesculap, B. Braun, Germany
Thermo flask (Mega®)	MA043	Megatrade Instruments, USA
Ultrapure water purification system (Milli-Q PF Plus)	-	Millipore Corporation, USA
Vapour pressure osmometer	Vapro 5520	WESCOR, USA
Vortex mixer	VTX3000L	LMS Co LTD, Tokyo, Japan
Water bath	GMP-GC-19	Memmert, Germany
Water purification system	-	Millipore, Bedford, MA,

(Milli RO 10 plus)		USA
Styrofoam box	-	-
Portable incubator		
Digital timer	-	Seiko, Japan

Appendix Table 1.2: List of Glassware/Labware/Disposables

Glassware/labware/disposables	Manufacturer
Aluminium foil	Reynolds Consumer Products, Richmond, Virginia, USA
Autoclavable disposal bag	Megalab Supplies, Malaysia
Razor blade	Gilette, USA
Borosilicate glass tubing (Microcaps)	Drummond Scientific Co., Broomal, PA, USA
Cryoleaf	Medicult, Denmark
Cover slips	Hirschmann®Laborgerete, Germany
Culture Petri dishes (35 mm, 60 mm, 4-well dish)	Nunc, Roskilde, Denmark
Disposable glass Pasteur pipette	John poulten Ltd., Essex, England
Disposable gloves	Protex, Malaysia
Disposable hand tissues	Megalab Supplies, Malaysia
Disposable plastic pipette (1-, 5-, 10-, 25-ml)	LP Italiana SPA, Milano, Italy
Falcon™ polystyrene round-bottom test tube, snap cap (5 ml, 14 ml)	Becton, Dickinson, USA
Glass (beakers, flasks, measuring cylinders etc.)	Pyrex, Iwaki Glass, Japan
Glass bottle (50 ml, 250 ml, 500 ml)	Schott Duran, UK
Haematocrit capillary tube (75 mm/µl)	Hirschmann® Laborgerete, Germany
ICSI Holding pipette	Self-made
ICSI Injection needle	Self made
Lens tissue	Kimwipes, Kimberly-Clark, Canada
Microcentrifige tubes (0.5 ml, 1.5 ml)	Elkay, Costelloe
Micropipette tip without filter (100 µl, 200 µl, 1000 µl – white, yellow, blue)	Axygen Scientific, USA

Microscope slide	Shanghai Machinary Import and Export Co., Shanghai, China
Parafilm	Pechiney Plastic Packaging, Chicago, USA
Serological pipette (1 ml, 5 ml, 10 ml, 25 ml)	LP Italiana SPA, Italy
Straw (Pailette cristal, 0.25 ml, 0.5 ml)	Cryo Bio System, France
Syringe (1 ml, 5 ml, 25 ml, 50 ml)	Terumo® Syringe, Philippines
Syringe filter (0.22 μm)	Schleicher & Schuell, Germany

Appendix Table 1.3: List of Chemicals, Reagents and Media

Chemicals, reagents and media	Catalogue no.	Manufacturer
70% ethanol	-	Prepared from absolute ethanol
7X®-PF non toxic detergent	-	FlowLab™, Sydney, Australia
Absolute ethanol (C_2H_2OH)	1322219	HmbG Chemicals, Germany
Absolute methanol (CH_3OH)	-	R&M Chemicals, Essex, UK
Basal Media Eagle (BME) amino acids solution (50x)	B6766	Sigma-Aldrich Co. St Louis, USA
Bovine serum albumin, BSA	A-9418	Sigma-Aldrich Co. St Louis, USA
Calcium chloride dehydrate ($CaCl_2.2H_2O$)	C-7902	Sigma-Aldrich Co. St Louis, USA
Calcium ionophore	10634	Sigma-Aldrich Co. St Louis, USA
Calcium lactate	2376	Sigma-Aldrich Co. St Louis, USA
D-(+)-Glucose, $C_6H_{12}O_6$	G-7021	Sigma-Aldrich Co. St Louis, USA
Dimethyl sulphoxide (DMSO), C_2H_6SO	D-5879	Sigma-Aldrich Co. St Louis, USA
DPX mountant	Prod 36029	BDH Laboratory, UK, England
Dulbecco's phosphate buffered saline (DPBS, Dulbecco tablet A tablet)	BR0014G	Oxoid, UK
Folligon (PMSG, 1000 IU)	-	Intervet International,

		Holland
Gentamicin sulphate	G1264	Sigma-Aldrich Co. St Louis, USA
Glycerol, anhydrous ($CH_3H_8O_3$)	0411-00	R&M Chemicals, Essex, UK
Heparin, sodium salt	H0777	Sigma-Aldrich Co. St Louis, USA
HEPES sodium salt	H7006	Sigma-Aldrich Co. St Louis, USA
Hepes-buffered medium 199	M7528	Sigma-Aldrich Co. St Louis, USA
Hoechst 33342	B2261	Sigma-Aldrich Co. St Louis, USA
Hyaluronidase (Type IV-S: from bovine testes)	H4272	Sigma-Aldrich Co. St Louis, USA
Hydrochloric acid (HCl)	-	HmbG Chemicals, Germany
Hydrofluoric acid (HFl)	1301030	HmbG Chemicals, Germany
Lactic acid	L-4263	Sigma-Aldrich Co. St Louis, USA
L-glutamine ($C_5H_{10}N_2O_3$)	G8540	Sigma-Aldrich Co. St Louis, USA
Liquid nitrogen	-	MOX gases Bhd, PJ, Selangor, Malaysia
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	M2393	Sigma-Aldrich Co. St Louis, USA
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	M-8150	Sigma-Aldrich Co. St Louis, USA
Mineral oil	M8410	Sigma-Aldrich Co. St Louis, USA

Minimum Essential Media (MEM) non essential amino acid solution, 100x	M7145	Sigma-Aldrich Co. St Louis, USA
Natrium EDTA	E-5143	Sigma-Aldrich Co. St Louis, USA
Natrium pyruvate	P-3662	Sigma-Aldrich Co. St Louis, USA
Oestradiol-17β ($C_{18}H_{24}O_2$)	E4389	Sigma-Aldrich Co. St Louis, USA
Ovagen™ (70 mg FSH)	oFSH	ICPbio Limited, Auckland, New Zealand
Penicillin	-	Sigma-Aldrich Co. St Louis, USA
Phenol red solution (0.5%)	15100-043	Gibco BRL, Life Technologies, USA
Potasium chloride (KCl)	P-5404	Sigma-Aldrich Co. St Louis, USA
Potasium hydrogen phosphate ($KH_2PO_{4)}$	P-5655	Sigma-Aldrich Co. St Louis, USA
PVP medium (Polyvinylpyrrolidone, 10%)	108900001	Sigma-Aldrich Co. St Louis, USA
Pyruvic acid, sodium salt ($C_3H_3O_3Na$)	P3662	Sigma-Aldrich Co. St Louis, USA
Silicone oil		R&M Chemicals, UK
Sodium bicarbonate ($NaHCO_3$)	S-5761	Sigma-Aldrich Co. St Louis, USA
Sodium chloride (NaCl)	S-5886	Sigma-Aldrich Co. St Louis, USA
Sodium DL-lactate (60% w/w syrup)	L4263	Sigma-Aldrich Co. St Louis, USA
Sodium phosphate monobasic	S5011	Sigma-Aldrich Co. St

($NaH_2PO_4.H_2O$)		Louis, USA
Steer serum (SS, heat inactivated)	-	Prepared ‘in house’
Streptomycin	S-9137	Sigma-Aldrich Co. St Louis, USA
Sucrose	S-1888	Sigma-Aldrich Co. St Louis, USA
Taurine	T-7146	Sigma Chem. Co., USA
Triton®X-100	93420	Fluka Chemie GmbH, Germany

Appendix Table 1.4: List of Media

Maturation Medium (TCM 199)

Chemicals/stock	Quantity (10 ml)
TCM 199 stock	9 ml
Natrium pyruvate stock	20 µl
Gentamycin stock	10 µl
Steer serum	1 ml
Oestradiol	10 µl/10 ml medium
FSH stock	10 µl

Fertilisation Medium (Brackett-Oliphant)

Medium BO (Stock A)

Chemicals/stock	Quantity (100 ml)
NaCl	6.5453 g
KCl	0.2997 g
$CaCl_2.2H_2O$	0.3307 g
$NaH_2PO_4.H_2O$ or $NaH_2PO_4.2H_2O$	0.1130 g or 0.1295 g
$MgCl_2.6H_2O$	0.1057 g
Fenol red stock	100 µl

Medium BO (Working Solution, WS)

Chemicals/stock	Quantity (100 ml)
Stock A	10 ml
Stock $NaHCO_3$	37 ml
Glucose	0.250 g
Natrium pyruvate	0.0137 g
Gentamycin stock	0.1 ml

BO-Theo-BSA

Chemicals/stock	Quantity (100 ml)
BO medium WS	10 ml
Theophylline (T-1633)	0.0090 g

BSA	0.0600 g
Heparin stock	10 µl
Calcium ionophore	20 µl

Culture Medium (mSOF)

mSOF (Stock B)

Chemicals/stock	Quantity (100 ml)
NaCl	6.2940 g
KCl	0.5338 g
$CaCl_2.2H_2O$	0.2514 g
KH_2PO_4	0.1618 g
$MgCl_2.6H_2O$	0.0996 g
Fenol red stock	0.05 ml

mSOF (Working Solution, WS)

Chemicals/stock	Quantity (100 ml)
Stock B	10 ml
Stock $NaHCO_3$	25.07 ml
Natrium pyruvate	0.0036 g
Na-Lactate	0.0548 ml
Gentamycin stock	0.1 ml
Glutamine stock	0.0146 g
BME (50X) aa solution	2 ml
MEM (100X) non aa solution	1 ml

mSOF-BSA

WS	10 ml
BSA	0.03 g

APPENDIX 2.0: STATISTICAL DATA

Appendix Table 2.1: Percentages of cleavage rate after IVF in different oocyte grades (Experiment 1)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
twocell	A homo	5	78.3080	3.91485	1.75078	73.4471	83.1689	72.72	83.33
	A hetero	5	50.9060	11.23779	5.02569	36.9524	64.8596	40.00	66.66
	B homo	5	65.9200	15.18376	6.79039	47.0669	84.7731	53.33	84.61
	B hetero	5	30.4820	9.00578	4.02751	19.2999	41.6641	16.66	41.66
	C homo	5	24.0220	12.10139	5.41191	8.9961	39.0479	12.50	40.00
	C hetero	5	11.6660	16.24355	7.26433	-8.5030	31.8350	.00	33.33
	Total	30	43.5507	26.33406	4.80792	33.7174	53.3840	.00	84.61
fourcell	A homo	5	69.7040	10.23318	4.57642	56.9978	82.4102	54.54	83.33
	A hetero	5	38.5720	10.97642	4.90880	24.9430	52.2010	26.66	54.54
	B homo	5	53.9700	18.11539	8.10145	31.4768	76.4632	33.33	80.00
	B hetero	5	19.8520	9.22010	4.12335	8.4037	31.3003	8.33	33.33
	C homo	5	12.6880	7.62885	3.41172	3.2155	22.1605	.00	20.00
	C hetero	5	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	30	32.4643	26.41825	4.82329	22.5996	42.3291	.00	83.33
eightcell	A homo	5	60.5460	8.29183	3.70822	50.2503	70.8417	50.00	70.00
	A hetero	5	33.7540	7.09883	3.17469	24.9396	42.5684	26.66	45.45
	B homo	5	44.8940	15.80172	7.06674	25.2736	64.5144	26.66	70.00
	B hetero	5	14.6480	13.14281	5.87764	-1.6710	30.9670	.00	33.33
	C homo	5	9.3560	8.97907	4.01556	-1.7930	20.5050	.00	20.00
	C hetero	5	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	30	27.1997	23.42099	4.27607	18.4541	35.9452	.00	70.00
morula	A homo	5	46.9760	12.61853	5.64318	31.3080	62.6440	35.71	66.66
	A hetero	5	20.1180	10.64092	4.75876	6.9056	33.3304	6.66	33.33
	B homo	5	24.8160	6.23136	2.78675	17.0787	32.5533	16.66	30.76
	B hetero	5	5.3320	7.67145	3.43078	-4.1934	14.8574	.00	16.66

	C homo	5	2.5000	5.59017	2.50000	-4.4411	9.4411	.00	12.50
	C hetero	5	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	30	16.6237	18.21815	3.32616	9.8209	23.4264	.00	66.66
cmorula	A homo	5	36.5660	8.03227	3.59214	26.5926	46.5394	30.00	50.00
	A hetero	5	9.4820	2.47588	1.10725	6.4078	12.5562	6.66	13.33
	B homo	5	20.6120	6.38742	2.85654	12.6810	28.5430	13.33	30.00
	B hetero	5	1.6660	3.72529	1.66600	-2.9596	6.2916	.00	8.33
	C homo	5	.0000	.00000	.00000	.0000	.0000	.00	.00
	C hetero	5	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	30	11.3877	14.24415	2.60061	6.0688	16.7065	.00	50.00
blastocyst	A homo	5	28.5560	4.53625	2.02867	22.9235	34.1885	21.42	33.33
	A hetero	5	2.6640	3.64783	1.63136	-1.8654	7.1934	.00	6.66
	B homo	5	14.4060	4.96186	2.21901	8.2450	20.5670	6.66	20.00
	B hetero	5	.0000	.00000	.00000	.0000	.0000	.00	.00
	C homo	5	.0000	.00000	.00000	.0000	.0000	.00	.00
	C hetero	5	.0000	.00000	.00000	.0000	.0000	.00	.00
	Total	30	7.6043	11.21823	2.04816	3.4154	11.7933	.00	33.33

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
twocell	Between Groups	16656.755	5	3331.351	23.146	.000
	Within Groups	3454.245	24	143.927		
	Total	20111.000	29			
fourcell	Between Groups	17453.487	5	3490.697	30.067	.000
	Within Groups	2786.307	24	116.096		
	Total	20239.793	29			
eightcell	Between Groups	13418.947	5	2683.789	25.880	.000
	Within Groups	2488.797	24	103.700		
	Total	15907.743	29			
morula	Between Groups	8019.574	5	1603.915	23.976	.000
	Within Groups	1605.550	24	66.898		
	Total	9625.124	29			

cmorula	Between Groups	5382.685	5	1076.537	51.540	.000
	Within Groups	501.297	24	20.887		
	Total	5883.983	29			
blastocyst	Between Groups	3415.598	5	683.120	70.058	.000
	Within Groups	234.017	24	9.751		
	Total	3649.615	29			

Post Hoc Test

Two cell

Duncan[a]

		Subset for alpha = 0.05			
treatment	N	1	2	3	4
C hetero	5	11.6660			
C homo	5	24.0220	24.0220		
B hetero	5		30.4820		
A hetero	5			50.9060	
B homo	5			65.9200	65.9200
A homo	5				78.3080
Sig.		.116	.403	.059	.116

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Four cell

Duncan[a]

		Subset for alpha = 0.05				
treatment	N	1	2	3	4	5
C hetero	5	.0000				
C homo	5	12.6880	12.6880			
B hetero	5		19.8520			
A hetero	5			38.5720		
B homo	5				53.9700	
A homo	5					69.7040
Sig.		.075	.304	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Eight cell

Duncan[a]

		Subset for alpha = 0.05			
treatment	N	1	2	3	4
C hetero	5	.0000			
C homo	5	9.3560	9.3560		
B hetero	5		14.6480		
A hetero	5			33.7540	
B homo	5			44.8940	
A homo	5				60.5460
Sig.		.159	.419	.097	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

morula

Duncan[a]

		Subset for alpha = 0.05		
treatment	N	1	2	3
C hetero	5	.0000		
C homo	5	2.5000		
B hetero	5	5.3320		
A hetero	5		20.1180	
B homo	5		24.8160	
A homo	5			46.9760
Sig.		.341	.373	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Compact morula

Duncan[a]

treatment	N	Subset for alpha = 0.05			
		1	2	3	4
C homo	5	.0000			
C hetero	5	.0000			
B hetero	5	1.6660			
A hetero	5		9.4820		
B homo	5			20.6120	
A homo	5				36.5660
Sig.		.592	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Blastocyst

Duncan[a]

treatment	N	Subset for alpha = 0.05		
		1	2	3
B hetero	5	.0000		
C homo	5	.0000		
C hetero	5	.0000		
A hetero	5	2.6640		
B homo	5		14.4060	
A homo	5			28.5560
Sig.		.229	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix Table 2.2: Percentages of fertilisation rate of different IVM duration after IVF after IVF in different oocyte grades (Experiment 2)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
twocell	A homo 20	5	58.57400	7.457096	3.334915	49.31479	67.83321	50.000	70.000
	A homo 24	5	78.30800	3.914852	1.750775	73.44707	83.16893	72.720	83.330
	A homo 28	5	58.88600	12.785964	5.718057	43.01013	74.76187	50.000	77.770
	A hetero 20	5	45.65200	3.610924	1.614854	41.16845	50.13555	40.000	50.000
	A hetero 24	5	50.90600	11.237792	5.025694	36.95244	64.85956	40.000	66.660
	A hetero 28	5	30.77200	5.994157	2.680669	23.32927	38.21473	25.000	37.500
	B homo 20	5	38.32600	6.486573	2.900884	30.27186	46.38014	30.000	45.450
	B homo 24	5	65.92000	15.183764	6.790386	47.06687	84.77313	53.330	84.610
	B homo 28	5	49.16400	11.540651	5.161136	34.83439	63.49361	33.330	62.500
	B hetero 20	5	39.64400	12.242342	5.474942	24.44313	54.84487	22.220	55.550
	B hetero 24	5	30.48200	9.005777	4.027506	19.29985	41.66415	16.660	41.660
	B hetero 28	5	28.82400	4.084952	1.826846	23.75186	33.89614	22.220	33.330
	C homo 20	5	15.85600	9.641767	4.311929	3.88416	27.82784	.000	25.000
	C homo 24	5	24.02200	12.101393	5.411908	8.99614	39.04786	12.500	40.000
	C homo 28	5	14.00000	12.942179	5.787918	-2.06984	30.06984	.000	25.000
	C hetero 20	5	21.66600	21.730227	9.718053	-5.31564	48.64764	.000	50.000
	C hetero 24	5	11.66600	16.243546	7.264335	-8.50303	31.83503	.000	33.330
	C hetero 28	5	4.00000	8.944272	4.000000	-7.10578	15.10578	.000	20.000
	Total	90	37.03711	22.485351	2.370164	32.32765	41.74658	.000	84.610
fourcell	A homo 20	5	45.08800	12.304555	5.502764	29.80988	60.36612	25.000	58.330
	A homo 24	5	69.70400	10.233178	4.576416	56.99783	82.41017	54.540	83.330
	A homo 28	5	38.55400	9.730724	4.351712	26.47171	50.63629	25.000	50.000
	A hetero 20	5	35.42400	11.112249	4.969549	21.62632	49.22168	20.000	50.000
	A hetero 24	5	38.57200	10.976417	4.908803	24.94298	52.20102	26.660	54.540

	A hetero 28	5	28.77200	7.732394	3.458032	19.17096	38.37304	20.000	37.500
	B homo 20	5	28.22200	12.342492	5.519730	12.89677	43.54723	10.000	40.000
	B homo 24	5	53.97000	18.115393	8.101450	31.47677	76.46323	33.330	80.000
	B homo 28	5	33.45000	7.070958	3.162229	24.67025	42.22975	25.000	42.850
	B hetero 20	5	18.20600	6.964961	3.114825	9.55786	26.85414	9.090	25.000
	B hetero 24	5	19.85200	9.220101	4.123355	8.40373	31.30027	8.330	33.330
	B hetero 28	5	21.74400	9.672535	4.325689	9.73396	33.75404	11.110	33.330
	C homo 20	5	13.00000	12.041595	5.385165	-1.95161	27.95161	.000	25.000
	C homo 24	5	12.68800	7.628848	3.411724	3.21553	22.16047	.000	20.000
	C homo 28	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C hetero 20	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C hetero 24	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C hetero 28	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	90	25.40256	21.176123	2.232159	20.96730	29.83781	.000	83.330
eightcell	A homo 20	5	42.08800	15.721389	7.030819	22.56732	61.60868	16.660	58.330
	A homo 24	5	60.54600	8.291826	3.708217	50.25034	70.84166	50.000	70.000
	A homo 28	5	29.83200	10.608380	4.744212	16.65996	43.00404	20.000	44.440
	A hetero 20	5	19.96800	8.995069	4.022717	8.79915	31.13685	9.090	30.760
	A hetero 24	5	33.75400	7.098826	3.174691	24.93964	42.56836	26.660	45.450
	A hetero 28	5	11.13600	11.087267	4.958376	-2.63066	24.90266	.000	25.000
	B homo 20	5	20.88800	13.936274	6.232491	3.58383	38.19217	.000	36.360
	B homo 24	5	44.89400	15.801721	7.066744	25.27357	64.51443	26.660	70.000
	B homo 28	5	25.23600	7.013803	3.136668	16.52721	33.94479	14.280	33.330
	B hetero 20	5	11.66600	7.892761	3.529750	1.86584	21.46616	.000	22.220
	B hetero 24	5	14.64800	13.142811	5.877644	-1.67095	30.96695	.000	33.330
	B hetero 28	5	10.41000	6.384857	2.855395	2.48215	18.33785	.000	16.660
	C homo 20	5	5.00000	11.180340	5.000000	-8.88223	18.88223	.000	25.000
	C homo 24	5	9.35600	8.979069	4.015562	-1.79299	20.50499	.000	20.000
	C homo 28	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C hetero 20	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C hetero 24	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C hetero 28	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	90	18.85678	19.219011	2.025862	14.83143	22.88212	.000	70.000

morula	A homo 20	5	23.63400	7.518762	3.362493	14.29822	32.96978	16.660	33.330
	A homo 24	5	46.97600	12.618527	5.643177	31.30803	62.64397	35.710	66.660
	A homo 28	5	17.88800	5.001352	2.236673	11.67800	24.09800	12.500	22.220
	A hetero 20	5	9.35400	2.565254	1.147217	6.16882	12.53918	6.660	13.330
	A hetero 24	5	20.11800	10.640920	4.758764	6.90555	33.33045	6.660	33.330
	A hetero 28	5	7.80200	7.934029	3.548206	-2.04940	17.65340	.000	18.180
	B homo 20	5	12.73000	11.625580	5.199117	-1.70506	27.16506	.000	30.000
	B homo 24	5	24.81600	6.231363	2.786750	17.07874	32.55326	16.660	30.760
	B homo 28	5	11.54600	6.620837	2.960928	3.32515	19.76685	.000	16.660
	B hetero 20	5	4.72200	6.484514	2.899963	-3.32959	12.77359	.000	12.500
	B hetero 24	5	5.33200	7.671448	3.430776	-4.19336	14.85736	.000	16.660
	B hetero 28	5	7.55400	7.342907	3.283848	-1.56342	16.67142	.000	16.660
	C homo 20	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C homo 24	5	2.50000	5.590170	2.500000	-4.44111	9.44111	.000	12.500
	C homo 28	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C hetero 20	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C hetero 24	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C hetero 28	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	90	10.83178	13.436644	1.416347	8.01753	13.64603	.000	66.660
cmorula	A homo 20	5	14.30200	14.306164	6.397911	-3.46145	32.06545	.000	33.330
	A homo 24	5	36.56600	8.032271	3.592141	26.59262	46.53938	30.000	50.000
	A homo 28	5	9.44400	5.324907	2.381371	2.83226	16.05574	.000	12.500
	A hetero 20	5	4.20200	3.858863	1.725736	-.58941	8.99341	.000	7.690
	A hetero 24	5	9.48200	2.475878	1.107246	6.40779	12.55621	6.660	13.330
	A hetero 28	5	5.30200	8.052100	3.601009	-4.69600	15.30000	.000	18.180
	B homo 20	5	4.00000	8.944272	4.000000	-7.10578	15.10578	.000	20.000
	B homo 24	5	20.61200	6.387423	2.856542	12.68097	28.54303	13.330	30.000
	B homo 28	5	5.35600	7.360956	3.291920	-3.78383	14.49583	.000	14.280
	B hetero 20	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	B hetero 24	5	1.66600	3.725289	1.666000	-2.95956	6.29156	.000	8.330
	B hetero 28	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C homo 20	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C homo 24	5	.00000	.000000	.000000	.00000	.00000	.000	.000

	C homo 28	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C hetero 20	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C hetero 24	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C hetero 28	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	90	6.16289	10.634228	1.120946	3.93559	8.39018	.000	50.000
blastocyst	A homo 20	5	3.48400	4.778225	2.136887	-2.44895	9.41695	.000	9.090
	A homo 24	5	28.55600	4.536246	2.028671	22.92351	34.18849	21.420	33.330
	A homo 28	5	2.50000	5.590170	2.500000	-4.44111	9.44111	.000	12.500
	A hetero 20	5	1.53800	3.439073	1.538000	-2.73217	5.80817	.000	7.690
	A hetero 24	5	2.66400	3.647832	1.631360	-1.86538	7.19338	.000	6.660
	A hetero 28	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	B homo 20	5	2.00000	4.472136	2.000000	-3.55289	7.55289	.000	10.000
	B homo 24	5	14.40600	4.961863	2.219012	8.24503	20.56697	6.660	20.000
	B homo 28	5	2.50000	5.590170	2.500000	-4.44111	9.44111	.000	12.500
	B hetero 20	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	B hetero 24	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	B hetero 28	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C homo 20	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C homo 24	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C homo 28	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C hetero 20	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C hetero 24	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C hetero 28	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	90	3.20267	7.558690	.796756	1.61953	4.78580	.000	33.330

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
twocell	Between Groups	35853.162	17	2109.010	16.606	.000
	Within Groups	9144.438	72	127.006		
	Total	44997.600	89			
fourcell	Between Groups	33448.741	17	1967.573	21.925	.000
	Within Groups	6461.368	72	89.741		
	Total	39910.109	89			
eightcell	Between Groups	26245.113	17	1543.830	16.768	.000
	Within Groups	6628.852	72	92.067		
	Total	32873.965	89			
morula	Between Groups	12758.687	17	750.511	16.327	.000
	Within Groups	3309.675	72	45.968		
	Total	16068.363	89			
cmorula	Between Groups	7775.701	17	457.394	14.387	.000
	Within Groups	2289.024	72	31.792		
	Total	10064.726	89			
blastocyst	Between Groups	4382.256	17	257.780	26.414	.000
	Within Groups	702.652	72	9.759		
	Total	5084.908	89			

Post Hoc Tests

Two cell

Duncan[a]

		Subset for alpha = 0.05									
treatment	N	1	2	3	4	5	6	7	8	9	10
C hetero 28	5	4.00000									
C hetero 24	5	11.66600	11.66600								
C homo 28	5	14.00000	14.00000	14.00000							
C homo 20	5	15.85600	15.85600	15.85600	15.85600						
C hetero 20	5		21.66600	21.66600	21.66600						
C homo 24	5		24.02200	24.02200	24.02200	24.02200					
B hetero 28	5			28.82400	28.82400	28.82400					
B hetero 24	5				30.48200	30.48200	30.48200				
A hetero 28	5				30.77200	30.77200	30.77200				
B homo 20	5					38.32600	38.32600	38.32600			
B hetero 20	5					39.64400	39.64400	39.64400			
A hetero 20	5						45.65200	45.65200	45.65200		
B homo 28	5							49.16400	49.16400		
A hetero 24	5							50.90600	50.90600	50.90600	
A homo 20	5								58.57400	58.57400	
A homo 28	5								58.88600	58.88600	
B homo 24	5									65.92000	65.92000
A homo 24	5										78.30800
Sig.		.134	.127	.066	.070	.057	.060	.121	.102	.057	.086

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Four cell

Duncan[a]

treatment	N	Subset for alpha = 0.05							
		1	2	3	4	5	6	7	8
C homo 28	5	.00000							
C hetero 20	5	.00000							
C hetero 24	5	.00000							
C hetero 28	5	.00000							
C homo 24	5	12.68800	12.68800						
C homo 20	5	13.00000	13.00000						
B hetero 20	5		18.20600	18.20600					
B hetero 24	5		19.85200	19.85200					
B hetero 28	5		21.74400	21.74400	21.74400				
B homo 20	5			28.22200	28.22200	28.22200			
A hetero 28	5			28.77200	28.77200	28.77200			
B homo 28	5				33.45000	33.45000	33.45000		
A hetero 20	5					35.42400	35.42400		
A homo 28	5					38.55400	38.55400		
A hetero 24	5					38.57200	38.57200		
A homo 20	5						45.08800	45.08800	
B homo 24	5							53.97000	
A homo 24	5								69.70400
Sig.		.060	.185	.121	.078	.136	.087	.143	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Eight cell

Duncan[a]

treatment	N	Subset for alpha = 0.05								
		1	2	3	4	5	6	7	8	9
C homo 28	5	.00000								
C hetero 20	5	.00000								
C hetero 24	5	.00000								
C hetero 28	5	.00000								
C homo 20	5	5.00000	5.00000							
C homo 24	5	9.35600	9.35600	9.35600						
B hetero 28	5	10.41000	10.41000	10.41000						
A hetero 28	5	11.13600	11.13600	11.13600						
B hetero 20	5	11.66600	11.66600	11.66600						
B hetero 24	5		14.64800	14.64800	14.64800					
A hetero 20	5			19.96800	19.96800	19.96800				
B homo 20	5			20.88800	20.88800	20.88800	20.88800			
B homo 28	5				25.23600	25.23600	25.23600			
A homo 28	5					29.83200	29.83200	29.83200		
A hetero 24	5						33.75400	33.75400	33.75400	
A homo 20	5							42.08800	42.08800	
B homo 24	5								44.89400	
A homo 24	5									60.54600
Sig.		.108	.171	.105	.116	.143	.055	.059	.086	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

morula

Duncan[a]

treatment	N	Subset for alpha = 0.05						
		1	2	3	4	5	6	7
C homo 20	5	.00000						
C homo 28	5	.00000						
C hetero 20	5	.00000						
C hetero 24	5	.00000						
C hetero 28	5	.00000						
C homo 24	5	2.50000	2.50000					
B hetero 20	5	4.72200	4.72200	4.72200				
B hetero 24	5	5.33200	5.33200	5.33200				
B hetero 28	5	7.55400	7.55400	7.55400				
A hetero 28	5	7.80200	7.80200	7.80200				
A hetero 20	5	9.35400	9.35400	9.35400	9.35400			
B homo 28	5		11.54600	11.54600	11.54600	11.54600		
B homo 20	5			12.73000	12.73000	12.73000		
A homo 28	5				17.88800	17.88800	17.88800	
A hetero 24	5					20.11800	20.11800	
A homo 20	5						23.63400	
B homo 24	5						24.81600	
A homo 24	5							46.97600
Sig.		.072	.071	.112	.072	.071	.146	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Compact morula

Duncan[a]

treatment	N	Subset for alpha = 0.05				
		1	2	3	4	5
B hetero 20	5	.00000				
B hetero 28	5	.00000				
C homo 20	5	.00000				
C homo 24	5	.00000				
C homo 28	5	.00000				
C hetero 20	5	.00000				
C hetero 24	5	.00000				
C hetero 28	5	.00000				
B hetero 24	5	1.66600	1.66600			
B homo 20	5	4.00000	4.00000			
A hetero 20	5	4.20200	4.20200			
A hetero 28	5	5.30200	5.30200			
B homo 28	5	5.35600	5.35600			
A homo 28	5		9.44400	9.44400		
A hetero 24	5		9.48200	9.48200		
A homo 20	5			14.30200	14.30200	
B homo 24	5				20.61200	
A homo 24	5					36.56600
Sig.		.222	.061	.204	.081	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Blastocyst

Duncan[a]

treatment	N	Subset for alpha = 0.05		
		1	2	3
A hetero 28	5	.00000		
B hetero 20	5	.00000		
B hetero 24	5	.00000		
B hetero 28	5	.00000		
C homo 20	5	.00000		
C homo 24	5	.00000		
C homo 28	5	.00000		
C hetero 20	5	.00000		
C hetero 24	5	.00000		
C hetero 28	5	.00000		
A hetero 20	5	1.53800		
B homo 20	5	2.00000		
A homo 28	5	2.50000		
B homo 28	5	2.50000		
A hetero 24	5	2.66400		
A homo 20	5	3.48400		
B homo 24	5		14.40600	
A homo 24	5			28.55600
Sig.		.156	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix Table 2.3: Percentages of cleavage rate after serum addition on blastocyst production (Experiment 4)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
twocell	A coc	5	44.53800	13.533430	6.052334	27.73403	61.34197	30.000	57.140
	B coc	5	38.94400	18.143888	8.114193	16.41539	61.47261	20.000	62.500
	C coc	5	7.85600	11.405423	5.100660	-6.30570	22.01770	.000	25.000
	Total	15	30.44600	21.506303	5.552904	18.53621	42.35579	.000	62.500
fourcell	A coc	5	42.53800	16.608544	7.427566	21.91577	63.16023	20.000	57.140
	B coc	5	22.72200	9.659654	4.319929	10.72796	34.71604	11.110	37.500
	C coc	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	15	21.75333	20.714645	5.348498	10.28195	33.22472	.000	57.140
eightcell	A coc	5	33.51800	21.488849	9.610105	6.83607	60.19993	10.000	57.040
	B coc	5	4.22200	5.794516	2.591387	-2.97284	11.41684	.000	11.110
	C coc	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	15	12.58000	19.482502	5.030360	1.79095	23.36905	.000	57.040
morula	A coc	5	16.65800	8.348330	3.733487	6.29218	27.02382	10.000	28.570
	B coc	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C coc	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	15	5.55267	9.272624	2.394181	.41766	10.68767	.000	28.570
cmorula	A coc	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	B coc	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C coc	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	15	.00000	.000000	.000000	.00000	.00000	.000	.000
blastocyst	A coc	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	B coc	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	C coc	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	15	.00000	.000000	.000000	.00000	.00000	.000	.000

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
twocell	Between Groups	3905.543	2	1952.771	9.119	.004
	Within Groups	2569.752	12	214.146		
	Total	6475.295	14			
fourcell	Between Groups	4530.741	2	2265.370	18.410	.000
	Within Groups	1476.611	12	123.051		
	Total	6007.352	14			
eightcell	Between Groups	3332.562	2	1666.281	10.092	.003
	Within Groups	1981.388	12	165.116		
	Total	5313.950	14			
morula	Between Groups	924.963	2	462.482	19.907	.000
	Within Groups	278.778	12	23.232		
	Total	1203.742	14			
cmorula	Between Groups	.000	2	.000	.	.
	Within Groups	.000	12	.000		
	Total	.000	14			
blastocyst	Between Groups	.000	2	.000	.	.
	Within Groups	.000	12	.000		
	Total	.000	14			

Post Hoc Tests

Two cell

Duncan[a]

treatment	N	Subset for alpha = 0.05	
		1	2
C coc	5	7.85600	
B coc	5		38.94400
A coc	5		44.53800
Sig.		1.000	.557

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Four cell

Duncan[a]

treatment	N	Subset for alpha = 0.05		
		1	2	3
C coc	5	.00000		
B coc	5		22.72200	
A coc	5			42.53800
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Eight cell

Duncan[a]

treatment	N	Subset for alpha = 0.05	
		1	2
C coc	5	.00000	
B coc	5	4.22200	
A coc	5		33.51800
Sig.		.613	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Morula

Duncan[a]

treatment	N	Subset for alpha = 0.05	
		1	2
B coc	5	.00000	
C coc	5	.00000	
A coc	5		16.65800
Sig.		1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix Table 2.4: Percentages of different sperm quality on fertilisation and cleavage rate after ICSI (Experiment 5)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
twocell	Dead A	5	28.33200	11.057175	4.944919	14.60270	42.06130	16.660	40.000
	Dead B	5	33.66600	11.925719	5.333344	18.85826	48.47374	20.000	50.000
	Dead C	5	30.66600	11.820228	5.286166	15.98925	45.34275	20.000	50.000
	Intact A	4	24.58250	6.289983	3.144992	14.57373	34.59127	20.000	33.330
	Intact B	5	39.33200	15.706262	7.024054	19.83010	58.83390	20.000	60.000
	Intact C	5	29.71400	11.743465	5.251837	15.13256	44.29544	20.000	50.000
	Immobilised A	5	63.42600	12.630684	5.648614	47.74293	79.10907	50.000	83.330
	Immobilised B	5	43.23600	7.075537	3.164277	34.45056	52.02144	33.330	50.000
	Immobilised C	5	23.82400	8.774214	3.923948	12.92937	34.71863	10.000	33.330
	Sham A	5	39.99600	14.905629	6.666000	21.48822	58.50378	33.330	66.660
	Sham B	5	46.66200	18.255593	8.164149	23.99469	69.32931	33.330	66.660
	Sham C	5	33.33000	.000000	.000000	33.33000	33.33000	33.330	33.330
	Total	59	36.59746	15.146876	1.971955	32.65016	40.54476	10.000	83.330
fourcell	Dead A	5	19.33200	14.220799	6.359735	1.67455	36.98945	.000	40.000
	Dead B	5	18.00000	17.535678	7.842194	-3.77342	39.77342	.000	40.000
	Dead C	5	11.66600	16.243546	7.264335	-8.50303	31.83503	.000	33.330
	Intact A	4	14.16500	9.573691	4.786846	-1.06888	29.39888	.000	20.000
	Intact B	5	27.99800	13.865465	6.200824	10.78175	45.21425	16.660	50.000
	Intact C	5	7.85600	11.405423	5.100660	-6.30570	22.01770	.000	25.000
	Immobilised A	5	57.23400	10.542532	4.714764	44.14372	70.32428	42.850	66.660
	Immobilised B	5	27.15800	3.434694	1.536042	22.89326	31.42274	22.220	30.000
	Immobilised C	5	10.41000	6.384857	2.855395	2.48215	18.33785	.000	16.660
	Sham A	5	13.33200	18.255593	8.164149	-9.33531	35.99931	.000	33.330
	Sham B	5	19.99800	18.255593	8.164149	-2.66931	42.66531	.000	33.330
	Sham C	5	6.66600	14.905629	6.666000	-11.84178	25.17378	.000	33.330
	Total	59	19.57475	18.184751	2.367453	14.83577	24.31372	.000	66.660

eightcell	Dead A	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Dead B	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Dead C	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Intact A	4	5.00000	10.000000	5.000000	-10.91223	20.91223	.000	20.000
	Intact B	5	17.33200	20.466830	9.153045	-8.08093	42.74493	.000	50.000
	Intact C	5	2.85600	6.386210	2.856000	-5.07353	10.78553	.000	14.280
	Immobilised A	5	37.04600	8.306505	3.714782	26.73211	47.35989	28.570	50.000
	Immobilised B	5	18.07800	5.437832	2.431873	11.32604	24.82996	11.110	25.000
	Immobilised C	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Sham A	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Sham B	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Sham C	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	59	6.72136	13.134862	1.710013	3.29839	10.14432	.000	50.000
morula	Dead A	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Dead B	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Dead C	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Intact A	4	.00000	.000000	.000000	.00000	.00000	.000	.000
	Intact B	5	4.00000	8.944272	4.000000	-7.10578	15.10578	.000	20.000
	Intact C	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Immobilised A	5	33.71200	12.471915	5.577610	18.22607	49.19793	16.660	50.000
	Immobilised B	5	6.50000	6.020797	2.692582	-.97581	13.97581	.000	12.500
	Immobilised C	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Sham A	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Sham B	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Sham C	5	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	59	3.74678	10.366288	1.349576	1.04531	6.44825	.000	50.000

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
bil	Between Groups	190.800	11	17.345	21.915	.000
	Within Groups	37.200	47	.791		
	Total	228.000	58			
twocell	Between Groups	6664.820	11	605.893	4.287	.000
	Within Groups	6641.996	47	141.319		
	Total	13306.816	58			
fourcell	Between Groups	10311.420	11	937.402	4.968	.000
	Within Groups	8868.320	47	188.688		
	Total	19179.740	58			
eightcell	Between Groups	7473.455	11	679.405	12.607	.000
	Within Groups	2532.971	47	53.893		
	Total	10006.426	58			
morula	Between Groups	5145.482	11	467.771	20.222	.000
	Within Groups	1087.195	47	23.132		
	Total	6232.676	58			

Post Hoc Test

Two cell

Duncan[a,,b]

		Subset for alpha = 0.05			
treatment	N	1	2	3	4
Immobilised C	5	23.82400			
Intact A	4	24.58250			
Dead A	5	28.33200	28.33200		
Intact C	5	29.71400	29.71400	29.71400	
Dead C	5	30.66600	30.66600	30.66600	
Sham C	5	33.33000	33.33000	33.33000	
Dead B	5	33.66600	33.66600	33.66600	
Intact B	5	39.33200	39.33200	39.33200	
Sham A	5	39.99600	39.99600	39.99600	
Immobilised B	5		43.23600	43.23600	
Sham B	5			46.66200	
Immobilised A	5				63.42600
Sig.		.076	.099	.060	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.898.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Four cell

Duncan[a.,b]

treatment	N	Subset for alpha = 0.05		
		1	2	3
Sham C	5	6.66600		
Intact C	5	7.85600	7.85600	
Immobilised C	5	10.41000	10.41000	
Dead C	5	11.66600	11.66600	
Sham A	5	13.33200	13.33200	
Intact A	4	14.16500	14.16500	
Dead B	5	18.00000	18.00000	
Dead A	5	19.33200	19.33200	
Sham B	5	19.99800	19.99800	
Immobilised B	5	27.15800	27.15800	
Intact B	5		27.99800	
Immobilised A	5			57.23400
Sig.		.053	.057	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.898.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Eight cell

Duncan[a.,b]

treatment	N	Subset for alpha = 0.05		
		1	2	3
Dead A	5	.00000		
Dead B	5	.00000		
Dead C	5	.00000		
Immobilised C	5	.00000		
Sham A	5	.00000		
Sham B	5	.00000		
Sham C	5	.00000		
Intact C	5	2.85600		
Intact A	4	5.00000		
Intact B	5		17.33200	
Immobilised B	5		18.07800	
Immobilised A	5			37.04600
Sig.		.374	.874	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.898.

Morula

Duncan[a,,b]

treatment	N	Subset for alpha = 0.05	
		1	2
Dead A	5	.00000	
Dead B	5	.00000	
Dead C	5	.00000	
Intact A	4	.00000	
Intact C	5	.00000	
Immobilised C	5	.00000	
Sham A	5	.00000	
Sham B	5	.00000	
Sham C	5	.00000	
Intact B	5	4.00000	
Immobilised B	5	6.50000	
Immobilised A	5		33.71200
Sig.		.082	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.898.

APPENDIX 3.0: RESULTS' FIGURE

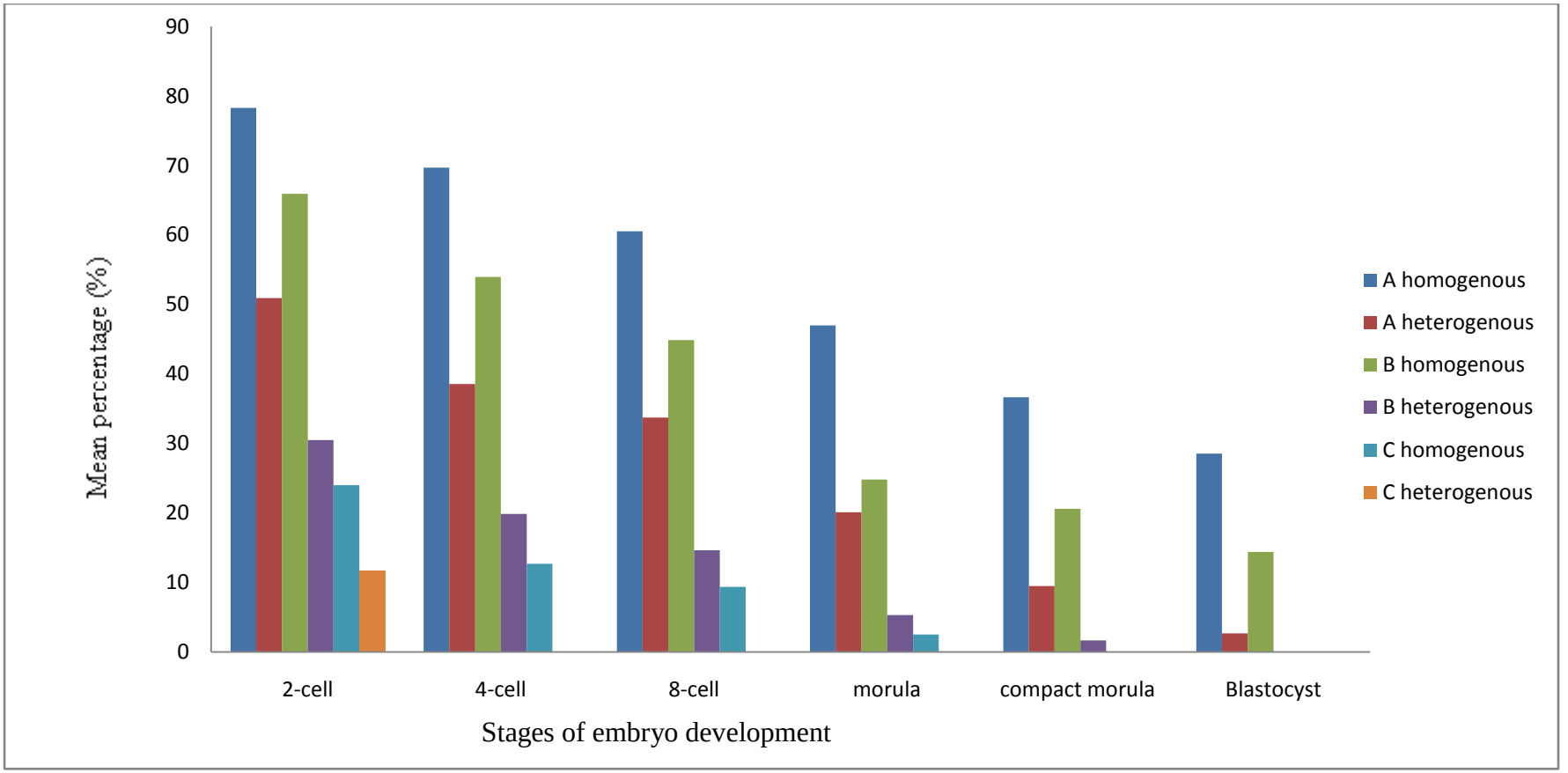

Figure 4.1: The mean percentage of cleavage rate after IVF in different oocyte grades.

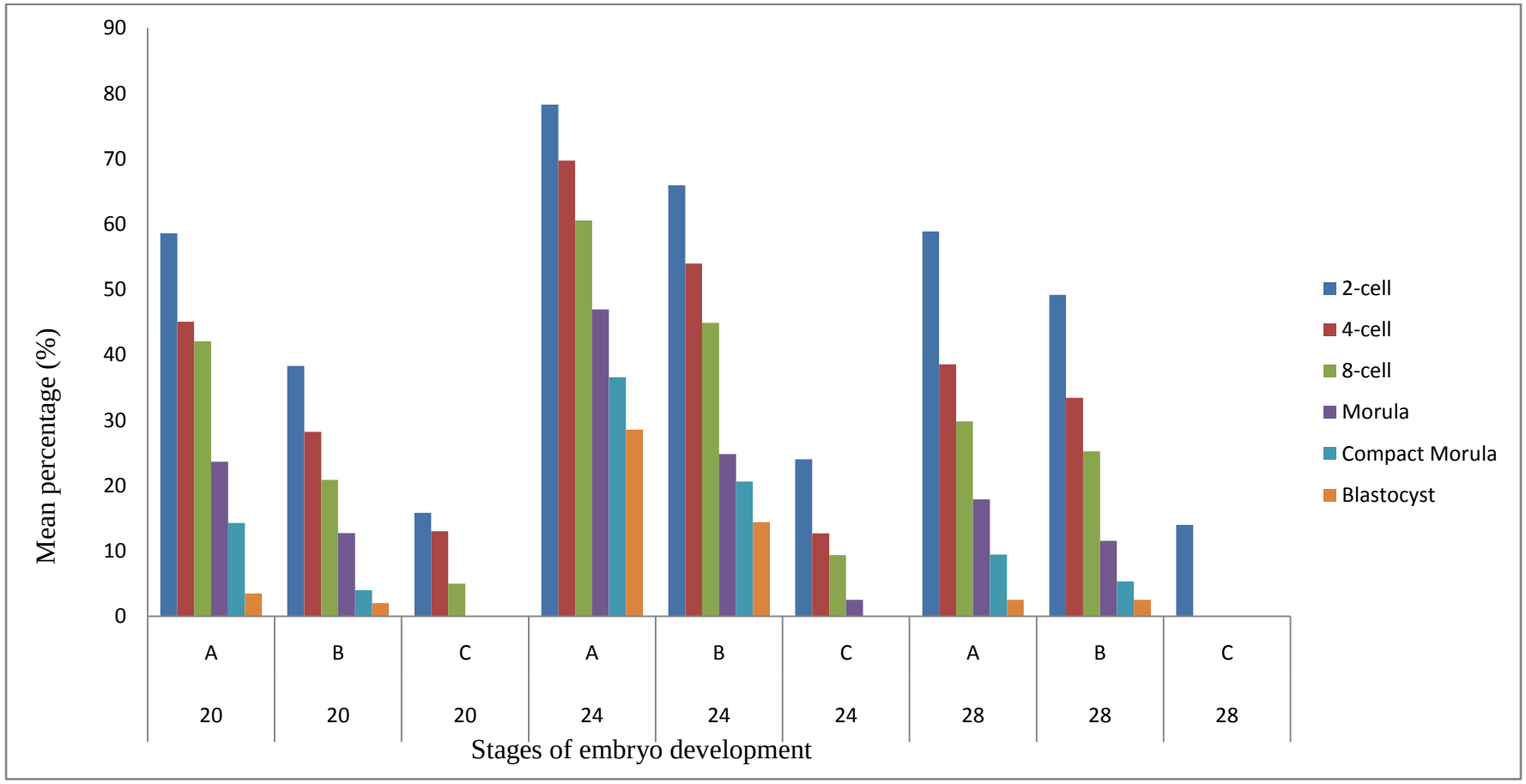

Figure 4.2: Embryonic development after IVF for different IVM durations of different COC group with homogenous cytoplasm.

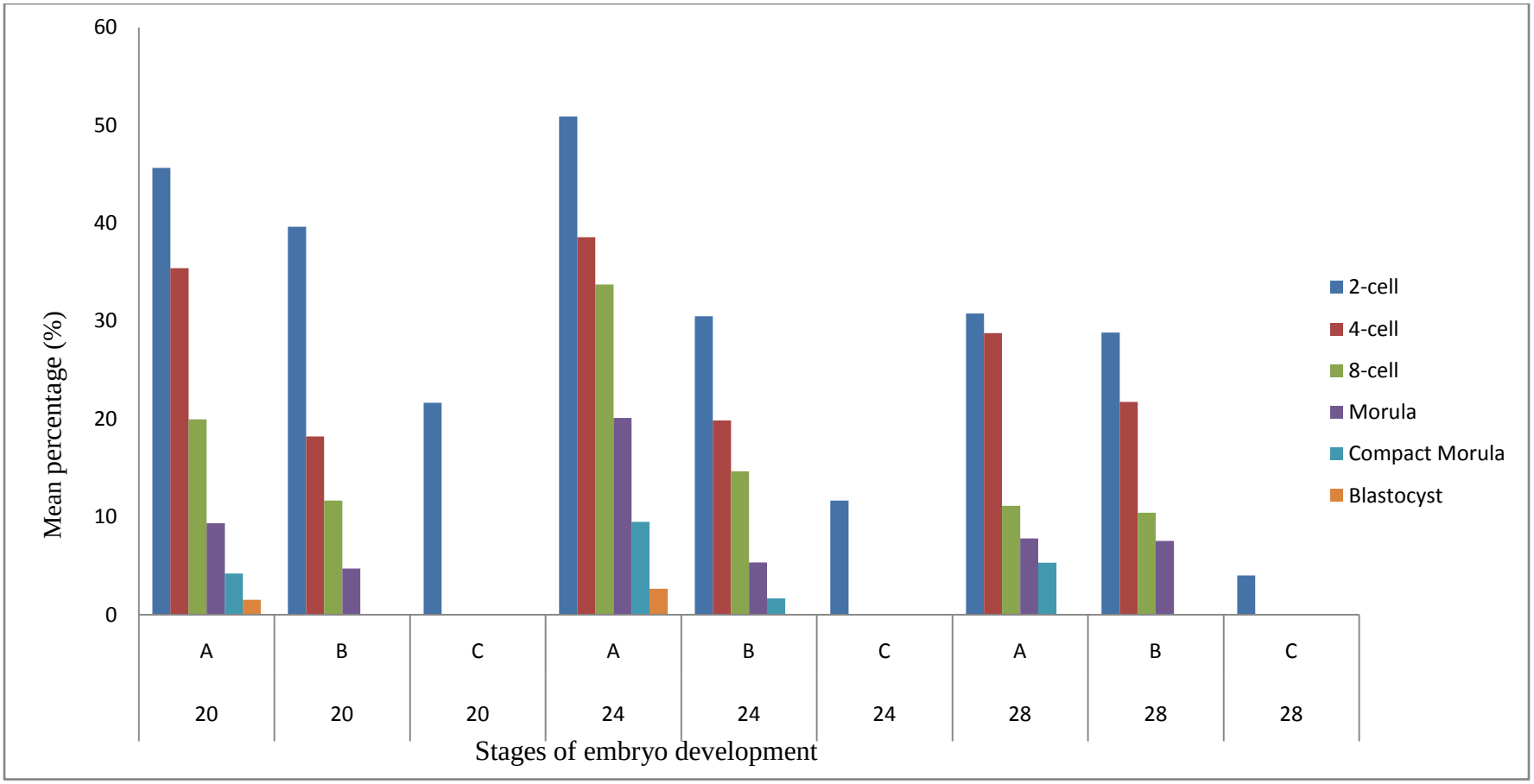

Figure 4.3: Embryonic development after IVF for different IVM durations of different COC group with heterogenous cytoplasm.

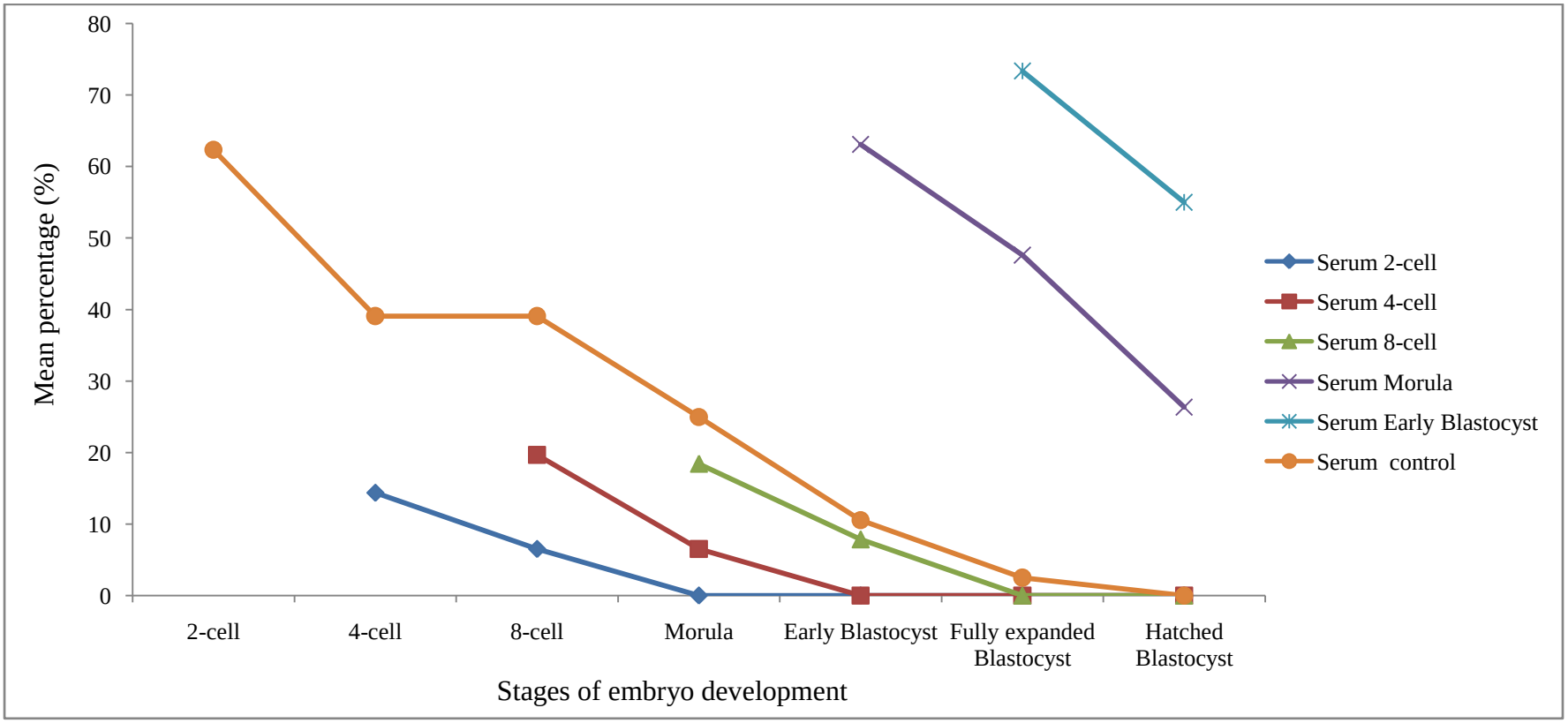

Figure 4.7: The mean percentage on effect of serum addition on blastocyst production after IVF.

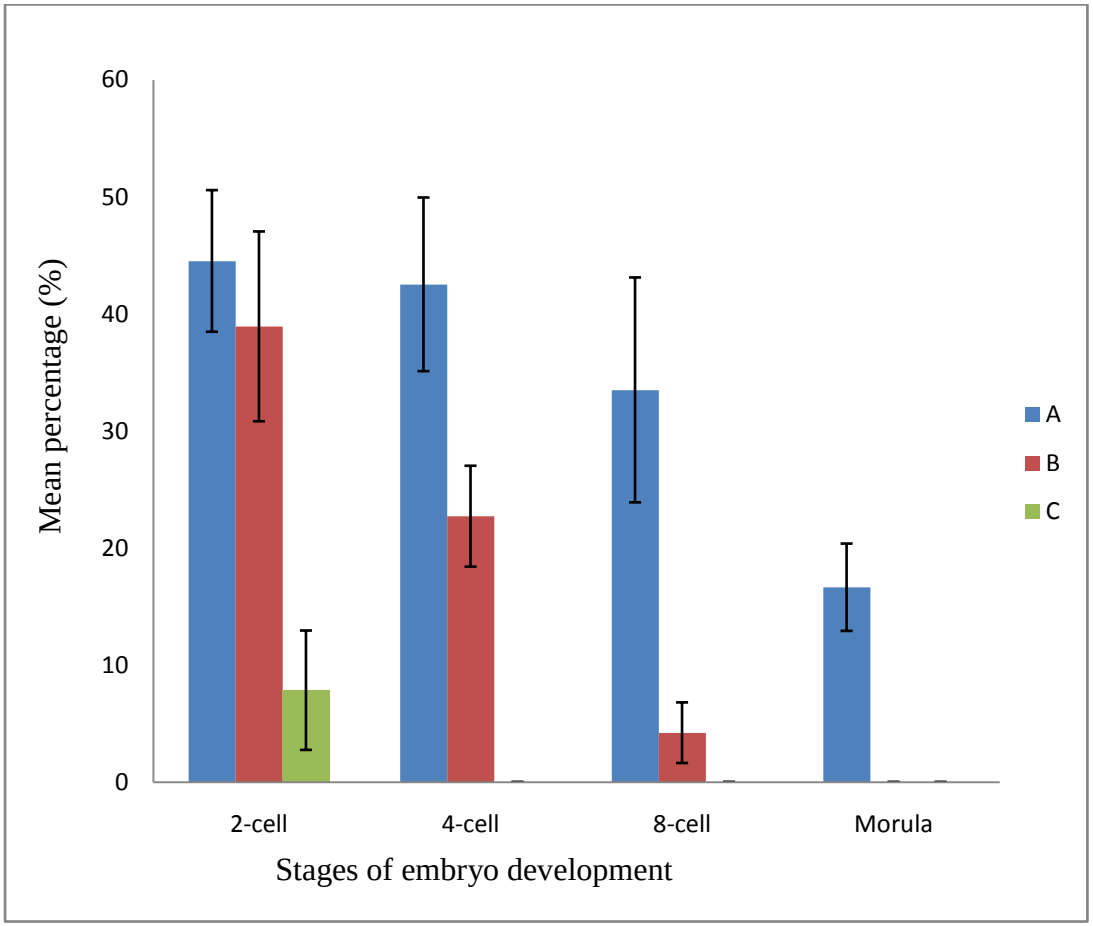

Figure 4.9: The mean percentage on effect of COC quality on fertilisation and cleavage rate after ICSI.

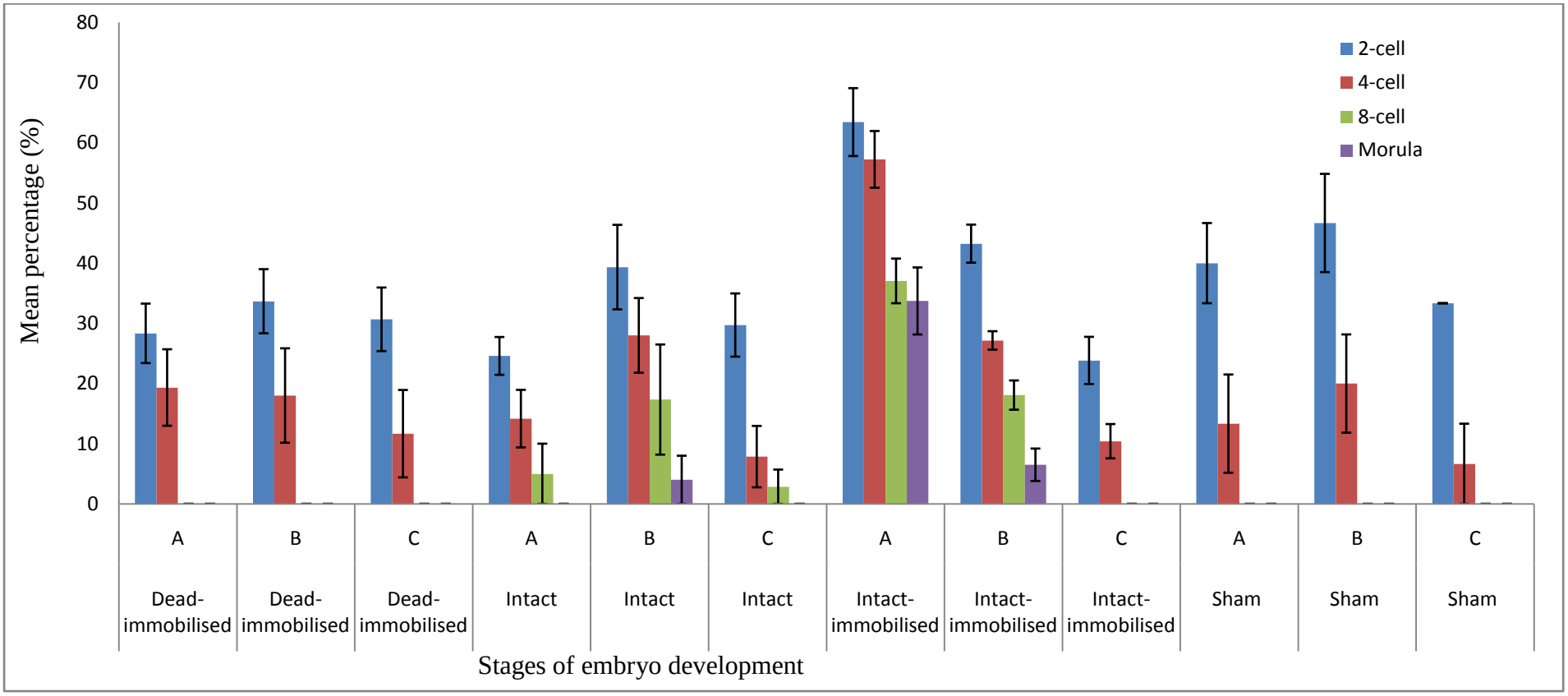

Figure 4.10: The mean percentage on the effect of sperm quality on fertilisation and cleavage rate after ICSI.

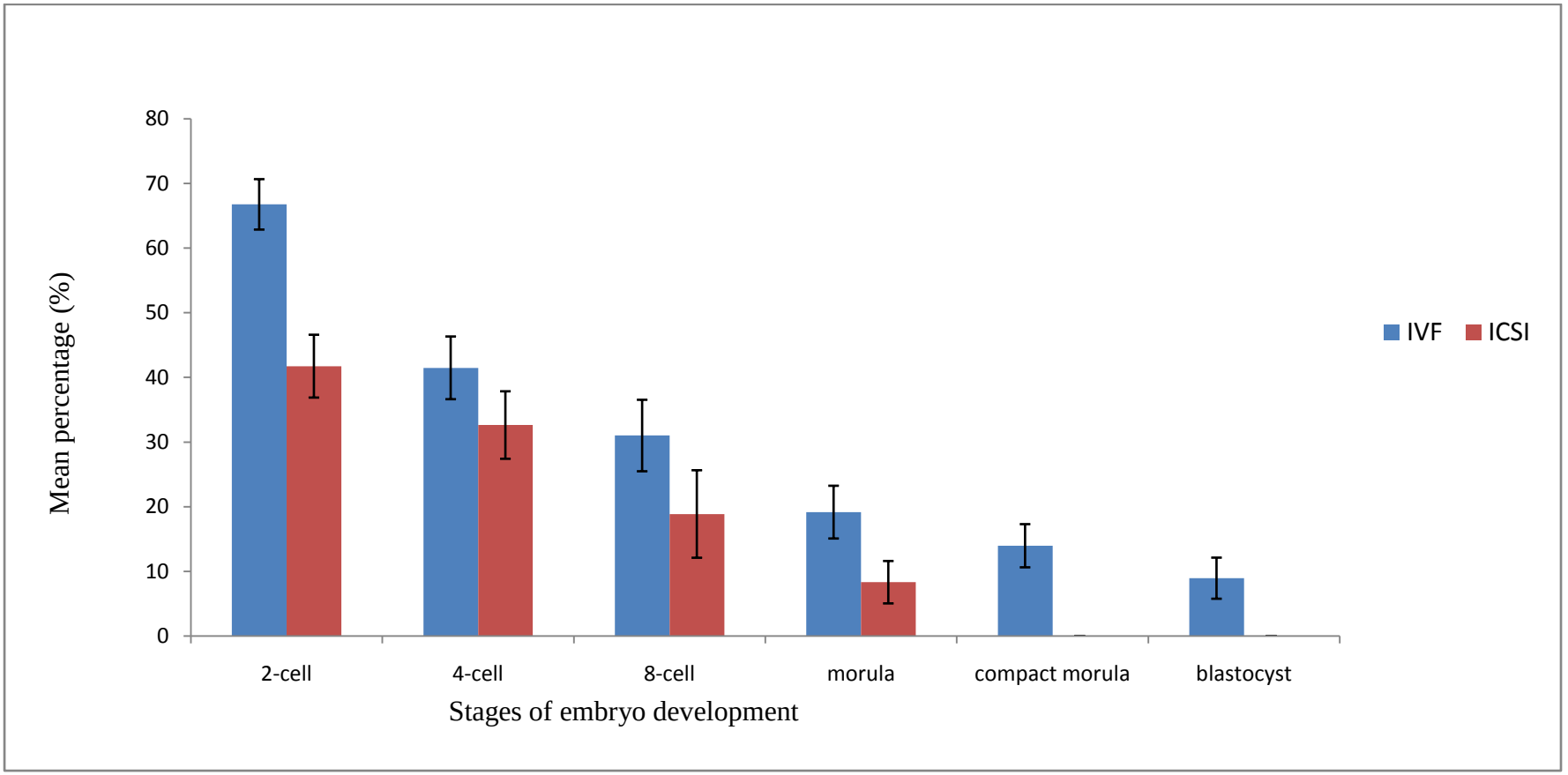

Figure 4.11: The mean percentage on the comparison between IVF and ICSI on fertilisation and cleavage rate.