EFFECT OF CUMULUS CELLS AND β-MERCAPTOETHANOL SUPPLEMENT DURING *in vitro* MATURATION ON BOVINE OOCYTE COMPETENCY

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EFFECT OF CUMULUS CELLS AND β-MERCAPTOETHANOL SUPPLEMENT DURING *in vitro* MATURATION ON BOVINE OOCYTE COMPETENCY

ABSTRACT

The oocyte source of livestock animals in eastern tropical region was relatively scarce and of unknown history, with the low-quality oocytes outnumbered the good quality. Hence, there was a need to develop more objective criteria or indicators for oocyte selection and modification of the microenvironment to improve the outcome of bovine *in vitro* embryo production. The objective of the study was to investigate the effects of: I) different groups of bovine oocytes according to the compactness of cumulus cells and II) β-mercaptoethanol (BME) supplement (50, 100, 150 and 200 μM) during *in vitro* maturation (IVM), on a) early apoptosis; b) intracellular GSH contents; c) oocyte maturation rate, and d) cleavage and subsequent embryo development rates after *in vitro* fertilisation (IVF). Bovine oocytes were retrieved from local slaughterhouses and classified into three groups, namely, Groups A (compact and dense cumulus cells), B (compact but less dense cumulus cells) and C (thin or little remnant of cumulus cells); and subsequently cultured *in vitro* in CO_2 (5%) in air at 38.5ºC in humidified atmosphere. Early apoptosis in oocytes before and after maturation was assessed using Annexin-V staining and GSH contents using GSH assay. Maturation rate, i.e. resumption to metaphase II was evaluated using Giemsa staining. Embryo development was assessed for 9 days after IVF. Analysis of Variance, Duncan's Multiple Range Tests and Independent T-test were used to analyse the data. The findings of Experiment I: cumulus cells influenced early apoptosis rates and GSH contents before and after *in vitro* maturation of bovine oocytes. *In vitro* maturation increased rates of early apoptosis and intracellular GSH was synthesised during maturation only in Group A oocytes. The compactness of cumulus cell layers exerted no effect on the bovine oocyte maturation rate after IVM. Cumulus cells promoted early embryo development up to

16-cell stage. In Experiment II, BME supplement during IVM increased GSH synthesis and early apoptosis in oocytes with less compact cumulus cells (Groups B and C) contradictory to Group A oocytes. In a nutshell, supplementation of BME in IVM medium did not exert any effect on oocyte developmental competency of good quality oocytes, except the addition of 50 μM BME increased maturation rate of Group A oocytes. The supplementation of BME above concentration 100 μM appeared to be detrimental to early embryo development. Group A oocytes, considered as good quality oocytes, with compact and dense cumulus cells, displayed a particular characteristic compared with Groups B and C. β-mercaptoethanol supplement during IVM played a role in promoting some factors in oocytes with less cumulus cells (Groups B and C). It is recommended that a specific *in vitro* embryo production protocols and procedures be established for the different bovine oocyte groups.

Keywords: cumulus cells, β-mercaptoethanol, oocyte maturation, early apoptosis, intracellular GSH

KESAN SEL KUMULUS DAN SUPLEMEN β-MERKAPTOETANOL SEMASA PEMATANGAN *in vitro* KE ATAS KOMPETENSI OOSIT BOVIN

ABSTRAK

Sumber oosit haiwan ternakan di kawasan tropika timur agak sukar didapati dan tidak diketahui asal-usulnya di mana bilangan oosit yang berkualiti rendah melebihi oosit yang berkualiti baik. Justeru, terdapat keperluan untuk membangunkan kriteria atau penanda pemilihan oosit yang lebih objektif dan juga pengubahsuaian persekitaran mikro bagi menambahbaik hasil embrio *in vitro* bovin. Objektif kajian adalah untuk menyelidiki kesan: I) kumpulan berbeza oosit bovin dari segi kemampatan sel kumulus dan II) suplemen β-merkaptoetanol (BME) (50, 100, 150 dan 200 μM) semasa pematangan *in vitro* (IVM), ke atas a) apoptosis awal; b) kandungan GSH dalam sel; c) kadar pematangan oosit dan d) kadar pembelahan dan perkembangan embrio selepas persenyawaan *in vitro* (IVF). Oosit lembu telah diperolehi dari rumah sembelih tempatan dan dikelaskan kepada tiga kumpulan, iaitu Kumpulan A (sel kumulus padat dan mampat), Kumpulan B (sel kumulus mampat dan kurang padat) dan Kumpulan C (sel kumulus nipis atau atau sedikit) dan seterusnya dikultur secara *in vitro* dalam inkubator CO_2 (5%) pada suhu 38.5°C dalam atmosfera lembap. Apoptosis awal dalam oosit sebelum dan selepas pematangan telah dinilai menggunakan pewarnaan Annexin-V manakala kandungan GSH menggunakan asai GSH. Kadar pematangan, iaitu perkembangan ke peringkat metafasa II telah dinilai menggunakan pewarnaan Giemsa. Perkembangan embrio telah dinilai selama 9 hari selepas IVF. Analisa Varians, Ujian Julat Ganda Duncan dan Ujian-T Tak Bersandar telah digunakan untuk menganalisis data. Dapatan Eksperimen I: sel kumulus mempengaruhi kadar apoptosis awal dan kandungan GSH, sebelum dan selepas pematangan *in vitro* oosit bovin. Pematangan *in vitro* telah meningkatkan kadar apoptosis awal dan GSH dalam sel telah disintesis semasa pematangan oosit hanya dalam Kumpulan A sahaja. Kemampatan lapisan sel kumulus tidak memberi kesan kepada kadar pematangan oosit bovin selepas IVM. Sel

kumulus menggalakkan perkembangan awal embrio sehingga peringkat 16-sel. Dalam Eksperimen II, suplemen BME semasa IVM meningkatkan sintesis GSH dan apoptosis awal dalam oosit dengan sel kumulus kurang padat (Kumpulan B dan C) berbeza daripada oosit Kumpulan A. Intipatinya, suplemen BME dalam medium IVM tidak memberi sebarang kesan ke atas kompetensi perkembangan oosit yang berkualiti tinggi, kecuali penambahan 50 μM BME meningkatkan kadar pematangan oosit Kumpulan A. Penambahan suplemen BME melebihi kepekatan 100 μM kelihatan menjejaskan perkembangan awal embrio. Oosit Kumpulan A yang dianggap sebagai berkualiti tinggi, mempunyai sel kumulus yang mampat dan padat mempamerkan ciri-ciri tertentu berbanding Kumpulan B dan C. Suplemen BME semasa IVM memainkan peranan dalam meransang beberapa faktor dalam oosit yang kurang sel kumulus (Kumpulan B dan C). Adalah disyorkan bahawa protokol dan prosedur penghasilan embrio *in vitro* yang spesifik seharusnya dibangunkan bagi kumpulan oosit bovin yang berbeza.

Kata kunci: sel kumulus, β-merkaptoetanol, pematangan oosit, apoptosis awal, GSH dalam sel

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	:	Degree Celsius
µg/ml	:	Microgramme per milliliter
µl	:	Microliter
µM	:	Micromolar
ACS	:	Anoestrous cow serum
AI	:	Artificial insemination
ANOVA	:	Analysis of variance
BME	:	Beta-mercaptoethanol
BO	:	Brackett-Oliphant
BOEC	:	Bovine oviduct epithelial cells
BRL	:	Buffalo rat liver
BSA	:	Bovine serum albumin
BSO	:	Buthioninesulfoximide
CaI	:	Calcium ionophore
CC	:	Cumulus cells
COC	:	Cumulus-oocyte-complex
CR1aa	:	Charles Rosenkrans 1 amino acid
Cys	:	Cysteamine
CZB	:	Chatot Ziomek Bavister
DPBS	:	Dulbelcco`s phosphate buffer saline
dH_2O	:	Distilled water
DMSO	:	Dimethyl sulphoxide
DTNB	:	5, 5'-dithiobis (2-nitrobenzoic acid)
DVS	:	Department of Veterinary Services

eCG	:	Equine chorionic gonadotrophin
ECS	:	Oestrus cow serum
EDTA	:	Ethylenediaminetetraacetic acid
EGA	:	Embryonic genome activation
EGF	:	Epidermal growth factor
FCS	:	Foetal calf serum
FGF	:	Fibroblast growth factor
FSH	:	Follicle stimulating hormone
g	:	Gramme
hCG	:	Human chorionic gonadotrophin
GPX	:	Glutathione peroxidase
GS	:	Glutathione synthetase
GSH	:	Glutathione
GSSG	:	Glutathione disulphide
GSTp	:	Glutathione S-transferase pi
GV	:	Germinal vesicle
HCI	:	Hydrochloric acid
HECM-6	:	Hamster embryo culture medium-6
HFI	:	Hydrofluoric acid
ICSI	:	Intracytoplasmic sperm injection
IGF	:	Insulin-like growth factor
ITS	:	Insulin transferrin sodium selenite
IVC	:	*In vitro* culture
IVF	:	*In vitro* fertilisation
IVM	:	*In vitro* maturation
IVEP	:	*In vitro* embryo production

LH	:	Luteinising hormone
MARDI	:	Malaysian Agricultural Research and Development Institute
mg/ml	;	Milligramme per milliliter
MI	:	Metaphase I
MII	:	Metaphase II
ml	:	milliliter
Mm	:	Millimolar
MOET	:	Multiple ovulation and embryo transfer
MPF	:	Maturation-promoting factor
NaCI	:	Sodium chloride
NADPH	:	Nicotinamide adenine dinucleotide phosphate
NBCS	:	New born calf serum
NT	:	Nuclear transfer
PB	:	Polar body
PI	:	Propidium iodide
PN	:	Pronuclear
PVP	:	Polyvinyl-pyrrolidone
SE	:	Standard error
SCNT	:	Somatic cell nuclear transfer
SOF	:	Synthetic oviductal fluid
SPSS	:	Statistical Package for Social Services
SS	:	Steer serum
TCM 199	:	Tissue culture medium 199
TUNEL	:	Tunel deoxynucleotidyl transferase dUTP nick end labeling
ZP	:	Zona pellucida

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 THE CATTLE INDUSTRY IN MALAYSIA

The livestock industry in Malaysia especially beef and dairy cattle, dairy buffaloes, sheep and goats are still produced in small-scale by small-holder farmers. Although favorable progress has been observed in recent years due to the initiatives of the government, but it is still unable to meet the local demand. The major challenges in ruminant livestock industry in Malaysia are lack of quality breeds, the high cost of animal feeds, and lack of grazing land area. The major breeds of beef cattle (*Bos indicus*) in Malaysia are Kedah-Kelantan (KK), Brahman Crosses and the European-KK-crossbreeds (Ariff *et al*., 2015). Malaysia relies heavily on imports of beef due to the high demand by Malaysian consumers and its cheaper price compared to the local one. This is based on the Malaysia Department of Veterinary Services (DVS) Statistics on the imports of beef and cattle for the year 2011 to 2015 (Table 1.1), it was shown that beef imports have been increasing every year with India currently being the major suppliers followed by Australia.

Table 1.1: Imports of cattle and beef into Malaysia (2011 – 2015)

Imports	2011	2012	2013	2014	2015
Cattle for breeding (nos.)	70,273	37,053	30,454	24,836	491
Cattle for slaughter (nos.)	63,258	80,536	62,971	114,692	68,047
Beef (metric tons)	128,046	134,032	153,991	164,612	177,763

(Source: Malaysia Department of Veterinary Services)
(Retrieved from: http://www.dvs.gov.my/index.php/pages/view/1498)

The number of cattle imported for breeding showed a reduction from 2011 to 2014, and drastically in 2015 (Table 1.1). This is attributable to the above challenges in the livestock industry, and probably due to the economic downturn, the interest in cattle breeding was not favourable leading to a review of the cattle breeding plan in the country. The imports of cattle for slaughter showed a stable increments and reductions every other year (Table 1.1). The imports of beef were shown to increase every year to meet the demands of consumers in Malaysia.

The Malaysia Department of Veterinary Services Statistics on Malaysia's total beef output and consumption from 2011 to 2016 is laid out in Table 1.2. Malaysian beef output showed a constant production amount around 50,000 metric tons yearly. The increase in beef consumption is the result of the increase in the number of population (Ariff *et al*., 2015). The production of beef in Malaysia was mainly contributed by the implementation of artificial insemination (AI) service provided by DVS. The National Institute of Veterinary Biodiversity (NIVB) under DVS has its own program of producing frozen semen mainly from the Mafriwal (synthetic dairy-beef breed) but also from individual acquired animal from beef or dairy breeds. Even so, most of the uptake of frozen semen is from imported stock (Malaysia Department of Veterinary Services). In Malaysia, AI has had a tremendous effect on both the dairy and beef industries in producing crossbreds with higher productivity, while being reasonably adjusted to the tropical environment and sufficiently disease resistant as compared to the pure temperate breeds. This has improved the economic status of farmers and had a positive impact on the livestock industry (Raymond & Saifullizam, 2010).

Table 1.2: Total local beef output and consumption in Malaysia (2011 – 2016)

	2011	2012	2013	2014	2015	2016
Output (metric tons)	48,835	51,277	51,715	52,901	50,493	50,283
Consumption (metric tons)	167,388	181,479	201,533	209,152	218,937	224,387

(Source: Malaysia Department of Veterinary Services)
(Retrieved from: http://www.dvs.gov.my/index.php/pages/view/1847)

The Department of Statistics Malaysia (2015) report on the Supply and Utilization Accounts (SUA) of Selected Agricultural Commodities, Malaysia showed the indicators of self-sufficiency ratio (SSR), import dependency ratio (IDR) and per capita consumption (PCC) for selected agricultural commodities. Table 1.3 highlights the important SSR, IDR and PCC in 2013 and 2014 of selected livestock. These indicators are important indicators to the national agriculture situation, particularly in determining sufficient food supply security. Self-sufficiency ratio explains the extent to which a country's supply of agricultural commodities to meet domestic demands. Self-sufficiency ratio that reaches 100% or more indicates production is sufficient to meet domestic needs. Supply of beef and mutton is shown to be very low compared to pork and poultry meat (Table 1.3). Import dependency ratio explains a country's dependence on imports of agricultural commodities to meet domestic needs. The higher IDR shows the more supply of agricultural commodities to be imported. Malaysia still depends highly on imports of beef and mutton (Table 1.3). The IDR for beef showed increment in 2014 and the values are expected to increase further. Per capita consumption refers to the amount of food consumed by each person per year. Malaysians consumed more pork and poultry meat compared to beef and mutton.

Table 1.3: The Indicators of the Supply and Utilization Accounts (SUA) of Selected Agricultural Commodities, Malaysia

Selected livestock	Self-sufficiency ratio (SSR)		Import dependency ratio (IDR)		Per capita consumption (PCC)	
	2013	2014	2013	2014	2013	2014
Beef	27.8	27.9	74.0	76.8	5.9	5.9
Mutton	14.5	12.8	85.6	87.2	1.0	1.2
Pork	96.0	95.3	4.5	5.1	18.5	18.3
Poultry meat	98.4	98.4	3.1	3.2	46.0	46.6

(Source: Malaysia Department of Statistics)
(Retrieved from: https://www.dosm.gov.my/v1/index.php)

The development of *in vitro* embryo production (IVEP) in Malaysia is still progressing. The low efficiency and high cost of IVEP research hampers the progress of the technology for practical applications in the livestock industry. Moreover, poor recovery of total and good quality immature oocytes still represents a major limitation of techniques for embryo manipulation in Malaysia. Limited availability of cattle ovaries was due to the low number of female cattle slaughtered compared to males and almost no females were slaughtered during festive seasons such as, Hari Raya Qurban (Aidil Adha). In addition to that, from time to time there are orders issued by DVS to cease female slaughter for a period of time. Only old or non-productive females are slaughtered while the young ones are still used for breeding. Some female cattle slaughtered were bred for their meat and their reproductive systems were suppressed, therefore they possessed very small ovaries with no oocytes or sometimes no ovary at all.

1.2 THE TECHNOLOGY OF *in vitro* EMBRYO PRODUCTION

Animal reproductive technology has been established mainly to accelerate the genetic improvement of farm animals by increasing the offspring of selected males and females and reducing generation intervals. Artificial insemination, Multiple Ovulation Induction and Embryo Transfer (MOET), *in vitro* fertilisation, semen and embryo cryopreservation and sexing, cloning by nuclear transfer, transgenics and genomics all are components of the reproductive technologies used for present and future applications (Betteridge, 2006).

Artificial insemination (AI) and preservation of semen are the main technologies used extensively both in developing and in developed countries. Embryo transfer involves a hormonal manipulation of the cow reproductive cycle, inducing multiple ovulations, coupled with AI, embryo collection, and embryo transfer to obtain multiple offspring from genetically superior females, by transferring their embryos into recipients of lesser genetic merit. The high genetic merit embryos can be frozen for later transfer (Tonamo, 2015). The use of MOET in developing countries is mainly for the identification of superior females of local breeds for use in a field programme of breed improvement (Barros & Nogueira, 2001; Kahi & Rewe, 2008).

In vitro fertilisation (IVF) requires the collection of unfertilised oocytes from ovaries of live donor animals by "ovum pick up" technique or from slaughtered animals by aspiration or slicing. The oocytes are matured in an incubator (*in vitro* maturation (IVM)) then fertilised with sperm (*in vitro* fertilisation (IVF)). The resulting zygotes are incubated in the laboratory to the blastocyst stage (*in vitro* culture (IVC)) and can be transferred fresh or frozen (Sejian *et al*., 2010). The cryopreservation of semen and embryo have been used for conserving rare livestock breeds (Long, 2008). The technology semen sexing enables sexed semen to be used for producing embryos of the desired sex, which is more efficient and less complicated than the Y chromosome

probe-based approach. However, the practical use of IVEP is limited by high production costs and the low overall efficiency under field conditions (Madan, 2005).

The technique of cloning by nuclear transfer is mainly applied for experimental purposes (Polejaeva & Campbell, 2000; Meenambigai *et al*., 2009). Cloning using somatic cells nuclear transfer (SCNT) provide the opportunities to select and multiply animals of specific merits (Arat *et al.,* 2003). The sheep "Dolly", was the first animal obtained by somatic cloning (Willmut *et al*., 1997). Since then, SCNT was used successfully for cloning cattle (Cibelli *et al*., 1998).

The development of IVEP technology is very important for production of high quality embryos. The IVEP process begins with harvesting oocytes from live animals or slaughtered animals' ovaries. Slaughterhouse animals being the most common source of ovaries constitute an economical source of oocyte which allows for large scale and economical production of embryos. However, the quality and quantity of oocytes which can be retrieved from a given number of ovaries are highly variable and these factors affect the developmental competences of the embryos derived from *in vitro* fertilisation (Habsah Bidin, 2006; Nor Fadillah Awang, 2013).

Laboratory protocols and procedures in *in vitro* embryo production include the following steps: *in vitro* oocyte maturation (IVM), *in vitro* fertilisation (IVF) and the development of the fertilised oocyte to the blastocyst (*in vitro* embryo culture, IVC). It is crucial that each of these steps has to be completed successfully in order for the embryos obtained to produce viable pregnancy that results in normal offspring. So far, the rate of expected transferable blastocysts after oocyte *in vitro* maturation, fertilisation and embryo culture only achieves 30% to 40% and, generally the major problem of IVEP procedure is reduced viability of *in vitro* embryos compared with *in vivo* (Ayman *et al*., 2016).

Mammalian oocytes are known to spontaneously resume meiosis once extracted from their follicular environment and maintained in *in vitro* (Edwards, 1965). The first introduced IVM medium components (cell culture medium, serum and antibiotics) and incubation conditions (5% CO_2 in humidified air) are still used today with only slight modifications (Edwards, 1965). TCM-199 and RPMI-1640 are chemically defined IVM media that are commercially available and widely used (Gliedt *et al*., 1996). Appropriate preparation of both sperm and oocyte is important for IVF, as well as the culture conditions for the insemination of the male and female gametes (Wright & Bondioli, 1981). Sperm separation procedures for bovine spermatozoa include Percoll density gradient, swim-up, sephadex and glass wool (Parrish *et al*., 1995). During IVF**,** capacitation of fresh and frozen bovine semen can be achieved by treatment of semen with a medium of High Ionic Strength (HIS) such as Brackett and Oliphant (BO) medium (Brackett *et al*., 1982) or through induction by heparin (Parrish *et al*., 1989) with an influx of extra-cellular Ca^{2+} using calcium ionophore (A23187) (Fukuda *et al*., 1988). For IVC of most ruminants, the culture media Synthetic Oviductal Fluid (SOF) are used widely as basis with some modifications (Restall & Wales, 1996; Tervit *et al*., 1972).

The handling of oocytes and the *in vitro* culture conditions exposed oocytes and embryos to oxidative stress resulting from events such as exposure to light, elevated oxygen concentrations and unusual concentrations of metabolites and substrates. High O_2 concentration due to increased reactive oxygen species (ROS) accumulation in the cytoplasm during *in vitro* culture caused reduction in developmental ability (Goto *et al*., 1993; Yang *et al*., 1998). Reactive oxygen species, such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet}$) anions, or hydroxyl radical ($^{\bullet}OH$), damage cell membranes and DNA and play a role in apoptosis (Nasr-Esfahani *et al*., 1990). Cell death by apoptosis is a physiological phenomenon occurring during several processes that occur in cells. The

investigation on apoptosis and its influence in oocyte and embryo quality is important, especially in *in vitro* technologies because the oocytes collected for use in embryo production comprised of a mixture of oocytes at various stages of growth and atresia (Kruip & Dieleman, 1982; Lee *et al*., 2001; Zeuner *et al*., 2003; Yuan *et al*., 2005).

Adequate glutathione (GSH) is necessary for viability in both oocytes and early embryos and has beneficial effects on subsequent embryo development (Calvin *et al*., 1986; Eppig, 1996; Knappen *et al.,* 1999: de Matos & Furnus, 2000; Fuji *et al.,* 2005). Glutathione is the most abundant non-protein thiol in biological systems and one important role of GSH is to maintain the redox state in cells by protecting them against free radicals and ROS generated during metabolism i.e. oxidative stress. The *in vitro* culture environment during bovine and ovine oocyte maturation plays a crucial role in promoting or depleting oocyte GSH content. Supplementation of IVM medium with antioxidants such as β-mercaptoethanol (BME), cysteine and cysteamine stimulated the synthesis of intracellular GSH, which in turn plays an anti-oxidative role and enhances the viability of IVF embryos (de Matos & Furnus, 2000; de Matos *et al*., 2002; Donnay *et al*., 2004).

1.3 *In vitro* EMBRYO PRODUCTION IN MALAYSIA

The application of animal reproductive biotechnology is a need to meet the worldwide demand as well as for the genetic improvement in the animal diversity (Ramli Abdullah *et al*., 2011). Some reports on bovine IVEP in Malaysia went back to the 1990s, for instance, Habsah Bidin (1996) studied some factors affecting the meiotic resumption *in vitro* in bovine oocytes. Then, the ultrastructural changes in bovine oocytes induced during *in vitro* production of embryos were observed (Kanwal *et al*., 1999). In terms of oocyte grade, Sianturi (2001) reported that cumulus expansion rates and maturation rates were better in oocytes surrounded by cumulus cells than in

denuded oocytes and fibrinated oocytes. And, the addition of hormones to the maturation medium enhanced cumulus expansion and maturation rates. Further study observed the efficient method of oocyte recovery where more oocytes were recovered by slicing with a surgical blade (29.3 oocytes per ovary) than by aspiration with a disposable syringe and needle (12.0 oocytes per ovary) (Sianturi *et al*., 2002).

Habsah (2006) studied the influence of oocyte quality and the culture system used on the developmental competence of bovine IVEP. The study found that oocyte quality had no influence on developmental competence. These oocytes were also found to perform better in culture system consisting the medium Charles Rosenkrans (CR1aa) + serum compared to CR1aa + serum co-cultured with bovine oviduct epithelial cell (BOEC). Another study reported that the compactness of cumulus cells surrounding the oocytes influenced the pronuclear formation of cattle oocytes but not cleavage and blastocyst rates. The presence of cumulus cells promoted normal fertilisation with proper pronuclear (2PN) formation (Habsah *et al*., 2009). In the recent years, IVEP research in Malaysia was focused more on the effects of adding supplements into the IVEP medium. These include cysteamine and BME (Habsah *et al.*, 2008; 2009; Nor Azlina *et al.,* 2009), insulin (Dashtizad *et al*., 2010a) and ghrelin (Dashtizad *et al*., 2010b; 2011) added to the IVM medium.

The development of IVEP of bovine and other small ruminants in Malaysia still has a long way to go. Although hampered by the factors mentioned earlier the development of bovine IVEP for the mass production of embryos in the future will have a great impact on cattle production and breeding plans in Malaysia.

1.4 PROBLEMS STATEMENT

A competent oocyte is defined as able to sustain embryonic development after fertilisation to term. However, oocytes are commonly recovered from ovaries of

slaughtered animals, which results in a mixture of oocytes at various stages of the oestrous cycle. The oocytes retrieved also showed variable conditions of the layers of the surrounding cumulus cells (CC). CC is structurally and metabolically linked with the oocytes by gap junctional communications. It is known that cumulus cells are important in oocyte maturation by its role in regulating the meiotic progression and supporting cytoplasmic maturation whereby cytoplasmic glutathione concentrations are maintained through the gap junctional communications with the oocytes (Moor *et al*., 1980; Eppig, 1982; Furger *et al*., 1996; Mori *et al*., 2000, Assidi *et al*., 2010; Ikeda & Yamada, 2014). Therefore, immature oocytes with many layers and compact cumulus cells surrounding the oocytes have been considered as good quality oocytes as they showed relatively higher competency compared to oocytes having fewer layers and less dense CC (Modina *et al*., 2004; Wongsrikeao *et al.,* 2005; Schoevers, *et al.,* 2007; Dey *et al.*, 2012; Hammad *et al*., 2015). In order to produce viable embryos in an IVEP programme,

i) is compact cumulus cell investments a good selection criterion for high quality oocytes?

ii) do these presumed good quality oocytes ensure high rates of maturation and embryonic development?

iii) Furthermore, do compact cumulus cells investments signifies the low rates of apoptosis and adequate GSH contents in oocytes, which will enhance oocyte competency?

Oocyte maturation is one of the most important and critical stages for successful IVEP. For IVM, both TCM and modified synthetic oviduct fluid (mSOF) may be used as a basic medium supplemented with fetal bovine serum (FBS), oestrus cow serum (OCS) or bovine serum albumin (BSA) as protein source, and follicle stimulating

hormone (FSH) may be used as a hormone supplement in basic medium for optimum IVM rate of indigenous Zebu cow's oocytes (Rahman *et al*., 2018). Efficient techniques for maturing and fertilising farm animal oocytes in *in vitro* culture would facilitate the application of embryo technologies in the production of embryos for the livestock industry. On the contrary, there are still many inadequacies in IVM of oocytes in domestic species. In comparison, the *in vitro* maturation (IVM) conditions are simpler than *in vivo* maturation condition whereby only limited materials are used for IVM process that lead to serious effect on the oocyte maturation status (Ayman *et al*., 2016). Studies on many different free radical scavengers have been carried out to examine their potential protection against oxidative stress on mammalian embryos subjected to *in vitro* culture systems. These include extra cellular enzymes, such as superoxide dismutase or catalase, other culture systems including taurine/hypotaurine, thioredoxin pyruvate or low molecular weight thiols such as β-mercaptoethanol (BME), glutathione (GSH) and cysteine (Cetica *et al*., 2001; Schnabel *et al*., 2006). However, the results on the effects of culture media supplementation with different antioxidants or free radical scavengers on oocyte and/or embryo quality and consequent development are not consistent and contradictory (Shan Wang *et al*., 2017). In developing an efficient technique of the culture media for an IVEP programme,

i) is the addition of certain low molecular weight thiols such as β-mercaptoethanol during IVM will enhance oocyte competency?

ii) what is the optimum concentration of β-mercaptoethanol that can be recommended to be supplemented in the maturation medium?

ii) do different qualities of oocytes perform differently when matured in IVM medium added with β-mercaptoethanol?

1.5 JUSTIFICATION OF STUDY

The proper selection of developmentally competent oocytes is crucial for successful IVEP because oocytes obtained from slaughterhouse derived ovaries of animals are highly variable in their developmental competence. The average percentage of considerably good oocytes harvested from local slaughterhouses is about 20% to 30% whereas low quality oocytes make up more than 70% of the total oocyte recovered (Habsah Bidin, 2006; Nor Fadillah Awang, 2013). In terms of homogeneity of the oocyte ooplasm, more than 80% of the oocytes recovered were of heterogenous ooplasm. Although oocytes with homogenous ooplasm were considered as good quality, there were studies that showed no difference between the two conditions of ooplasm (Nagano *et al*., 1999). Since low quality oocytes contribute a large portion of the total oocytes collected, therefore there is a need to determine whether oocyte selection based on CC surrounding the oocytes affect the outcome of *in vitro* embryo production in terms of maturation and embryo development rate, early apoptosis rates and GSH level in the oocyte.

Embryos may be protected from oxidative stress by antioxidants produced during *in vivo* culture in addition to the antioxidants present in both follicular and oviductal fluid. However, the physiological antioxidants production during *in vitro* culture is inadequate to prevent oxidative stress (Khazaei & Aghaz, 2017). Therefore, it is important to protect oocytes from oxidative stress during IVM. One approach is to supplement the medium with antioxidant compounds, for example, β-mercaptoethanol (Nikseresht *et al*., 2017). Many different methods have been tried but *in vitro* maturation is still not as efficient as *in vivo* maturation. Therefore, there is a need to develop an efficient maturation technique through the modifications in the culture system during IVM by supplementing the maturation medium with BME to reduce oxidative stress and maximize the oocyte maturation rate and improve the quality of *in*

vitro matured oocytes, especially the low-quality oocytes. The effect of supplementing BME in the maturation medium on oocyte maturation, embryo development, apoptosis rates and GSH levels in oocytes may be determined.

1.6 RESEARCH OBJECTIVES

1.6.1 Effects of Cumulus Cells on Oocyte and Embryo Developmental Competency (Experiment I)

To develop more objective criteria or indicators for oocyte selection based on the cumulus cells surrounding the oocyte to improve the outcome of bovine IVEP, the following investigations were carried out:

a) The effect of cumulus cells on early apoptosis in bovine oocytes.

b) The effect of cumulus cells on the intracellular GSH in bovine oocytes.

c) The effects of cumulus cells on nuclear maturation of bovine oocytes.

d) The effects of cumulus cells on cleavage and subsequent embryonic development after *in vitro* fertilisation (IVF).

1.6.2 Effects of β-mercaptoethanol (BME) Supplement during IVM on Oocyte and Embryo Developmental Competency (Experiment II)

To develop an efficient *in vitro* maturation technique of cattle oocyte by supplementing the maturation medium with BME, a thiol compound that may induce intracellular glutathione synthesis, the following effects were evaluated:

a) The effect of BME supplement during IVM on early apoptosis in bovine oocytes.

b) The effect of BME supplement during IVM on intracellular GSH in bovine oocytes.

c) The effect of BME supplement during IVM nuclear maturation of bovine oocytes.

d) The effect of BME supplement during IVM on cleavage and subsequent embryonic development after IVF.

CHAPTER 2: LITERATURE REVIEW

2.1 *In vitro* PRODUCTION OF BOVINE EMBRYOS

Great attention for the last four decades was directed to in vitro production of bovine embryos in the world. The birth of the first calf produced by IVF marked the beginning of IVF as a tool for production in the cattle industry (Brackett *et al*., 1982). *In vitro* embryo production is currently one of the most important biotechnologies in cattle breeding and husbandry. Developing countries such as South America and Asia, has been showing an increment in the IVEP. Although it is more expensive to produce embryos with this method than with conventional embryo transfer programs, mass production of *in vitro* embryos has been carried out in some countries such as Japan and Italy for the commercial production of calves for beef production (Galli & Lazzari, 1996; Galli *et al.,* 2003; Hasler, 2003; Thibier, 2005; Hamano *et al*., 2006). The challenges and prospects of embryo transfer industry as reviewed by Thibier (2016) showed that Asia ranked second after South America of the worldwide total number of *in vitro* produced embryos transferred, particularly from Japan.

Despite all the improvements in oocyte and embryo culture, developmental rate and viability of the *in vitro* produced embryos was found to be lower than *in vivo* produced embryos (Leibfried-Rutledge *et al*., 1987; Rizos *et al.,* 2002). Due to the differences between *in vivo* and *in vitro* environment, the rate of *in vitro* embryo production is still low with only 30-40% of oocytes developing into blastocysts, and the produced embryos also found to have altered morphology and gene expression (Camargo *et al*., 2006). Furthermore, the rate of *in vitro* produced embryos that survived after cryopreservation is less than embryos produced *in vivo*, indicating that the IVEP still needs considerable improvement (Do *et al.,* 2011).

In vitro production of embryos consists of three steps: oocyte *in vitro* maturation (IVM), *in vitro* fertilisation (IVF), and *in vitro* embryo culture (IVC). The events that occur during oocyte maturation, fertilisation and subsequent development strongly affect the development of embryo *in vitro.* Therefore, it is important to improve the efficiency and identify the sources of variations between different IVEP systems or laboratories' settings in order to develop a specific culture regime which are able to support *in vitro* culture to the blastocyst stage. The significant findings in the development of cattle IVEP research in Malaysia is laid out in Table 2.1.

Table 2. 1: Significant findings in cattle IVEP research in Malaysia

Year	Author(s)	IVEP Findings in Malaysia
1999	Kanwal	Changes in ultrastructure induced during various stages of *in vitro* embryo production contribute towards low viability of *in vitro* produced embryos. - Holding of oocytes in commonly used transport medium induced ultrastructural changes in the cumulus cells and in the oocytes. The severity of changes was higher in oocytes transported at 2-4º C than those transported at 35-37º C. None of the cleaved oocytes (0/49) in the former while an average of 18.20% (10/55) of the oocytes in the latter developed to morulae. - Oocytes submitted to IVM, exhibited incomplete cumulus expansion, swelling of mitochondria, reduced incidence of cortical granules and accumulation of lipid droplets. - 28.90% (35/121) of *in vitro* produced morula showed the presence of a large of lipid droplets, vacuoles and numerous mitochondria undergoing the process of degeneration.
1999	Yaakub *et al.*	Dietary treatment used prior to oocyte recovery did not significantly influence the developmental competence of the oocytes *in vitro*. - No overall effect of diet (P>0.05) on P_4 concentrations. Number of follicles grown in heifers on silage+concentrate supplement (18.8±3.3), silage (23.5±3.4) or hay (18.1±2.6) were not affected by the dietary treatment (P>0.05).

Table 2.1, continued

Year	Author(s)	IVEP Findings in Malaysia
		- Percentage of oocytes fertilised from heifers on hay (88.0%) was higher compared to oocytes from heifers on silage (79.0%; $P<0.05$), but was not different ($P>0.05$) compared to the proportion of oocytes from heifers on silage+concentrate supplement (86.0%). - Percentage of fertilised oocytes cleaved was higher from heifers on silage (94.0%; $P<0.01$) compared with oocytes from heifers on hay (82.0%) or silage+concentrate supplement. (86.0%). - Proportion of embryos developed to blastocyst was not different ($P>0.05$) between groups of oocytes from heifers on silage+concentrate supplement (8.0%), silage (14.0%) or hay (15%). Heifers on silage produced numerically more blastocysts (silage: 19 from 14 heifers; silage+concentrate supplement: 8 from 14 heifers; hay: 12 from 14 heifers).
2001	Sianturi	*In vitro* production of embryos from abbatoir-derived cattle oocytes. - More oocytes per ovary recovered by slicing with a surgical blade (29.3 oocytes) than by aspiration with a disposable syringe and needle (12.0 oocytes). - Cumulus expansion rate and maturation rate were better in oocytes surrounded by cumulus cells than in denuded oocytes and fibrinated oocytes. - Addition of hormones to maturation medium enhanced cumulus expansion rate and maturation rate. In the absence of hormones, 20% serum level rendered better cumulus expansion than with 10% serum but had no effect on the maturation rate. - No significant differences on the effect of serum level (10.0% or 20.0%) and hormones supplementation in the maturation medium on the cleavage rate and developmental competence of embryos. Cleavage and blastocyst rates were 71.20% and 6.20% for cumulus-intact oocytes whereas the rates were 47.20% and 1.90% for cumulus-free oocytes, respectively.
2009	Habsah *et al.*	Presence of cumulus cells promoted normal fertilisation with proper pronuclear (2PN) formation but not subsequent cleavage and blastocyst formation. Grade A (compact and dense cumulus cells) Grade B (compact and less dense cumulus cells) Grade B' (thin and little remnants of cumulus cells, almost naked)

Table 2.1, continued

Year	Author(s)	IVEP Findings in Malaysia
		- Fertilisation rates were significantly different (P<0.05) among 3 grades of oocytes. (Grade A (55.0%), Grade B (44.0%) and Grade B' (30.2%)). - The mean % of cleavage and blastocyst rates were not significant (p>0.05) in Grades A, B and B' (71.6, 74.9 and 73.6%, respectively, and 10.5, 10.4 and 7.4%, respectively).
2010	Hadi *et al.*	Vitrified immature bovine oocytes can be equilibrated (maintained) successfully at 37°C while higher or lower temperature can significantly decrease their subsequent viability and development. - Oocytes equilibrated at 37°C had significantly higher (p<0.05) viability than 41°C, but no significant difference was found between 41 and 32°C. Maturation rate in 37°C group was significantly higher compared with other groups. - Highest percentage of degenerated and germinal vesicle stage oocytes obtained from 41°C than 32 and 37°C. - Cleavage rate of 37°C group (38.77%) was greater than other groups (30.84 and 28.95% for 32 and 41°C, respectively). - Highest blastocyst rate produced when oocytes equilibrated at 37°C (6.00%).
2011	Wahid *et al.*	Ethylene glycol-based freezing and vitrification solutions are suitable choices for cryopreservation of immature oocytes and most organelles can retain their normal morphology following cryopreservation and thawing processes. - Higher incidence of abnormalities in the frozen-thawed and vitrified-warmed oocytes compared to those in freezing solution and vitrification solution-exposed groups (P <0.05) - Marked alterations in the perivitelline space, microvilli and vesicles of frozen thawed and vitrified-warmed oocytes characterized by loss of elasticity and integrity of cytoplasmic processes and microvilli following cooling and warming.
2011	Dashtizad *et al.*	*In vitro* mass production of gaur-cattle hybrid embryo (hybrid between the Indian bison (*Bos gaurus*) and cattle (*Bos taurus*). IVF- and ICSI-derived hybrid embryos could successfully progress to the transferable stage embryos.

Table 2.1, continued

Year	Author(s)	IVEP Findings in Malaysia
		- The proportion IVF-derived purebred embryos that cleaved and developed to the blastocyst stage was greater than those observed in corresponding hybrid embryos (82.95% vs. 74.17% and 42.66% vs. 33.83%, respectively). - In ICSI-derived hybrid embryos, the percentages of cleavage and morula were remarkably inferior to those developed in purebred group (47.19% vs. 60.01% and 21.01% vs. 30.29%, respectively). - No significant differences were detected regarding total cell number and the ratio of inner cell mass to total cell number in IVF- and ICSI-derived hybrid and purebred embryos.
2013	Nor Fadillah Awang	Quality of oocytes, sperm, IVM duration and culture supplement influenced the maturation, fertilisation and culture of IVF-derived embryos. Grade A (compact and dense cumulus cells > 3 layers) Grade B (< 3 layers and partially naked) Grade C (naked) - Homogenous cytoplasm, Grade A gave highest 2-cell cleavage rate (78.30%) followed by Grade B (65.92%) and Grade C (24.02%) whereas heterogenous cytoplasm, Grade A (50.90%), Grade B (30.48%) and Grade C (11.66%) - 24 hours IVM duration gave better results compared to 20 and 28 hours for homogenous ooplasm - Serum addition at morula stage could be beneficial to embryo development.
2013	Khairul Osman *et al*.	Fertilisation rate between Annexin-V selected COC with morphologically healthy selected COC. - Oocyte selection using annexin-V-FITC showed significant differences in cleavage rate compared with oocytes assumptive as morphologically healthy which is a mixture of viable and nonviable oocytes.
2014	Nor Azlina *et al.*	Early apoptosis incidence in different groups of bovine oocytes. Group A: oocytes with compact and dense cumulus cells (CC) Group B: oocytes with compact but less dense CC Group C: oocytes with thin CC or almost naked - Before maturation, the incidences of early apoptosis in Groups B (19.00%) and C (20.60%) were significantly high ($p<0.05$) compared to Group A (10.20%). - After maturation, no significant difference was found in the incidence of early apoptosis between all groups of

Table 2.1, continued

Year	Author(s)	IVEP Findings in Malaysia
		oocytes (Group A (28.40%), B (18.20%) and C (23.00%).
2014	Azizah *et al*.	*In vitro* embryo production for germplasm preservation in *Bos indicus* crossbred cows. - Only follicles of 4 mm and bigger in diameter were suitable for oocyte recovery. - Both FSH- and PMSG-stimulated group have better follicle development with more follicles available for OPU on both collection days of 24-h and 72-h post CIDR removal.
2015	Azizah *et al*.	Repeated removal of two consecutive days (48-hr) of OPU averted the development of dominant follicle i.e reduction of follicle diameter and recovery rate. It was suggested that OPU to be carried out later than 48 hour so that the follicle has more time to increase the diameter size. - 24-h OPU showed significantly greater numbers of medium and large follicles than small categories ($P<0.05$). - 48-hr of OPU showed no differences of follicles categories ($P>0.05$). - Mean total number of follicles and immature oocytes recovered were higher ($P<0.05$) in 24-hr OPU (13.76±1.2 and 7.38 ± 1.7) compared to 48-hr OPU (9.08 ± 1.5 and 3.54 ± 1.00) with oocyte retrieval rate of 51.22% and 38.17%, respectively. - Morphological classification indicated the 24-hr oocyte retrieval produced 62% of suitable immature oocytes that can be used for *in vitro* embryo production.
2016	Noraina Mohd Bakri *et al.*	Apoptosis expression in the embryo can occur regardless of oocyte quality and cleavage stage - No significant difference ($p> 0.05$) of late apoptosis status among grade A (1.5%), B (0.5%) and C (10.4%) embryos. - Early apoptosis was not seen in grade A embryo. No significant difference ($p> 0.05$) of apoptosis status between 2, 4, 8 and >8-cell embryo stage. - Early apoptosis was not seen in >8-cell stage. Although no differences in apoptosis expression between the 3 classes, the cleavage rate of grade A oocytes was significantly higher ($p< 0.01$) than grade B and C.

2.2 *In vitro* MATURATION (IVM) OF OOCYTES

In most *in vitro* experiments, immature oocytes arrested at the first prophase of meiotic division are used. Maturation of the oocytes, defined as the transition to the second metaphase of meiotic division, takes place during a 24-hour *in vitro* (Smiljakovic & Tomek, 2006). It refers to the sequence of events occurring from the germinal vesicle stage to completion of the second meiotic division with formation of the first polar body (McGaughey, 1983). Maturation of mammalian oocytes is a complex process and comprises nuclear maturation and cytoplasmic maturation (Duszewska *et al*., 2010). Complete nuclear and cytoplasmic maturation of *in vitro*-matured oocytes has the utmost importance in supporting the successful development of *in vitro* produced embryos. It is well known that incomplete oocyte maturation leads to the developmental failure of IVF and somatic cell nuclear transfer (SCNT) embryos, and it also influences nuclear remodelling and reprogramming in SCNT embryos (Takano *et al*., 1993).

Nuclear maturation is characterised by the oocyte's ability to resume meiotic division up to metaphase II during IVM and can be visualised by the extrusion of the first polar body and the appearance of the metaphase plate using a nuclear staining technique such as Hoechst 33342 (Pursel *et al*., 1985) or orcein (Liu *et al*., 2003; Talukder *et al*., 2009). Cytoplasmic maturation is dictated by the entire array of maternal mRNAs, proteins, substrates, nutrients and mitochondrial accumulation in the ooplasm during folliculogenesis (Blondin *et al*., 1997; Gandolfi & Gandolfi, 2001; Watson, 2007). The embryonic genome activation (EGA) phase, whereby certain genes are expressed and new proteins are synthesised is important to the success of embryogenesis in the pre-implantation stage because the progression of early embryo development to the eight-cell stage especially in cattle depends upon the transcript and proteins stored in the cytoplasm of oocytes (Meirelles *et al.,* 2004). Cytoplasmic

maturation enables the oocytes to control the first cleavage divisions until the embryonic genome is activated and takes over this responsibility (Fair, 2003). Although they are distinct processes, nuclear maturation and cytoplasmic maturation is an interlinked event that occurs simultaneously at determined times, even though the molecular programming of the cytoplasm may have already started during the phase of oocyte growth (Ferreira *et al.*, 2009).

Research activities in different laboratories have their own protocol for maturation *in vitro*. The most widely used media employed to perform IVM such as Ham's F10a, tissue culture medium 199 with and without serum and synthetic oviductal fluid (SOF) are complex and may be supplemented with foetal calf serum (FCS), oestrus cow serum (ECS), new born calf serum (NBCS), superovulated cow serum (SCS), anoestrus cow serum (ACS) or bovine serum albumin (BSA) (Gandhi *et al.,* 2000). Maturation media are also supplemented with pituitary FSH and/or LH (gonadotrophins) with oestradiol-17α or with extra gonadotrophins like human chorionic gonadotrophin (hCG) or equine chorionic gonadotrophin (eCG), or growth factors like epidermal growth factor (EGF) (Nedambale *et al*., 2004), EGF plus fibroblast growth factor (FGF), insulin like growth factor (IGF), insulin, transferrin sodium selenite (ITS) for improvement of maturation *in vitro* (Galli *et al*., 2001; Suthar & Shah, 2009).

2.2.1 Factors Affecting Oocyte IVM

A competent oocyte means it is able to sustain embryonic development to term (Gandolfi & Gandolfi, 2001). Several oocyte criteria including the intracellular glutathione (GSH) level, the maturation promoting factor activity, and the ability to form a pronucleus, have been used as indicators to assess cytoplasmic maturity of IVM oocytes. Follicular diameter (Duarte *et al*., 2008; Lonergen, *et al*., 1994; Pavlok *et al*.,

1992), follicle status (Vassena *et al*., 2003), oocyte diameter (Otoi *et al*., 1997; Duarte *et al*., 2008), cumulus morphology (Yuan *et al*., 2005) and reproductive status of animals (Vassena *et al*., 2003) are some factors associated to the maturational competence of oocytes. Thus, these factors were proposed as selection criteria for good quality oocytes believed to be competent for fertilisation and embryo production *in vitro*.

It has been known that the characteristics considered to affect oocyte quality confer the oocyte capability to develop into an embryo, establish a pregnancy and produce healthy offspring. Also, it is generally accepted that the quality of embryos produced *in vitro* is significantly lower than the ones derived *in vivo* (Sirard, 1989; Cognie *et al*., 2003; Peterson & Lee, 2003). The use of inferior-quality bovine oocytes derived from abattoir-retrieved ovaries might partly be the reason for the low efficiency in the development of oocytes to the blastocyst stage (Rizos *et al*., 2002, 2003). Therefore, the proper selection of developmentally competent oocytes is crucial for successful *in vitro* embryo production. The following factors have been associated with the quality of oocytes retrieved for IVEP.

2.2.1.1 Follicle size and atresia

During oocyte growth inside the follicle (six months in cattle), the interaction between the oocyte and the theca and granulosa cells provide the competency of oocyte to undergo meiotic maturation (Lussier *et al*., 1987; Miyano, 2003). Also, transcripts and proteins that are important in the maturation, fertilisation, and initiation of embryo development are accumulated in bovine oocyte (Kruip *et al*., 2000). It was found in previous studies that as follicle size increases so is the competency of oocyte, indicating that the environment provided by the large follicles improve oocyte quality (Shabankareh *et al*., 2014). In *in vitro* system, cattle oocyte first acquired the

competency to develop into blastocysts in follicles with diameter of 2-3 mm. Bovine oocytes retrieved from ovaries carrying large follicles (greater than 8 mm) showed highly significant rates of maturation and blastocyst formation compared to ovaries carrying follicles of 2-5 mm in diameter (Gandolfi *et al*., 1997; Blondin *et al*., 1997; Kubota *et al.,* 1998; Hendriksen *et al.,* 2000).

Major changes that occur in the oocyte nuclei as bovine follicle grows from 1-20 mm may exert critical effect on the developmental potential of the oocyte. The nature of the follicle growth affects the accumulation of a stable mRNA during maturation, fertilisation and early embryonic development (Sirard *et al.*, 1992). In another study, the follicular environment and the structure of oocytes retrieved was compared between dominant follicles and subordinate follicles a few days after ovulation. It was discovered that the dominant follicle environment is dominated with oestrogen and the oocytes were surrounded by healthy cumulus cells, whereas the subordinate follicles environments was dominated with progesterone and displayed some features of atresia such as degenerated cumulus cells and also meiotic activation (Assey *et al*., 1994). It was suggested that oocytes originated from small follicles displayed low developmental rates because these oocytes may have not achieved complete meiotic and/or cytoplasmic competence, or the follicles are undergoing atresia (Hendriksen *et al.*, 2000; Nicholas *et al.*, 2005). However, it was found that some bovine oocytes retrieved from medium size follicles were able to produce embryos while oocytes from large follicles failed to do so, suggesting that follicular size may partly contribute to oocyte competence. Furthermore, follicles of the same diameter may be undergoing different stages of the oestrous cycle and may progress towards growth or atresia. Apparently, compared to the diameter of follicle, the health of the follicles appears to play a bigger role for the competence of oocyte than their diameter (Hyttel *et al*., 1997).

2.2.1.2 Time and temperature of ovary storage

Another important factor needs to be considered during the recovery of oocytes for *in vitro* maturation is the time interval between slaughtering of animals and the recovery of oocytes from the ovaries. Besides time interval, the temperature of ovary storage should also be considered. It was suggested that bovine oocytes must be recovered within 1 to 2 hours of animal slaughter and the ovaries should be stored at a temperature of about 30ºC (Sekine *et al.,* 1992). This is supported by a study showing that during oocyte recovery, when the oocytes were exposed to temperature below 35ºC, the quality and quantity of *in vitro* produced embryos were decreased (Pollard *et al.,* 1996).

2.2.1.3 Methods of oocyte retrieval

Follicle enclosed oocytes are arrested at the diplotene stage of prophase of the first meiotic division. For IVEP, oocytes are generally retrieved from slaughterhouse ovaries, from live animals by 'ovum pick up technique' (OPU) or from preantral follicles. Slaughterhouse ovaries provide a cheap and abundant source of oocytes. The most common method of bovine oocyte recovery is by aspiration of vesicular follicles, using an appropriate pipette or syringe and needle. This method has proven to be best among others, due to the speed of operation which is important particularly where embryos are commercially produced (Nandi *et al.*, 2006). However, the disadvantage of using this method is that oocytes were only retrieved from 30% to 60% of the punctured follicles (Katska, 1984). Cumulus-oocyte complexes (COC) recovered by follicle dissection were shown to be significantly of highest quality and quantity compared with by aspiration. One reason is that aspiration of oocyte caused greater disruption of surrounding cumulus cells, because the cumulus oophorus is still firmly attached to the stratum granulosum (Gordon, 1994). In cattle, the use of slicing procedures was found

to increase the number of oocytes retrieved at an average of 55 oocytes per animal, which was a threefold increase compared to aspiration. Moreover, the number of blastocyst produced after IVM, IVF and IVC were increased significantly using oocytes recovered by slicing (Carolan *et al.,* 1994). In another study, a combination of slicing after preliminary aspiration of follicles showed no difference in the quality and quantity of oocytes recovered (Takagi *et al.,* 1992).

Ovum pick up by ultrasonography allows for the repeated non-surgical collection of oocytes from superior donors cattle and buffalo without any noticeable effects on the future reproductive performances of the animals. (Bungartz *et al*., 1995; Galli *et al*., 2001). It is also very practical in countries where oocyte collection from slaughtered animals is not allowed due to religious reasons (Manik *et al*., 2003). The advantages of OPU is that the technique can be used to obtain oocytes from genetically selected cows that do not respond to AI, or superovulation in multiple ovulation embryo transfer (MOET) programmes, animals with blocked oviducts, nonpregnant adult cows and also from prepubertal and pubertal animals and from pregnant cows in the first three months of pregnancy (Armstrong *et al*., 1997; Galli *et al*., 2001; Faber *et al*., 2003; Hasler, 2003; Oropeza *et al*., 2004; Imai *et al*., 2006; van Wagtendonk-de Leeuw, 2006).

2.2.1.4 Cumulus cells and homogeneity of the ooplasm

During IVM, cumulus cells (CC) have been reported to affect the nuclear and cytoplasmic maturation of mammalian oocytes (Mattioli *et al*., 1988; Tatetomo *et al*., 2000; Somfai *et al*., 2004). In the antral ovarian follicle, cumulus cells surround the oocyte, while mural granulosa cells form the follicular wall. The oocyte and its surrounding cumulus/granulosa cells stay in close contact with each other via gap junctions, which serve for the bidirectional exchange of regulatory molecules and

metabolites (Moor *et al*., 1980; Eppig, 1982; Furger *et al*., 1996). This communication is required for the development of both cell types. Granulosa and cumulus cells are not only important for oocyte maturation and fertilisation but also influence embryonic development to the blastocyst stage and further (Hazeleger *et al.*, 1993; Janowski, 2012). It was found that the thicker the layers of cumulus cells surrounding the immature oocytes retrieved by aspiration, the probability of oocyte development was increased (Lonergan, 1992)**.** Oocytes having thin layers of cumulus cells were found to have aberrant protein synthesis resulting in lower frequency of meiosis completion (de Loos *et al.,* 1989; Kastrop *et al.,* 1990). The cumulus cells are important during oocyte growth as they provide nutrients, participate in the zona formation, and synthesise the matrix composed of proteins and hyaluronic acid important in oviductal transport or in sperm trapping, following the LH surge (Bedford & Kim, 1993).

Removal of CC before *in vitro* maturation has been reported to be detrimental to oocyte maturation in mice (Schroeder & Eppig, 1984), rats (Vanderhyden & Armstrong, 1989), cattle (Chian *et al*., 1994; Zhang *et al*., 1995), and pigs (Wongsrikeao *et al*., 2005). Yamauchi & Nagai (1999) reported that the denudation of cumulus cells from immature porcine oocytes decreased nuclear maturation rates, GSH concentrations and rates of sperm penetration and male pronucleus (MPN) formation. Co-culture with cumulus-oocyte complexes (COC) or CC has been found to partially restore the developmental potential of oocytes denuded of CCs in cattle (Zhang *et al*., 1995; Hashimoto *et al*., 1998; Luciano *et al*., 2005) and mice (Cecconi *et al*., 1996; Yamazaki *et al*., 2001; Ge *et al*., 2008). However, the mechanisms by which CC improve oocyte maturation are poorly understood. Removal of CC from oocytes or zygotes at various stages of development still represents a major limitation of techniques for embryo manipulation; hence, an efficient IVM system for denuded oocytes would greatly

facilitate such procedures as germinal vesicle (GV) transfer, somatic cell haploidisation, and oocyte cryopreservation at the GV stage.

Oocytes with homogeneous ooplasm and compact multilayered cumulus investment are known to be suitable for *in vitro* maturation (Bavister *et al.*, 1983). However, the number of oocytes with heterogeneous ooplasm having dark clusters collected from the ovary is approximately more than 25% of the oocytes with homogeneous ooplasm (Hazeleger *et al.,* 1993). Many studies had shown that compared to oocytes with homogeneous ooplasm, oocytes with heterogeneous ooplasm with dark clusters have a similar or higher capacity for IVM, IVF and IVC (de Loos *et al.*, 1989, 1992; Hawk & Wall, 1994; Blondin & Sirard, 1995; Momozawa & Fukuda, 1995). In another study, oocytes with heterogeneous ooplasm was found to have a higher capacity for normal fertilisation as a result of reduction in polyspermy. However, the nuclear maturation and fertilisation rates showed no significant difference between oocytes with homogenous and heterogenous ooplasm (Nagano *et al.,* 1999). These results were inconsistent due to the *in vitro* maturation, fertilisation, or subsequent development was separately studied by different groups under different culture conditions. Moreover, investigations concerning developmental competence of bovine oocytes based on their morphology found that COC showing early signs of atresia (e.g., slight expansion of the cumulus and slight granulation of the cytoplasm) have a higher developmental potential than oocytes showing healthy morphology. Oocytes showing increased signs of follicular atresia appeared to have increased percentage of development to blastocysts, except for highly atretic oocytes (Blondin & Sirard, 1995; de Wit *et al*., 2000; de Wit & Kruip, 2001; Bilodeau-Goeseels & Panich, 2002). Table 2.2 lists the different grading of oocytes in different research based on cumulus cells surrounding the oocyte and morphology of the ooplasm.

Table 2.2: Oocyte grading in IVEP studies

Year	Author	Grading of oocytes
1979	Leibfried-Rutledge & First	Oocytes grade based on CC cell layers, ooplasm and chromatin. Group 1: >3 complete and compact CC layers, Group 2: > 3 incomplete or <3 complete and compact CC layers, Group 3: expanded CC, Group 4: nude oocytes, only enclosed by zona pellucida.
1988	Shioya *et al.*	Oocytes grade based on CC cell layers. Class A: compact and dense CC layers, Class B: compact but not dense CC layers, some partially naked with compact CC layers, Class B': partially naked oocytes with thin CC layers or with small remnants of CCs, Class C: naked oocytes.
1989	de Loos *et al.*	Oocytes grade based on CC cell layers and ooplasm. Category 1: compact, multi-layered CC investment, homogenous ooplasm, total COC light and transparent. Category 2: compact, multi-layered CC investment, homogenous ooplasm but with a coarse appearance, and a darker zone at the periphery, total COC slightly darker and less transparent. Category 3: less compact CC investment, irregular ooplasm with dark clusters, total COC darker than Category 1 and 2. Category 4: expanded CC investment, scattered in dark clumps in a jelly matrix, irregular ooplasm with dark clusters, total COC dark and irregular.
1992	Wurth & Kruip	Oocytes grade based on CC layers and ooplasm. Class A: clear and compact CC and translucent ooplasm. Class B: dark and compact CC and dark ooplasm. Class C: dark and expanded CC and dark ooplasm.
1999	Nagano *et al.*	Oocytes grade based on CC and ooplasm. Category 1: oocytes with multi-layered CC investment and homogenous ooplasm. Category 2: oocytes with multi-layered CC investment and heterogenous ooplasm with dark clusters.
2000	Khurana & Niemann	Oocytes grade based on CC and ooplasm. Category 1: compact cumulus oophorus with more than 3 layers and having a homogenous evenly granulated cytoplasm. Category 2: fewer than 3 layers of CC or partially nude but having a homogenous evenly granulated cytoplasm. Category 3: oocytes surrounded by corona radiata cells only Category 4: nude oocytes.
2001	De Wit & Kruip	Oocytes grade based on CC investment. Class A: oocytes with bright, compact CC investment,

Table 2.2, continued

Year	Author	Grading of oocytes
		Class B1: oocytes with compact CC investments, but darker than Class A. Class B2: oocyte colour comparable with Class B1 but the corona radiata appeared to be associated with the rest of CC investment. Class B3: oocytes with almost black CC and corona radiata was almost completely dissociated from the rest of CC investment. Class C: oocytes with strongly expanded CC investment and dark spots of degenerated cells.
2001	Mayes & Sirard	Oocytes grade based on CC and ooplasm. Class 1: at least 5 layers of compact CC and a homogenous cytoplasm or showed a dark zone around the periphery. Class 2: a slight expansion of the outer layers of CC and slight granulation of the cytoplasm. Class 3: oocytes with atretic or incomplete CC.
2001	Seneda *et al.*	Oocytes grade based on CC layers. Grade 1: more than three layers of compact CC. Grade 2: at least one layer of CC. Grade 3, denuded. Grade 4, atretic with dark CC and signs of cytoplasm degeneration.
2002	Bilodeau-Goeseels & Panich	Oocytes grade based on CC and ooplasm. Group 1: more than 5 CC layers, compact and homogenous ooplasm. Group 2: more than 5 CC layers, slight expansion and/or slight granulation. Group 3: less than 5 CC layers with no or slight expansion and homogenous ooplasm. Group 4: less than 5 CC layers with no or slight expansion and granulation ooplasm. Group 5: no cumulus variable Group 6: full expansion with dark clumps and heavy granulation.
2005	Yuan *et al.*	Oocytes grade based on CC layers. Group 1: > 5 layers of compact CC. Group 2: 1 to 5 layers of compact CC. Group 3: expanded CC.
2006	Nagano *et al.*	Oocytes grade based on CC ooplasm. Grade 1: brown and homogenous ooplasm. Grade 2: brown and homogenous ooplasm with dark zone around the periphery. Grade 3: brown and heterogenous ooplasm with dark clusters. Grade 4: pale and heterogenous ooplasm. Grade 5: pale and heterogenous ooplam with dark clusters.

Table 2.2, continued

Year	Author	Grading of oocytes
		Grade 6: black and homogenous ooplasm. Grade 7: variable ooplasmic features and a diameter less than 115 μm.
2009	Jeong *et al.*	Oocytes with 2 or 3 layers of CC and homogenously granulated ooplasm grade based on colour of ooplasm. Group 1: pale colour Group 2 brown colour Group 3: dark colour

2.2.1.5 Oocyte diameter

Oocytes may grow to a diameter of more than 120 μm during the growth phase, and the critical diameter for an oocyte to acquire developmental competence is 110 μm, which corresponds to oocytes associated with follicles 3 mm in diameter (Fair *et al.,* 1995; Hytell *et al.,* 1997). Oocytes with a diameter of less than 110 μm are less able to develop after fertilisation because they are still in the growth phase, and are also prone to chromosome alterations during maturation, which may affect their development further (Lechniak *et al.*, 2002; Fair, 2003).

2.2.1.6 Age of donor

Prepubertal heifers demonstrate low oocyte development competence compared to cows. It was found that oocytes from 3-4-month-old calves less likely to develop into blastocysts after IVF than oocytes from heifers (Khatir *et al.*, 1996; Palma *et al.*, 2001). Other investigations reported no difference in oocyte developmental competency between 7-11 months old prepubertal Holstein heifers and adult cows (Presicce *et al.*, 1997; Majerus *et al.*, 1999). In another study, it was reported that 4-7-month-old crossbred *Bos indicus* heifers produced less competent oocytes compared to adult cows, but the competency of oocytes from 9-14-month-old crossbred heifers were similar to the adults' oocytes (Camargo *et al.*, 2005). The low developmental competence of

prepubertal oocytes may be due to the differences in energy metabolism and oocyte size, activity of cytoplasmic factors important for maturation between oocytes from 2-6-month-old calves and adult cows (Steeves & Gardner, 1999; Salamone *et al.*, 2001). The developmental competence of prepubertal oocytes may be enhanced by using hormonal stimulation of donors to improve cytoplasmic maturation and increase the number of follicles available for puncture on the ovarian surface, but the blastocyst rates were still lower than oocytes from mature cows (Presicce *et al.,* 1997; Armstrong, 2001)

2.2.1.7 Reactive oxygen species and oxidative stress

To achieve success in IVEP, the optimisation of the culture medium to produce an *in vitro* environment mimic the oviduct and uterus is a very important step (Yuan *et al*., 2003). Several factors can influence the *in vitro* culture environment such as media composition, protein supplementation, number of embryos present in the culture drop and gas atmosphere (Carolan *et al*., 1996; Hendricksen *et al*., 1999; Fukui *et al*., 2000; Khurana & Niemann, 2000). Apart from these factors, oxidative stress induced by greater oxygen tension has been the focus for the past few years (Ali *et al*., 2003; Bedaiwy *et al*., 2004; Fatehi *et al*., 2005). Accumulation of reactive oxygen species (ROS) in the cytoplasm of developing embryos caused a reduction in the developmental ability due to high O_2 concentration during the *in vitro* culture (Goto *et al*., 1993; Yang *et al*., 1998). Naturally, the oxygen concentration in the lumen of the female reproductive tract is between 3% and 7% (Fischer & Bavister, 1993). It was reported that when embryos from mice (Umaoka *et al*., 1992), sheep (Thompson *et al*., 1990), cattle (Fujitani *et al*., 1997; Takahashi *et al*., 2000), and humans (Dumoulin *et al*., 1999) were cultured *in vitro* under a low O_2 atmospheric concentration (5%), their rates of development were higher compared to the embryos cultured under 20% O_2 (Takahashi *et al*., 2000; Guérin *et al*., 2001; Yuan *et al*., 2003; Kitagawa *et al*., 2004). Many studies

have reported the use of SOFaaci medium with 5% O_2 in many culture systems for IVEP embryos (Lonergan *et al*., 1999a; Hashimoto *et al*., 2000; van Soom *et al*., 2002; Ali *et al*., 2003; Luciano *et al*., 2005).

Reactive oxygen species mainly superoxide (O_2), hydrogen peroxide (H_2O_2) and hydroxyl (OH^-) are formed in the intermediary steps of oxygen reduction during aerobic metabolism. The production of ROS by the embryo in the *in vitro* culture are increased as a result of the presence of dead sperm cells after fertilisation, the oxidation of proteins, and the presence of metallic ions (Goto *et al*., 1993). When the production of ROS is higher than the capacity of the cells to produce antioxidants, it will go through oxidative stress (Droge, 2002). Oxidative stress showed by the peroxidation of membrane lipids and modification of proteins and nucleic acids leads to cellular death through apoptosis and hence impairing embryo quality and viability (Nasr-Esfahani *et al*., 1990; van Soom *et al*., 2002). Therefore, the balance between ROS and the presence of antioxidants is important in the enhancement of embryonic development (de Lamirande *et al*., 1997).

During *in vivo* culture, the antioxidants synthesised by the embryo together with the ones in the follicular and oviductal fluid protect embryos from oxidative stress (Gardiner & Reed, 1995). However, the production of physiological antioxidants by the embryo during *in vitro* culture is not sufficient to prevent oxidative stress, leading to the requirement for exogenous antioxidant supplements (Ali *et al*., 2003). There are two groups of such antioxidant supplements for the culture medium, i.e. enzymatic antioxidants, such as catalase, superoxide dismutase and glutathione peroxidase; and non-enzymatic or metabolic antioxidants with low molecular weight, such as cysteine, β-mercaptoethanol, L-ascorbic acid (vitamin C) and α-tocopherol (vitamin E) (Nordberg & Arnér, 2001). In another study, it was suggested that the addition of vitamin E to the medium improved bovine embryo development and blastocyst

formation resulting from the inhibition of NADPH oxidase that protect cell membranes (Olson & Seidel, 2000).

2.3 *In vitro* FERTILISATION (IVF)

In vitro fertilisation process follows the same steps as *in vivo*. The first step of *in vitro* fertilisation is sperm capacitation, which enables sperm penetration through the zona pellucida (ZP) of the oocyte. Subsequently, the sperm fuses with the membrane of the oocyte and penetrates the ooplasm (Duszewska *et al*., 2010). The binding of the sperm to the ZP stimulates it to undergo an acrosome reaction that provide the sperm with an enzymatic drill to get through the ZP, hence enabling the sperm to penetrate and fertilise the egg (Deppe *et al*., 2008). Acrosome is a large modified lysosome containing enzymes for digesting zona which is located around the anterior part of the sperm head. Sperm penetration is a factor stimulating the oocyte to meiosis termination (oocyte activation), which is manifested by second polar body extrusion and formation of female pronucleus (Yanagimachi, 1994). At the same time, nucleus of the sperm transforms into male pronucleus. Thereafter, DNA in every pronucleus replicates, followed by a fusion of pronuclei and formation of the nucleus of the zygote. The envelopes of both pronuclei disperse and chromosomes arrange into the metaphase plate, and subsequently first mitotic division of the zygote occurs (Gilbert, 2000). Appropriate preparation of both sperm (capacitation) and oocyte (activation), as well as culture conditions favourable to the metabolic activity of the male and female gametes are important for a successful bovine IVF. Generally, TALP (Tyrode's modified medium) (Parrish *et al.*, 1988) or BO (Brackett and Oliphant medium) (Goto *et al*., 1988) is most widely used medium for *in vitro* fertilisation. The addition of protein such as BSA (bovine serum albumin) accelerates the formation of pronuclei during IVF (Chen-Lu & Lu, 1990; Tajik *et al*., 1994; Eckert & Niemann, 1995).

2.3.1 Sperm Capacitation

For successful fertilisation of oocytes, good sperm preparation is one of the essential and crucial steps. Different researchers attempted different methods for separation of good motile sperm like swim-up (Lopata *et al*., 1976) or Percoll based separation system. Sperm separated by swim-up penetrated more oocytes that those selected by Percoll, but the latter is a faster procedure and the recovery of motile sperm can be increased six-fold with higher cleavage rates and embryo production (Parrish *et al*., 1986; Mendes *et al*., 2003). Sperm used for fertilisation should pass through the process of capacitation. Capacitation involves alterations of the sperm plasma membrane including removal of inherent plasma protein, reorganisation of plasma membrane lipids and changes in some enzymatic activities such as protein kinase C, which causes it to become unstable and to undergo vesiculation with the outer acrosomal membrane. Capacitated sperm become motility hyperactive due to destabilisation of the sperm membrane in preparation for acrosome reaction (Yanagimachi & Usui, 1974; Furuya *et al*., 1993).

Capacitation in bovine occurs mainly in the oviduct during the period of oestrus and it is reported that it is caused by a heparin-like glycosaminoglycan in the oviductal fluid (First & Parrish, 1987). Many previous studies reported that heparin (Brackett & Zuelke, 1993; Breininger *et al.,* 2010) or its combination with penicillamine, hypotaurine and epinephrine (Gordon, 1994; Galli *et al*., 2003; Way & Killian, 2006), Ca^{2+} ionophore A23187 (Pereira *et al*., 2000; Januskauska *et al*., 2001) with or without caffeine (Niwa & Ohgoda, 1988; Breininger *et al*., 2010; Li & Funahashi, 2010) and high ionic strength media increased IVF rates (Brackett *et al*., 1982). Heparin have been shown to bind to cattle sperm and induces changes in the intracellular environment of sperm, including increases in cAMP, pH and tyrosine phosphorylation that are required

for capacitation (Parrish *et al*., 1988; Visconti & Kopf, 1988; Handrow *et al*., 1989; Parrish *et al*., 1994; Uguz *et al*., 1994; Vredenburgh-Wilberg & Parrish, 1995; Galantino-Homer *et al*., 1997).

2.3.2 Oocyte Activation

Mammalian oocytes (except of canine oocytes which are arrested at prophase of meiosis I) are arrested at metaphase I (MI) after ovulation and complete meiosis after fertilisation. When removed from secondary follicles, immature oocytes undergo spontaneous maturation and remain arrested at MII stage until fertilisation. Binding of sperm caused several metabolic and physical changes known as oocyte activation. This includes a rise in the intercellular concentration of calcium, completion of the second meiotic division i.e. cortical reaction which is a massive exocytosis of cortical granules seen shortly after sperm-oocyte fusion. The cortical granules contained proteases that diffuse into the zona pellucida, inducing zona reaction and interact with the oocyte plasma membrane (Tanghe, 2005; Tae *et al*., 2008). A mechanical block to polyspermy is established by cortical reaction whereby the zona pellucida is hardened, and the sperm in the middle of traversing the zona pellucida will be stopped in their tracks. (Wang *et al*., 1997; Way & Killian, 2006; Coy & Aviles, 2010). Furthermore, sperm receptors in the zona pellucida are destroyed and as a result, the sperm are unable to bind to the zona pellucida (Landim-Alvarenga *et al*., 2002).

2.4 *In vitro* CULTURE (IVC)

The final stage of *in vitro* production of bovine embryos is the culturing of embryos for 168 hours, from the zygote until the blastocyst stage (Duszewska *et al*., 2010). After 168 hours, embryos at various stages of development are recovered from early morula through late morula and early blastocyst to late or even hatching blastocyst

(Van Soom & De Kruif, 1996). The very early stage of development of bovine embryos is similar to that of human embryos, and therefore cattle have been considered as a model species to study embryogenesis (Niemann & Wrenżycki, 2000). Bovine embryos cultured *in vitro* develop slower than embryos *in vivo*, because in the former the cell cycle between eight-cell and sixteen-cell stage embryos is longer (Barnes & Eyestone, 1990; Grisard *et al*., 1994). In cattle, activation of the embryonic genome occurs at the eight-cell stage (Eystone & First, 1986). The maternal-embryonic control transition is associated with ultrastructural changes in the nucleus and nucleoli and with the protein synthesis based on expression of embryonic genes. However, the expression of some embryonic genes occurs much earlier in development, at the two-cell stage (Niemann & Wrenżycki, 2000).

There are various systems available for *in vitro* culture of zygotes that include co-culture with various types of cells such as bovine oviduct epithelial cells (BOEC) (Eyestone & First, 1989), cumulus cells or trophoblastic vesicles, established cell line, buffalo rat liver (BRL) cells or vero cells. Nowadays, more studies have resort to the use of chemically defined media like SOF, CR1aa, Chatot Ziomek Bavister medium (CZB), hamster embryo culture medium-6 (HECM-6), and G1.1/G2.2 (Chatot *et al.,* 1989; Krisher *et al*., 1999), with SOF being the most commonly used by different laboratories and researchers. The advantage of defined media is that higher blastocyst rates can be achieved with only low oxygen tension (5%) compared to 20% when bovine oocytes were cultured in SOF and in SOF plus BSA (Lonergan *et al*. 1999b; Vanroose *et al*., 2001). Amino acids secreted by the female reproductive tract were use used as energetic substrate by the embryo. Therefore, the addition of amino acids in serum-free culture medium would enhance embryo development probably through antioxidant action, pH and osmolality control, apart from reducing stress and cell

fragmentation resulting from culture *in vitro* (Bavister, 1995; Donnay *et al*., 1997; Gaedner, 1998; Lee *et al*., 2004).

2.5 APOPTOSIS IN CUMULUS CELLS AND OOCYTES

Reactive oxygen species may induce cell death by necrosis or by apoptosis, in different cell types, the origin and the rate of oxidative stress generation (Nasr-Esfahani *et al*., 1990). Apoptosis is a self-destruction process of cells which is a physiological phenomenon that occurs during several processes, including embryogenesis (Parchment, 1991; Pierce *et al*., 1991; Ameisen, 2002). Apoptotic cells usually undergo cell shrinkage, translocation of phosphatidyl-serine to the outer cytoplasmic membrane, DNA fragmentation, and segmentation of the cell into apoptotic bodies (Kerr *et al*., 1972).

Apoptosis incidence in cumulus cells were reported to affect oocyte quality and subsequent embryo development (Lee *et al*., 2001; Zeuner *et al*., 2003; Corn *et al*., 2005; Yuan *et al*., 2005). In the ovary, apoptosis is responsible for follicular atresia, whereby most follicles present at birth are lost during further development. Atresia can occur at any stage of follicular development, but the follicular compartment in which atresia is initiated differs. In antral follicles, atresia starts with the degeneration of granulosa cells and the oocyte is only affected during the last stage of follicular atresia (Driancourt *et al*., 1991; Yang & Rajamahendran, 2000, 2002; Irving-Rodgers *et al*., 2001). In contrast, in preantral follicles, cell death is often initially observed within the oocyte (Marion *et al*., 1968; Reynaud & Driancourt, 2000). In addition to the compartment initially affected, the molecular mechanism of granulosa cell death also varies according to follicle size (Alonso-Pozos *et al*., 2003). The process of atresia occurs continuously from birth until the complete depletion of the follicle pool (Hirshfield, 1991; Hsueh *et al*., 1994; Kaipia & Hsueh, 1997). At any given moment, over half of the follicles present in an ovary are at different stages of atresia. Therefore,

when oocytes recovered by slicing of slaughtered animals' ovaries, most of these oocytes will have arisen from atretic follicles which could affect blastocyst production (Kruip & Dieleman, 1982).

Cumulus cells induce oocyte maturation and fertilisation by releasing and mediating signals to oocytes (Tanghe *et al*., 2002). Although many investigations reported the occurrence of apoptosis in COC, the impact in COC on oocyte development are still unclear. In the studies of Lee *et al*. (2001), Mikkelsen *et al*. (2001), and Zeuner *et al*. (2003), it was reported that human and bovine cumulus cells were subjected to apoptosis, while others found no occurrence of apoptosis in cumulus cells of rats (Szoltys *et al*., 2000), pigs (Manabe *et al*., 1996), and cattle (Yang & Rajamahendran, 2000; Ikeda *et al*., 2003). A further study was carried out by Lee *et al*. (2001) investigating the relationship between apoptosis in cumulus cells and the outcome of human embryo *in vitro* culture. It was demonstrated that the incidence of cumulus cells apoptosis can be used as an indicator of oocyte quality, outcome of *in vitro* fertilisation-embryo transfer, and age-related decline in fertility.

The occurrence of apoptosis was studied on *in vivo*-and *in vitro*-derived embryos and was shown to be regulated through the production of ROS in the blastocoele fluid and the intracellular level of GSH (Feugang *et al*., 2004; Pierce *et al*., 1991). Earlier, the occurrence of apoptosis in mouse MII stage oocytes was reported by Takase *et al*. (1995) and Fujino *et al*. (1996). Then, Perez *et al*. (1999) succeeded in detecting DNA fragmentation and caspase activity i.e. proof of apoptosis in ovulated mouse oocytes.

Byrne *et al*. (1999) showed that apoptosis was detected in the 9–16 cell stage and increased in the morula stage in the bovine embryos. Although previous reports have investigated the occurrence of apoptosis in bovine ovarian follicles (Isobe & Yoshimura, 2007) and cumulus cells (Luciano *et al*., 2000), studies were limited and the

findings were inconsistent. Matwee *et al*. (2000), using Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining showed that apoptosis occurred in both mature and immature oocytes, while Yuan *et al*. (2005), using the same technique, detected no apoptotic oocytes before or after maturation. The research by Li *et al*. (2009) that found an association between early apoptosis with improved developmental potential in bovine oocytes defying the general view that oocytes undergoing apoptosis have poor developmental competence.

2.6 GLUTATHIONE IN LIVING CELLS

Glutathione (L-γ-glutamylcysteinylglycine; GSH, Figure 2.1), a tripeptide consisting of cysteine, glutamate and glycine is a ubiquitous low-molecular-weight non-protein thiol-containing molecule found at millimolar concentrations in eukaryotic cells (Meister & Anderson, 1983). A thiol is an organosulfur compound that contains a carbon-bonded sulfhydryl (-C-SH or R-SH) group. Thiol groups are reducing agents, and GSH reduces disulphide bonds formed within cytoplasmic proteins to cysteines by serving as an electron donor. In the process, glutathione is converted to its oxidised form glutathione disulphide (GSSG), also called L(-)-Glutathione. Once oxidised, glutathione can be reduced back by glutathione reductase, using NADPH as an electron donor (Figure 2.2) (Ilkhani *et al.,* 2016). Under normal conditions the balance of the equation is far in the direction of maintaining cellular glutathione in its reduced state (GSH >99%). Thus, GSH is the most abundant non-protein thiol in biological systems and has many important functions in intracellular physiology and metabolism. The ratio of reduced glutathione to oxidised glutathione within cells is often used as a measure of cellular toxicity (Pastore *et al*., 2003).

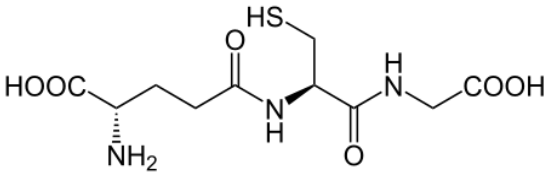

Figure 2.1: Glutathione structure (Source: Wikipedia, Glutathione)

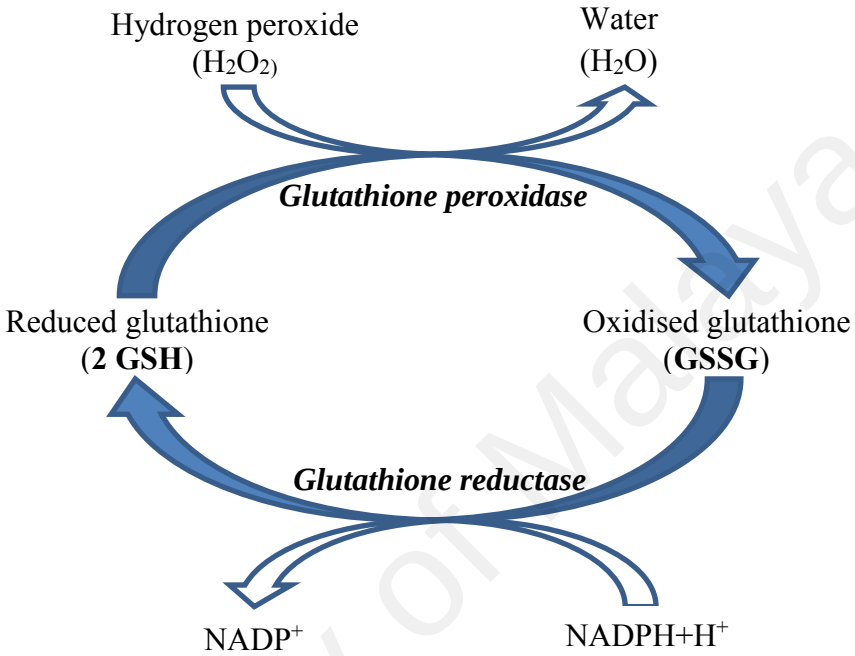

Figure 2.2: Oxidation and reduction of glutathione (Adapted from Ilkhani *et al.*, 2016)

One of the most important roles of GSH is to maintain the redox state in cells, protect against free radicals and reactive oxygen species (ROS) generated during metabolism i.e. oxidative stress (Simic, 1988; Anderson, 1997; Sipos *et al*., 2002; Pastore *et al*., 2003). Reactive oxygen species induce damage to the DNA of cells and lipid peroxidation which adversely affects membrane structure, fluidity and function (Freeman & Crapo, 1982). Glutathione is a major non-enzymatic antioxidant defence in embryos functioning as strong basal reactive oxygen species (ROS) scavenging activity (Johnson & Nasr-Esfahani, 1994; Gardiner *et al*., 1998; Dröge, 2002). It can react with many ROS and act as a cofactor for glutathione peroxidase that catalyses the reduction of toxic H_2O_2 and hydroperoxides (Bilodeau *et al.*, 2001). Glutathione participates in redox buffering (Schafera & Buettner, 2001), detoxification of organic and inorganic

hydroperoxides (Camel-Harel & Storz, 2000) and detoxification of xenobiotics (Griffith & Mulcahy, 1999; Hayes & McLellan, 1999; Pócsi & Penninckx, 2004).

Glutathione can be obtained from diet or can be synthesized *de novo* in the liver. The tripeptide is synthesized by combining cysteine with l-glutamate which is catalysed by gamma-glutamylcysteine synthetase (γ-GCS). Next, a glycine is added by the enzyme glutathione synthetase (GS). Other important glutathione-related enzymes include glutathione peroxidase (GPX), which reduces lipid hydroperoxides and H_2O_2 and converts GSH to the oxidised form (GSSG), glutathione reductase, which reduces GSH to GSSG, and glutathione S-transferase pi (GSTp), which catalyses the conjugation of GSH with deleterious compounds (Anderson, 1985; Arai *et al*., 1999; Bernardini *et al*.,1999). The antioxidant cytoprotective effects of GSH are derivative of its role as a co-factor for selenium-dependent glutathione peroxidases that reduce hydrogen peroxide, and lipid and phospholipid peroxides. These reactions lead to the oxidation of GSH and formation of glutathione disulphide (GSSG), which can be readily salvaged via reduction by glutathione reductase (Griffith & Mulcahy, 1999; Hayes & McLellan, 1999). The resulting oxidised glutathione (GSSG) is reduced to GSH by glutathione reductase using NADPH as the co-factor. The GSH/GSSG pair plays important roles as redox sensor and as protective agents against ROS-induced damages in many cell types (Halliwell & Gutteridge, 1999). Therefore, the ratio of both forms is crucial for the characterisation of the oxidative stress in living systems. Glutathione homeostasis is dependent on the rate of GSH synthesis, utilisation, and export. Most cells do not import significant quantities of GSH, therefore the *de novo* GSH biosynthesis in maintaining intracellular GSH levels during periods of enhanced GSH utilisation is important (Backos *et al.*, 2010).

2.6.1 Importance of Glutathione in Embryo Production

Adequate glutathione (GSH) is crucial for both oocytes and early embryos viability (Knappen *et al*., 1999; de Matos & Furnus, 2000; Fujii *et al*., 2005), and has beneficial effects on subsequent embryo development (Calvin *et al*., 1986). During maturation of the oocyte in the ovary GSH content increases as the time of ovulation is approached (Perreault *et al*., 1988), forming a reservoir pool which will protect the cell in the later stages of post-fertilisation development (Telford *et al*., 1990). Glutathione has been found to be synthesised during oocyte *in vitro* maturation (IVM) in mouse (Calvin *et al*., 1986), hamster (Perreault *et al*., 1988), pig (Yoshida *et al*., 1993), cattle (Miyamura *et al*., 1995) and buffalo (Gasparrini *et al*., 2006). The GSH content increased during oocyte maturation and furthermore, oocytes matured *in vivo* contained significantly more GSH than oocytes matured *in vitro* (de Matos & Furnus, 2000; de Matos *et al*., 2002; Yoshida *et al*., 1993; Zuelke *et al*., 2003).

It was also demonstrated that GSH concentration was lower in oocytes from prepubertal animals than in oocytes from adult animals (Rodriguez-Gonzalez *et al*., 2003; Donnay *et al*., 2004). Glutathione synthesis during *in vitro* maturation (IVM) has an important role in embryo development whereby increased intracellular concentration of GSH assisted the developmental competency of oocytes after fertilisation and cleavage (Eppig, 1996). Intracellular GSH which functions in DNA and protein synthesis and amino acid transport inside mammalian cells (Meister, 1973; Nagai, 2001), promotes the oocyte-spermatozoan complex to develop a male pronucleus (MPN) (Yoshida *et al*., 1993; Yamauchi & Nagai, 1999), thus enhances subsequent embryo development (Abeydeera *et al*., 1998). Glutathione is important for sperm function, chromatin decondensation and hence for male pronuclear formation following sperm penetration (Perreault *et al*., 1988; Yoshida *et al*., 1992; Yoshida, 1993; Grupen *et al*., 1995; Williams & Ford, 2005).

In the mouse, GSH is involved in different embryonic events, including cell proliferation and differentiation at later preimplantation stages and, as a constituent of co-enzymes, it contributes to energy generating metabolism (Gasparrini *et al*., 2003). When fertilised mouse oocytes were cultured *in vitro,* they underwent a 2-cell block and showed a decrease in GSH content compared with embryos that developed *in vivo*. Furthermore, GSH levels drop approximately 10-fold during preimplantation development between the unfertilised oocyte and the blastocyst stage. It was also found that the mouse blastocyst has the capacity to synthesise GSH, but early embryos up to morula stage have only a limited ability to synthesise it (Gardiner & Reed, 1995). In another study, Lim *et al.* (1996) suggested that in bovine embryos *de novo* synthesis of GSH begins to increase at the 9 to 16 cell stage, and embryos at this stage seem to be more resistant to oxidative stress than presumptive zygotes or blastocysts (Van Langendonckt *et al*., 1998). The increase in GSH concentrations during IVM of cattle oocytes was found to improve subsequent embryo development to blastocyst stage (de Matos *et al*., 1995; 1996; Miyamura *et al*., 1995; Furnus *et al*., 1998).

2.6.2 Thiol Compounds and Glutathione Synthesis

It was shown that glutathione content increases during development and oocyte maturation in the ovary as the oocyte approaches the time of ovulation (Perreault *et al*., 1988) and protects it in later stages of fertilisation (Telford *et al*., 1990). Addition of thiol containing precursors of GSH such as cysteine (CySH), cysteamine, β-mercaptoethanol or use of a cysteine-rich medium (TCM 199 or Waymouth MB 75211) increased GSH content of oocytes after maturation (de Matos *et al*., 2002). Studies have been carried out to examine the effects of adding thiol containing precursors of GSH including cysteamine, β-mercaptoethanol, cysteine, and cystine to media to assist in the *in vitro* production of embryos in bovine (de Matos & Furnus, 2000; Donnay *et al*.,

2004), ovine (de Matos *et al*., 2002), pig (Yoshida *et al*., 1993), goat (Rodriguez-Gonzalez *et al*., 2003), and hamster (Zuelke *et al*., 2003). In general, these compounds were found to increase the GSH concentration and improved sperm decondensation and pronucleus formation.

Studies had reported that low molecular weight thiol compounds such as β-mercaptoethanol and cysteamine enhance cysteine-mediated GSH synthesis in bovine embryos. An increase of intracytoplasmic GSH concentration caused by the addition of β-mercaptoethanol and cysteamine to the culture medium was found to be beneficial for the development of 6-8-cells stage bovine embryos to the blastocyst stage (Takahashi *et al*., 1993). de Matos and Furnus (2000) reported that addition of cysteamine during bovine IVM caused the high intracellular GSH level and improved bovine embryo development and quality, producing more embryos reaching the blastocyst stage on Day 6 than embryos matured in unsupplemented medium (de Matos *et al*., 1995; 1996). When present during IVM of bovine oocytes, cysteamine (Cys) or β-mercaptoethanol stimulate glutathione synthesis and decrease hydrogen peroxide levels, improving embryo development (de Matos *et al*., 1996; 1999; de Matos & Furnus, 2000). Extracellular cysteine can be transported into the oocyte and β-mercaptoethanol promoted the uptake of cysteine and synthesis of GSH (de Matos *et al*., 1996; 2002). The influence of intracellular GSH synthesis on embryo development was supported by the finding that buthioninesulfoximide (BSO), a specific inhibitor of GSH synthesis neutralises the induce effect of thiol compounds on development and GSH synthesis of bovine oocytes and embryos (Griffith & Meister, 1979; Takahashi *et al*., 1993; de Matos *et al*., 1996). Addition of cysteamine in maturation medium enhanced the glutathione (GSH) synthesis (de Matos & Furnus, 2000) and improved oocyte maturation by protecting the oocytes from oxidative stress (Meister, 1983; Gasparrini *et al*., 2003; Zicarelli & Gasparrini, 2004).

During IVM of ovine oocytes, cysteamine (Cys) or β-mercaptoethanol stimulates glutathione synthesis and decreases hydrogen peroxide levels, improving embryo development (de Matos *et al*., 1996; 1999; de Matos & Furnus, 2000). In addition, both Cys and β-mercaptoethanol stimulate glutathione synthesis during ovine maturation *in vitro*, but only Cys promotes embryo development and quality in this species, whereas β-mercaptoethanol was uninfluential (de Matos *et al*., 1999). Supplementation with β-mercaptoethanol and cysteine during the *in vitro* maturation of oocytes from adult sheep increased intracellular GSH concentrations (de Matos *et al*., 1996; 2002). Abeydeera *et al.* (1998) demonstrated that addition of β-mercaptoethanol to IVM medium increased intracellular pig oocyte GSH levels and improved embryo development. It was also reported that, in the presence of a thiol compound, such as β-mercaptoethanol, supplementation of IVM medium with cysteine increases the GSH level and improves developmental competence of pig oocytes following fertilisation (Abeydeera *et al*., 1999). Positive effects of cysteamine during IVM on developmental competence of pig oocytes were also observed (Grupen *et al*., 1995).

CHAPTER 3: MATERIALS AND METHODS

3.1 INTRODUCTION

In the present study, the oocytes used were retrieved from slaughtered cattle ovaries collected from local slaughterhouse in Shah Alam and Banting, Selangor and Senawang, Negeri Sembilan. All experiments in this study involving works on oocyte retrieval, IVM, IVF and IVC were conducted at the Gamete Physiology Laboratory, Strategic Livestock Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor. The laboratory experiments were conducted according to the animal and ethical guidelines of University of Malaya and MARDI. This research was conducted from 2010 till 2016.

3.2 MATERIALS

3.2.1 Laboratory Equipment

All equipment used in the study are listed in Appendix A. All surfaces and environment of the working place were kept sterilised with 70% ethanol (Merck, Germany) before and after each experiment.

3.2.2 Labware and Disposables

The labware and disposables used in the study are listed in Appendix B. All labware were immersed overnight in cleaning solution 7X-PF (ICN Biomedicals, Inc), rinsed with tap water then with distilled water and finally dried. They were then wrapped with aluminium foil (Diamond, USA), autoclaved with other laboratory disposables such as micropipette tips, microcentrifuge tubes and dried in an oven at 60^{o}C.

3.2.3 Chemicals and Reagents

The list of chemicals and reagents used in the study is given in Appendix C. All chemicals used were purchased from Sigma-Aldrich Co. from USA, unless stated otherwise.

3.2.4 Samples Source

Frozen Mafriwal semen was obtained from the National Institute of Veterinary Biodiversity, Jerantut, Pahang. Mafriwal (Malaysian-Friesian-Sahiwal) is cross-bred cows developed by the Kelantan DVS that are able to withstand warm weather and produce more milk than indigenous cows. Only straws with more than 60% post-thawed sperm motility were used.

The ovaries were collected from the Abbatoir Complex, Department of Veterinary Services in Shah Alam and Banting, Selangor and in Senawang, Negeri Sembilan, whenever available. The ovaries collected regardless of the phases of oestrous cycle and pregnancy were then transported to the laboratory within 1 to 2 hours after slaughter in a flask containing Dulbecco's Phosphate Buffered Saline (DPBS, Sigma) as a collecting medium. The temperature of DPBS was maintained between 30 to 37ºC.

3.3 METHODOLOGY

3.3.1 Preparation of Stock Solutions for Ovary Collection and Oocyte Retrieval

All stock solutions for ovary collection and oocyte retrieval were prepared under laminar flow and stored at 2 to 8ºC prior to use. The solutions were prepared according to Habsah Bidin (2006) with slight modifications.

3.3.1.1 Dulbecco's Phosphate Buffered Saline (DPBS)

Dulbecco's Phosphate Buffered Saline was prepared in a 1 litre bottle (Duran, Germany) by diluting a packet of DPBS powder in 1 litre milli-Q water. The DPBS was sterilised by autoclaving and kept at 2 to 8°C up to three months.

3.3.1.2 Collecting medium

The ovaries from the slaughterhouse were washed and kept in DPBS alone during transport to the laboratory. In the laboratory, the collecting medium used for washing, slicing, and rinsing of ovaries was DPBS supplemented with 10% steer serum (SS). The temperature of the collecting medium was maintained at 37°C in a water bath prior to each experiment.

3.3.1.3 Steer serum

Approximately 300 to 400 ml of blood were collected from the jugular vein of steers immediately after slaughter. The blood was allowed to clot before being transported to the laboratory. Next, the blood was centrifuged at 3000 rpm for 20 minutes. Serum was harvested and then placed in a glass conical flask. The serum was heat-treated at 56°C in a water bath for 30 minutes to denature blood proteins. Then, the serum was allowed to cool down to room temperature (25°C) before being centrifuged again at 1000 rpm for 10 minutes. Finally, the supernatant (serum) was collected and stored in 15 ml centrifuge tubes (Falcon) at -20°C for future use.

3.3.2 Stock Solutions for *in vitro* Maturation (IVM)

3.3.2.1 IVM medium

The IVM medium was prepared using tissue culture medium 199 (TCM 199, Gibco, Invitrogen Co., USA) supplemented with 25% steer serum, 100 mM L-

glutamine, 20 mM sodium-pyruvate and gentamycin solution according to Habsah (2006). It was then filter sterilised through a 0.22 μm Millipore membrane and the pH was adjusted between 7.35 and 7.45. Next, 1 μg/ml of oestradiol-17β was added to the IVM medium after filtration. The medium was usually prepared fresh prior to each experiment and calibrated for at least 3 hours in a humidified 5% CO_2 in air at 38.5°C.

3.3.2.2 Silicone oil

Silicone oil, 150 ml was filtered through 0.22 μm Millipore membrane and transferred to 50 ml centrifuge tubes and stored in the humidified 5% CO_2 in air at 38.5°C for future use.

3.3.2.3 Oestradiol-17β

The stock solution of oestradiol-17β was prepared by dissolving 1 mg of oestradiol-17β in 10 ml of absolute ethanol. It was then stored at -20°C in sealed vials for future use.

3.3.2.4 β-mercaptoethanol

The stock solution of β-mercaptoethanol was prepared by dissolving 0.78 mg of β-mercaptoethanol in 10 ml of dH_2O. It was then stored at -20°C in sealed vials for future use.

3.3.3 Stock Solutions for *in vitro* Fertilisation (IVF)

3.3.3.1 Preparation of IVF medium

The medium used for *in vitro* fertilisation in the study was Brackett-Oliphant (BO) medium (Table 3.1). The BO medium was filtered and divided into three portions / solution (SWS) and semen diluting solution (SDS). The SWS was prepared by

supplementing BO medium with 20 μg/ml heparin solution, while OWS and SDS were added with bovine serum albumin (BSA), 10 mg/ml and 20 mg/ml, respectively. All solutions were prepared fresh and calibrated at least 1 hour in the 5% CO_2 in air at 38.5°C prior to use.

Table 3. 1: Brackett-Oliphant (BO) medium

Chemical	Quantity
Solution A	25.3 ml
Na-pyruvate	4.6 mg
Penicillin-Streptomycin	33.3 μl
Solution B	8.0 ml
Solution A (stock solution)	
NaCl	2.15 g
KCI	0. 10g
$CaCl_2 2H_20$	0.10 g
$NaH_2PO_4.2H_2O$	0.04 g
$Mg\ Cl_2.6H_2O$	0.04 g
0.5 % Phenol red	50 μl
Added dH_2O up to 250 ml	
Solution B (stock solution)	
$NaHCO_3$	1.30 g
Added dH_2O up to 100 ml	

3.3.3.2 Heparin solution

Heparin solution was prepared by adding 50 mg of heparin in 2.5 ml of BO medium to obtain 100 U of heparin solution. The solution was stored at 2 to 8°C and can be kept up to three months.

3.3.3.3 Calcium-ionophore (CaI) solution

Calcium-ionophore (CaI) solution was prepared by diluting 1 mg of CaI in 1.9 ml medium containing dimethyl sulphoxide (DMSO) and absolute ethanol in 3:1 ratio (v/v). Next, aliquots of 20 µl of the solution dissolved in 1 ml of milli-Q water was prepared and stored in the refrigerator (4°C) until further use.

3.3.4 Stock Solutions for *in vitro* Culture (IVC)

The medium used in the IVC of fertilised oocytes was Charles Rosenkrans 1 amino acid (CRlaa), a chemically-defined medium (Table 3.2). The medium was prepared fresh and calibrated at least 1 hour in the 5% CO_2 in air at 38.5°C prior to use.

Table 3.2: Charles Rosenkrans 1 amino acid (CRlaa) medium

Chemicals	Quantity
NaCl	0.67 g
KCl	0.02 g
$NaHCO_3$	0.22 g
L-glutamine	0.02 g
Na-pyruvate	0.004 g
L(+)-lactate	0.055 g
	Added dH_2O up to 100 ml

3.3.5 Staining Solutions

3.3.5.1 Giemsa stain

Giemsa stain (Sigma) was used to evaluate the stages of nuclear maturation of bovine oocytes following *in vitro* maturation. The method used was based on Habsah Bidin (2006). A 4% Giemsa solution was prepared by adding 4 ml Giemsa stain in 100 ml dH_2O. The solution was usually prepared fresh prior to each experiment.

3.3.5.2 Annexin V FITC stain

Annexin V FITC stain was used to detect the incidence of early apoptosis in the oocytes. The detection kit used in the experiment was Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, Cat. No. A2214).

3.3.6 Preparation of Slides

Slides (Corning, NY, USA) were soaked in absolute ethanol for at least 1 hour to clean and remove dirt. The slides were air dried prior to use.

3.3.7 Procedures of *in Vitro* Bovine Embryo Production

3.3.7.1 Retrieval of oocytes

The cumulus oocytes complexes (COC) were retrieved from the follicles by slicing the ovaries in a checkerboard incisions manner using a surgical blade (No. 23) held on a scalpel. Next, the ovaries were rinsed and the follicles were flushed with the collecting medium using an 18-gauge needle (Terumo) attached to a 10-ml syringe (Terumo). The collecting medium containing COC were collected into a beaker (Pyrex) and the contents of the beaker were then poured onto a 90-mm culture dish (Nunc). The beaker was rinsed thoroughly to ensure that all oocytes were transferred into the culture dish. The COC were allowed to settle down in the medium for a few minutes. For washing the COC, about half of the medium was aspirated using a sterile Pasteur pipette before adding fresh medium into the culture dish. The washing procedure was repeated several times, depending on the clearness of the solution and each time, the COC were allowed to settle down in the collecting medium. The COC was retrieved from the collecting medium using a micropipette (Wiretrol II, Drummond Scientific Co.) under a stereomicroscope and finally transferred into a 35 x 10 mm culture dish (Nunc)

containing 3 ml of TCM 199 supplemented with 25% steer serum (Habsah Bidin, 2006).

3.3.7.2 Oocyte maturation

The COC were classified according to the morphology of the cumulus cells (Shioya *et al*., 1988) with modification before culture. Oocytes with compact and dense cumulus cell layers were classified as Grade A (Figure 3.1). Grade B consisted of oocytes with compact but less dense cumulus cell layers (Figure 3.2) and Grade C, oocytes with thin or little remnants of cumulus cell layers, some were partially naked (Figure 3.3). Groups of 10 to 20 COCs were transferred into droplets of 100 µl IVM medium covered with silicone oil in a 35 x 10 mm culture dish (Nunc) pre-equilibrated in a CO_2 incubator. The COC were cultured for 20 to 24 hours to achieve complete maturation in 5% CO_2 humidified incubator at 38.5°C. Most oocytes showed dispersion of cumulus cells after 24-hr IVM as shown in Figure 3.4. *In vitro* viability assessment of immature bovine oocytes recovered from local slaughterhouse in the study, were assessed using several indicators. These include determination of cumulus expansion, meiotic stages, early apoptosis, and cleavage and blastocyst rates.

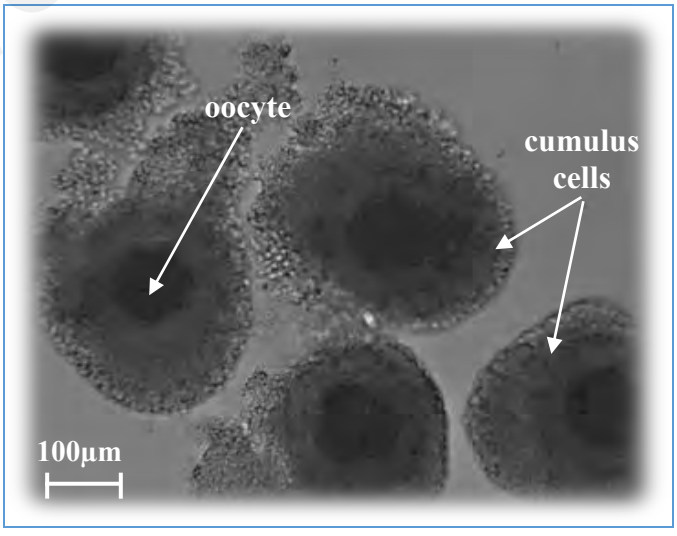

Figure 3.1: Group A oocytes (compact and dense cumulus cell layers) (200X magnification)

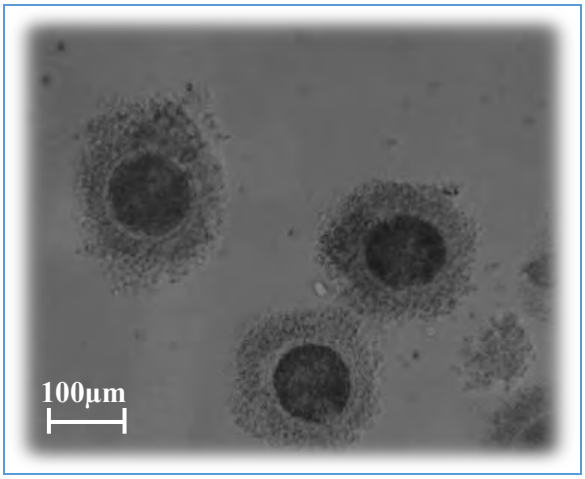

Figure 3.2: Group B oocytes (compact but less dense cumulus cell layers) (200X magnification)

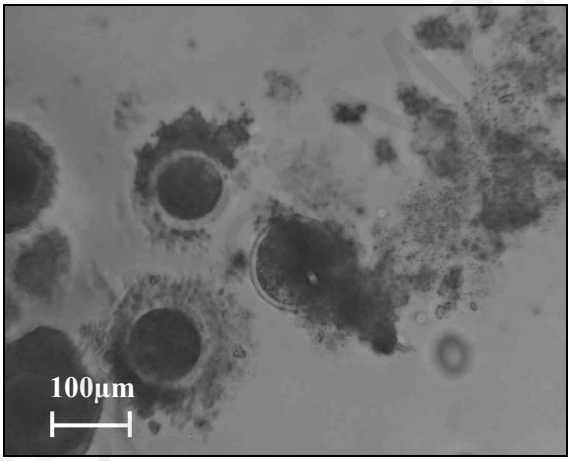

Figure 3.3: Group C oocytes (thin or little remnants of cumulus cell layers) (200X magnification)

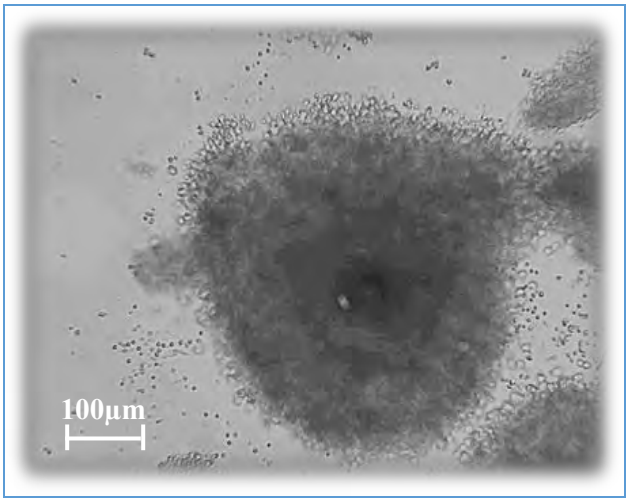

Figure 3.4: Oocytes showing dispersed cumulus cells after 24-hr IVM (200X magnification)

3.3.7.3 Chromosome staining

Chromosome staining of oocytes with dispersed cumulus cells was carried out 20 to 24 hours after IVM culture. The oocytes were completely denuded by pipetting and then treated individually with 3 ml of 1% hypotonic trisodium citrate solution (1 cm^3 sodium citrate solution in 99 cm^3 water) for 3 minutes in a 4-well plate (Nunc). The treatment made the oocytes swell without causing rupture. Oocytes were then transferred onto a clean microscopic slide and fixed with 1:1 ratio of methanol and acetic acid solution, and blow-dried. Next, the slides were immersed in fixative solution (3:1, methanol: acetic acid) in a Coplin jar and kept at 2 to 8^oC overnight. Then, the slides were air-dried and stained with 4% Giemsa solution for 3 minutes. Permanent mounts were prepared by immersing the slides in xylene for 5 to 10 minutes for cleaning purposes. Next, DPX Mountant (BDH Laboratories Supplies People, England) was used to hold the coverslip so that it is in contact with the oocytes without excessive pressure and allowed to dry. The slides were then observed under a phase contrast microscope (Zeiss) at 400X magnification.

The stages of nuclear maturation were determined as first metaphase (MI) and second metaphase (MII). Metaphase I was shown by co-orientation of the bivalents with centromeres at the opposite side of the equator of the spindle, whereas, metaphase II was characterised by a wide separation of the homologous chromatids. Zygotene/pachytene was seen as pairing of homologus chromosomes forming bivalents (Wasmann, 2013). Sample of photos from the study showing metaphase I and II were represented in Figure 3.5 and 3.6, respectively. The oocytes were scored as unidentified if the chromatin observed were clumped or condensed.

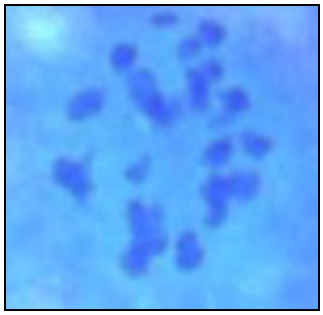

Figure 3.5: Metaphase I (400X magnification)

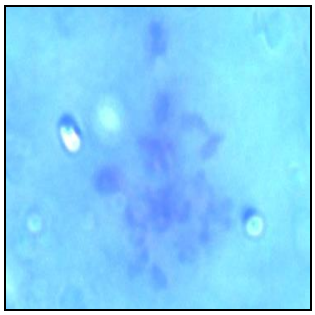

Figure 3.6: Metaphase II (400X magnification)

3.3.7.4 Early apoptosis detection

The procedure for apoptotic cell detection was carried out according to the instructions in the kit. Annexin V-FITC is a fluorescent probe which binds to phosphatidylserine in the presence of calcium (Andree *et al.,* 1990; Thiagarajan & Tait, 1990). At the onset of apoptosis, phosphatidylserine which is normally found on the internal part of the plasma membrane becomes translocated to the external portion of the membrane (Arur *et al*., 2003). Basically, COCs were denuded by pipetting and washed twice in PBS. Then the COCs were stained with 100 µl binding buffer containing Annexin V-FITC (5 µL of FITC Annexin V and 1 µL of the 100 µg/mL PI working solution) and propidium iodide (PI) (100 µg/mL) in a 35 x 10 mm culture dish (Nunc). The 100 µl droplets, covered with silicone oil were then incubated at room temperature for exactly 10 minutes in the dark. The fluorescence of the cells was immediately determined using a fluorescence microscope (Partec Syscope, excitation/emission wavelength - FITC: 490/525 nm, PI: 536/617 nm). Cells in the early apoptotic process were stained with Annexin V-FITC and detected as green fluorescence (Figure 3.7). Propidium iodide staining was used to distinguish live and dead cells, whereby live cells were not stained (Figure 3.9) and dead cells were detected as red fluorescence (Figure 3.8).

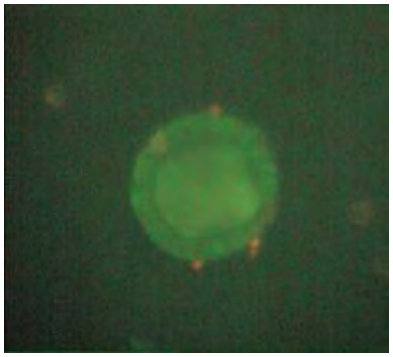

Figure 3.7: Oocytes showing early apoptosis (fluorescent green) (200X magnification)

Figure 3.8: Dead oocytes (fluorescent red) (200X magnification)

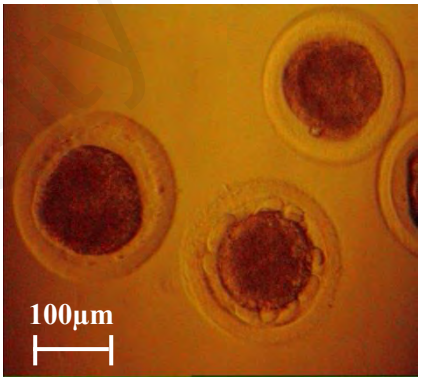

Figure 3.9: Live oocytes (not stained) (200X magnification)

3.3.7.5 Glutathione Assay

For GSH assay, the samples were prepared according to Furnus *et al*. (2008). First, the COC were denuded in HEPES buffered TCM199 medium. Next, they were washed three times in Mg^{2+}/Ca^{2+} free PBS containing 1 mg/ml polyvinylpyrrolidone (PVP). Then, more than 30 oocytes were placed in 10 µl PBS in microcentrifuge tubes

(Eppendorf) and were frozen at -20ºC. The oocytes were thawed and frozen again at -20ºC. The freezing and thawing steps were repeated three times. Prior to GSH assay, the oocytes were disrupted by pipetting in and out using a micropipette or by using a vortex. Finally, the samples were deproteinized using 5% sulfosalicylic acid and centrifuged to remove precipitated protein. The supernatant was collected and kept at 2 to 8ºC within 2 hours for GSH assay.

The recycling enzymatic method using 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) has frequently been used to measure the total amount of GSH (Tietze, 1969; Giustarini *et al*., 2013). In the study, GSH assay was carried out according to the instructions in the GSH Assay Kit (Sigma-Aldrich, Cat. No. CS0260). Basically, 150 µl of working mixture, i.e., assay buffer (100 mM potassium phosphate buffer with 1 mM ethylenediaminetetraacetic acid (EDTA)) containing 6 units/ml glutathione reductase and 1.5 mg/ml DTNB was added into the wells of a 96-well plate (Nunc). Then, 10 µl of sample was added to the working mixture and mixed well by pipetting up and down. The plate was incubated for 5 minutes at room temperature before adding 50 µl nicotinamide adenine dinucleotide phosphate (NADPH) solution (0.16 mg/ml) into each well and mixed. A plate reader was set up to 412 nm with kinetic read at 1-minute intervals for 5 minutes to measure the absorbance in each well. The amount of glutathione in the samples was determined using a standard curve of reduced glutathione.

3.3.7.6 Sperm preparation for IVF

For sperm preparation procedure, 1 ml of SWS in a sterile 15 ml centrifuge tube was pre-equilibrated in 5% CO_2 in air at 38.5ºC for at least 1 hour. Prior to fertilisation, frozen-thawed sperm was added gently into the SWS at the bottom of the centrifuge tube to avoid mixing and then kept in the incubator for half an hour to allow sperm

swim-up. At the end of the incubation period, approximately 800 µl of the upper portion of the SWS was aspirated and transferred into a sterile 15 ml centrifuge tube for washing. Sperm washing was done twice by adding SWS up to 6 ml volume and then centrifuged at 700 rpm for 5 minutes. The supernatant was removed each time and finally the concentration of sperm was adjusted with SDS to obtain at a concentration of 1 X 10^6 sperm per ml. Next, 5 µl/ml of CaI was added and the sperm suspension was incubated for 15 minutes. For IVF, 100 µl of sperm droplets were prepared and covered with silicone oil in 35 x 10 mm culture dish and then equilibrated for at least 1 hour in 5% CO_2 in air at 38.5ºC before insemination.

3.3.7.7 Oocyte fertilisation (IVF)

At the end of the cultivation period, oocytes after IVM culture were washed several times in 100 to 150 µl of OWS droplets in a 90-mm culture dish. Using a sterile micropipette, a group of 10 oocytes were transferred to the 100 µl of sperm droplets covered with silicone oil at a final concentration of 1 x 10^6 sperm per ml. Then the oocytes and sperm were co-incubated for 18 to 20 hours in 5% CO_2 in air at 38.5ºC.

3.3.7.8 Embryo culture (IVC)

At 18 to 20 hours of insemination, the oocytes were washed twice in 100 to 150 µl of OWS droplets and once in the same volume of CRlaa in a 90-mm culture dish without silicone oil. A total of 10 to 20 presumptive zygotes (indistinguishable from unfertilised ova at this stage) were placed in the 100 µl of CRlaa culture droplets medium covered in silicone oil that had been prepared and equilibrated earlier. The day of IVC is considered as Day 1. The culture medium was changed on Day 3, Day 5 and Day 7 by aspirating 50 ul of the medium and adding the same volume of fresh CR1aa medium. On Day 5, 4 µl of SS (approximately 4%) was added to each droplet as it was

shown to enhance development after morula stage (Nor Fadillah Awang, 2013). The embryo development was assessed daily for 9 days, as cleavage (2-cell), 4-cell, 8-cell, 16-cell, morula, compact morula (increased number of cells then morula and cell borders are less distinguishable) and blastocyst (Figure 3.10) (Giesert & Malayer, 2000).

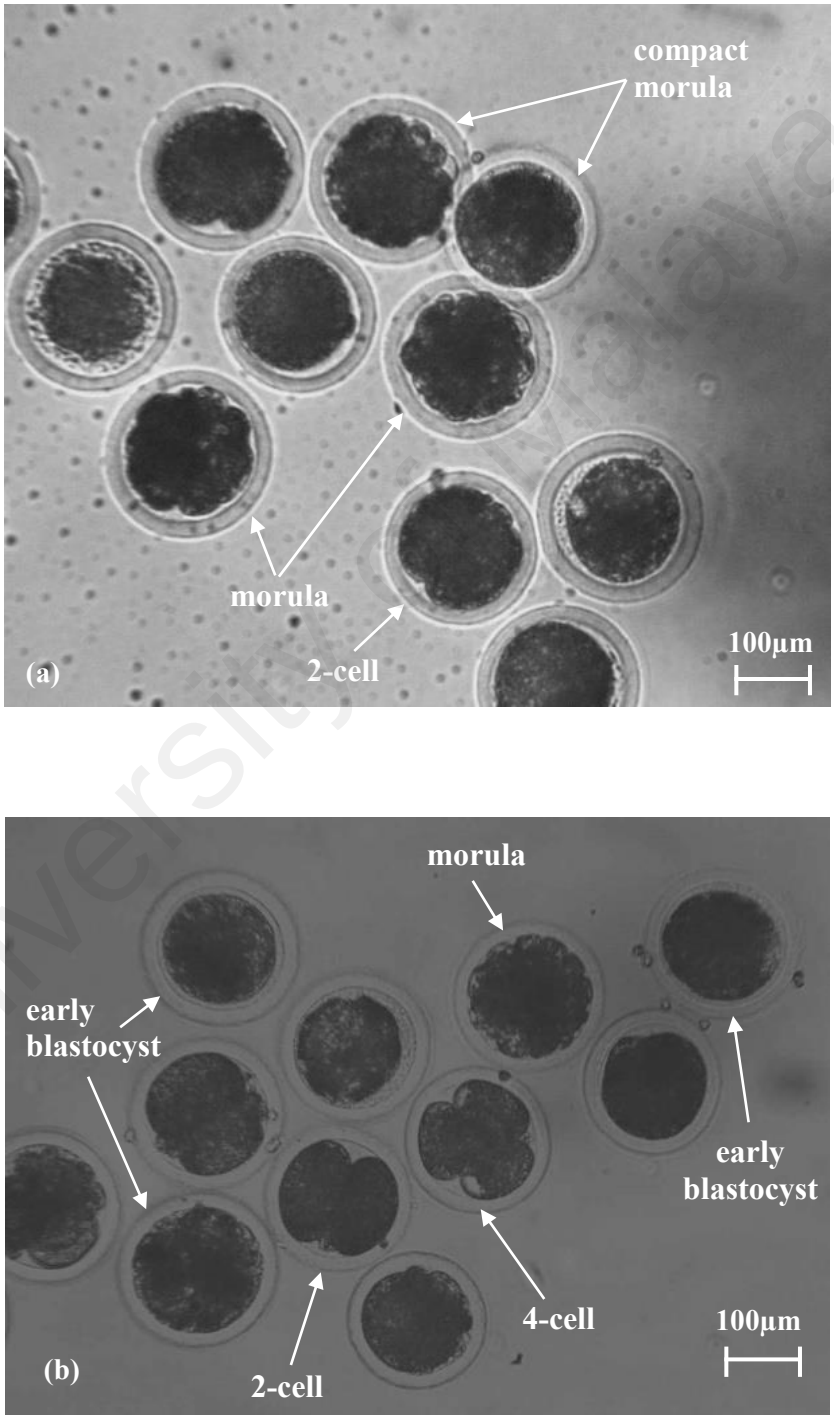

Figure 3.10: Stages of embryo development (a and b) (200X magnification)

3.4 EXPERIMENTAL DESIGN

3.4.1 Effects of Cumulus Cells on Oocyte and Embryo Developmental Competency (Experiment I)

3.4.1.1 Effect of cumulus cells on early apoptosis in bovine oocytes

The experiment was carried out to evaluate the effects of different groups of bovine oocytes based on the compactness of cumulus cells on early apoptosis rates before and after *in vitro* maturation. A total of 378 oocytes, classified into Grades A, B and C according to their quality were used in this experiment. The oocytes were matured in IVM media supplemented for 24 hours in a humidified 5% CO_2 in air at 38.5^{o}C. The numbers of oocytes which were in the early apoptosis process were determined before and after 24 hours IVM and the early apoptosis rates were analysed.

3.4.1.2 Effect of cumulus cells on intracellular GSH in bovine oocytes

The experiment was conducted to determine the effects of oocyte cumulus cells on the intracellular GSH level before and after *in vitro* maturation for 24 hours. A total of 857 oocytes were used and classified into Grades A, B, and C. The oocytes were matured in IVM media for 24 hours. The GSH levels in the three groups of oocytes were determined and analysed.

3.4.1.3 Effect of cumulus cells on nuclear maturation of bovine oocytes

The objective of this experiment was to determine the effects of oocyte cumulus cells on the nuclear maturation rate before and after *in vitro* maturation for 24 hours. A total of 251 oocytes were used and classified into Grades A, B and C according to their quality. The oocytes were matured in IVM media for 24 hours in a humidified 5% CO_2 in air at 38.5^{o}C and the different stages of meiotic resumption of the oocytes were recorded and analysed.

3.4.1.4 Effect of cumulus cells on cleavage and subsequent embryonic development after *in vitro* fertilisation (IVF)

The experiment was conducted to determine the effects of oocyte cumulus cells on the cleavage rates and subsequent embryo development of bovine oocytes. A total of 212 oocytes were used and classified into Grades A, B, and C. The oocytes were matured in IVM media for 20 to 24 hours and fertilised for 18 to 20 hours in 5% CO_2 at 38.5ºC. Presumptive zygotes were cultured up to 216 hours (Day 9) after insemination. Embryo developmental stages of the three groups of oocytes were recorded and evaluated daily.

3.4.2 Effects of β-mercaptoethanol (BME) Supplement during IVM on Oocyte and Embryo Developmental Competency (Experiment II)

3.4.2.1 Effect of BME supplement during IVM on early apoptosis in bovine oocytes

The experiment was conducted to determine the effects of supplementing different concentrations of BME in the *in vitro* maturation (IVM) medium of different oocyte grades on early apoptosis rates. A total of 1046 oocytes were used and classified into Grades A, B, and C. The oocytes were matured in IVM media supplemented with 0 (control), 50, 100, 15 and 200 μM BME for 24 hours. The early apoptosis rates in the three groups of oocytes before and after maturation with BME supplement were determined.

3.4.2.2 Effect of BME supplement during IVM on intracellular GSH in bovine oocytes

The aim of the experiment was to determine the effects of supplementing different concentrations of BME in the *in vitro* maturation (IVM) medium of different oocyte grades on the intracellular GSH level. A total of 2226 oocytes were used and classified into Grades A, B, and C. The oocytes were matured in IVM media

supplemented with 0 (control), 50, 100, 15 and 200 μM BME for 24 hours. The GSH levels in the three groups of oocytes before and after IVM were determined.

3.4.2.3 Effect of BME supplement during IVM on nuclear maturation of bovine oocyte

The objective of this experiment was to determine the effects of supplementing different concentrations of BME in the *in vitro* maturation (IVM) medium of different oocyte grades on the meiotic resumption after *in vitro* maturation for 24 hours. A total of 1255 oocytes were used in this experiment which were classified into Grades A, B and C according to their quality. The oocytes were matured in IVM media supplemented with 0 (control), 50, 100, 150 and 200 μM BME for 24 hours in a humidified 5% CO_2 in air at 38.5ºC and the stages of meiosis of the oocytes were recorded and analysed.

3.4.2.4 Effect of BME supplement during IVM on cleavage and subsequent embryonic development after IVF

The experiment was carried out to determine the effects of supplementing different concentrations of BME in the *in vitro* maturation (IVM) medium of different oocyte grades on cleavage and blastocyst rates. A total of 1533 oocytes were used and classified into Grades A, B, and C. The oocytes were matured in IVM media supplemented with 0 (control), 50, 100, 15 and 200 μM BME for 24 hours and fertilised for 18 to 20 hours in 5% CO_2 at 38.5ºC. Presumptive zygotes were cultured up to 216 hours (Day 9) after insemination. Embryo developmental stages of the three groups of oocytes matured in different concentrations of BME were recorded and evaluated daily.

3.5 STATISTICAL ANALYSIS

Data were statistically analysed using Analysis of Variance (ANOVA), Duncan's Multiple Range Test and Independent T-test. The statistical programme used was the Statistical Package for the Social Science (SPSS).

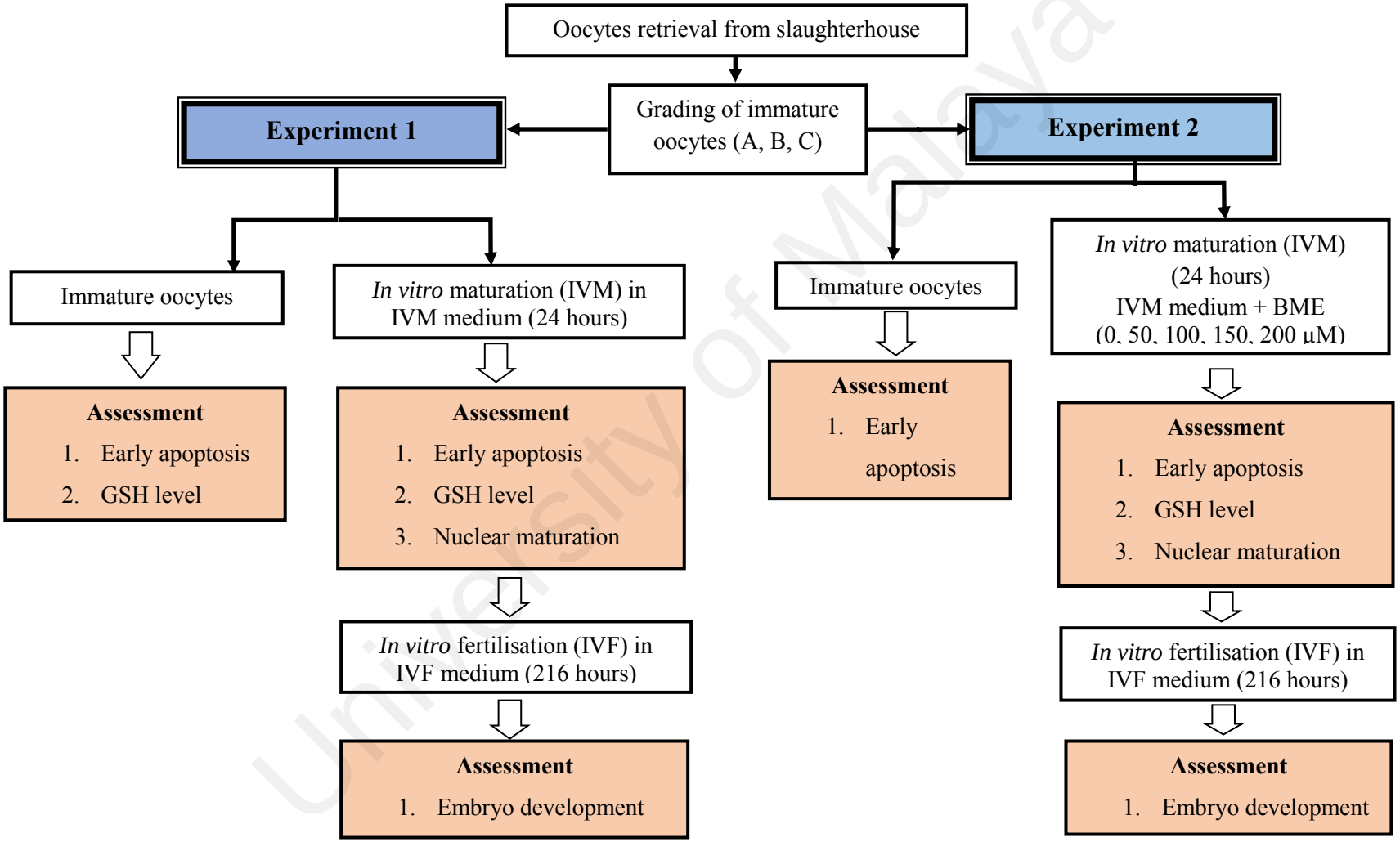

Figure 3.11: Experimental Design Flow Chart

CHAPTER 4: RESULTS

4.1 EFFECT OF CUMULUS CELLS ON OOCYTE AND EMBRYO DEVELOPMENTAL COMPETENCY (EXPERIMENT 1)

4.1.1 Effect of Cumulus Cells on Early Apoptosis in Bovine Oocytes

The early apoptosis rates in different cumulus cells morphology of bovine oocytes, before and after IVM culture are presented in Table 4.1 and Figure 4.1. Before IVM culture, the early apoptosis rates in Groups B (19.00%) and C (20.60%) were found to be significantly higher than Group A (10.20%) ($P<0.05$). After IVM culture, however, Group A (28.40%) showed significantly higher occurrence of early apoptosis than Groups B (18.20%) and C (23.00%) oocytes ($P<0.05$). Comparison between the early apoptosis rates before and after IVM culture among Group A oocytes showed a significant increase from 10.20% to 28.40% ($P<0.05$), whereas both Groups B and C oocytes retained similar rates of early apoptosis before and after IVM culture ($P>0.05$).

Table 4.1: Mean percentage of early apoptosis in different cumulus cells morphology of bovine oocytes before and after 24-hour IVM

Oocyte group	N	Early apoptosis (Mean % ± SEM) n(m)	
		Before IVM	After IVM
A	86	$10.20 \pm 2.82^{a,x}$ 4(42)	$28.40 + 3.59^{b,y}$ 14(46)
B	135	$19.00 \pm 2.28^{b,x}$ 14(72)	$18.20 \pm 3.09^{a,x}$ 12(63)
C	157	$20.60 \pm 2.36^{b,x}$ 18(86)	$23.00 \pm 3.49^{a,x}$ 16(71)

(A = oocyte with compact and dense cumulus cells; B = oocyte with compact but less dense cumulus cells; C = oocyte with thin or little remnants of cumulus cells layers)
N = total number of oocytes examined (at least 4 replicates each experiment)
n = number of oocytes showing early apoptosis
m = number of oocytes examined for the experiment
[a,b] Means in a column with different superscripts were significantly different (P<0.05)
[x,y] Means in a row with different superscripts were significantly different (P<0.05)

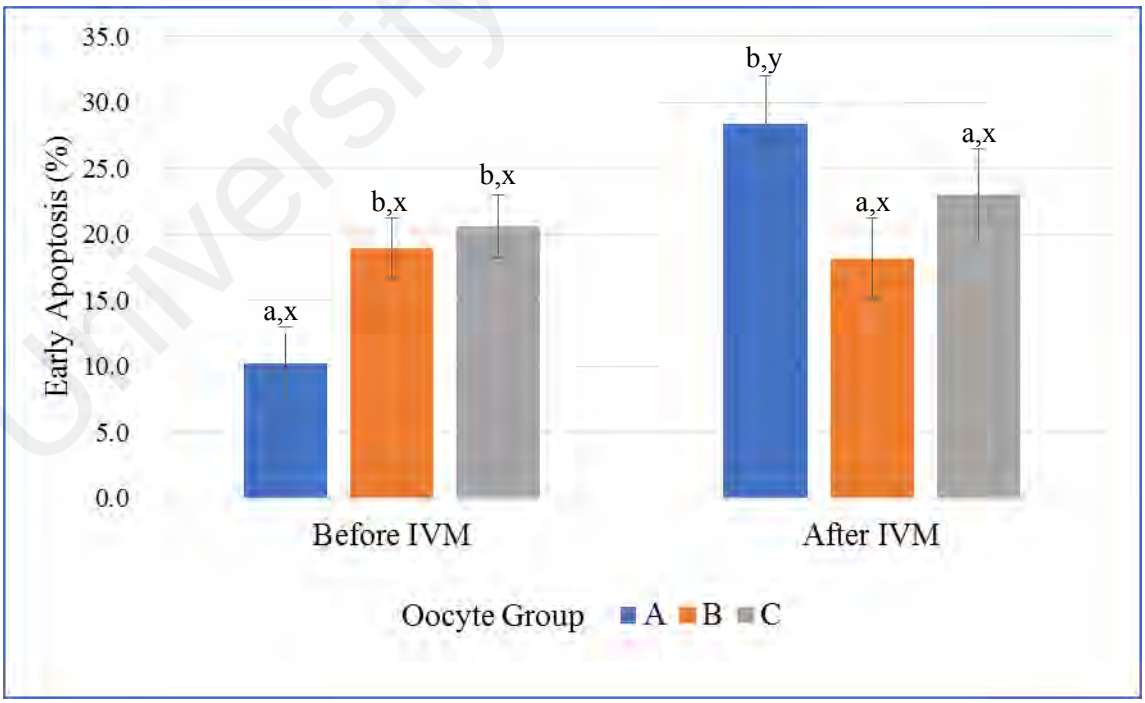

Figure 4.1: Mean percentage of early apoptosis in different cumulus cells morphology of bovine oocytes before and after 24-hour IVM.

4.1.2 Effect of Oocyte Cumulus Cells on Intracellular GSH in Bovine Oocytes

The data in Table 4.2 and Figure 4.2 include the GSH concentrations of different cumulus cells morphology of bovine oocytes before and after IVM for 24 hours. No difference was found in the intracellular GSH content between the different groups of oocytes before IVM culture ($P>0.05$). The intracellular GSH concentrations of Groups A, B and C oocytes were 3.08, 3.30 and 3.00 pmol/oocyte, respectively. After IVM culture however, the intracellular GSH concentration of Group A oocytes (4.26 pmol/oocyte) were significantly greater than Groups B (3.68 pmol/oocyte) and C (3.44 pmol/oocyte) ($P<0.05$). Comparison before and after maturation culture showed significant increase of intercellular GSH in Group A oocytes ($P<0.05$).

Table 4.2: Mean GSH concentrations in different cumulus cells morphology of bovine oocytes before and after 24-hour IVM

Oocyte group	**N**	**Mean GSH concentrations (pmol/oocyte ± SEM)**	
		Before IVM	After IVM
A	247	$3.08 \pm 0.28^{a,x}$	$4.26 \pm 0.28^{b,y}$
B	296	$3.30 \pm 0.26^{a,x}$	$3.68 \pm 0.35^{a,x}$
C	314	$3.00 \pm 0.18^{a,x}$	$3.44 \pm 0.28^{a,x}$

(A = oocyte with compact and dense cumulus cells; B = oocyte with compact but less dense cumulus cells; C = oocyte with little remnants of cumulus cells)
N = total number of oocytes used (minimum 30 oocytes per experiment, at least 4 replicates each experiment)
^{a,b} Means in a column with different superscripts were significantly different ($P<0.05$)
^{x,y} Means in a row with different superscripts were significantly different ($P<0.05$)

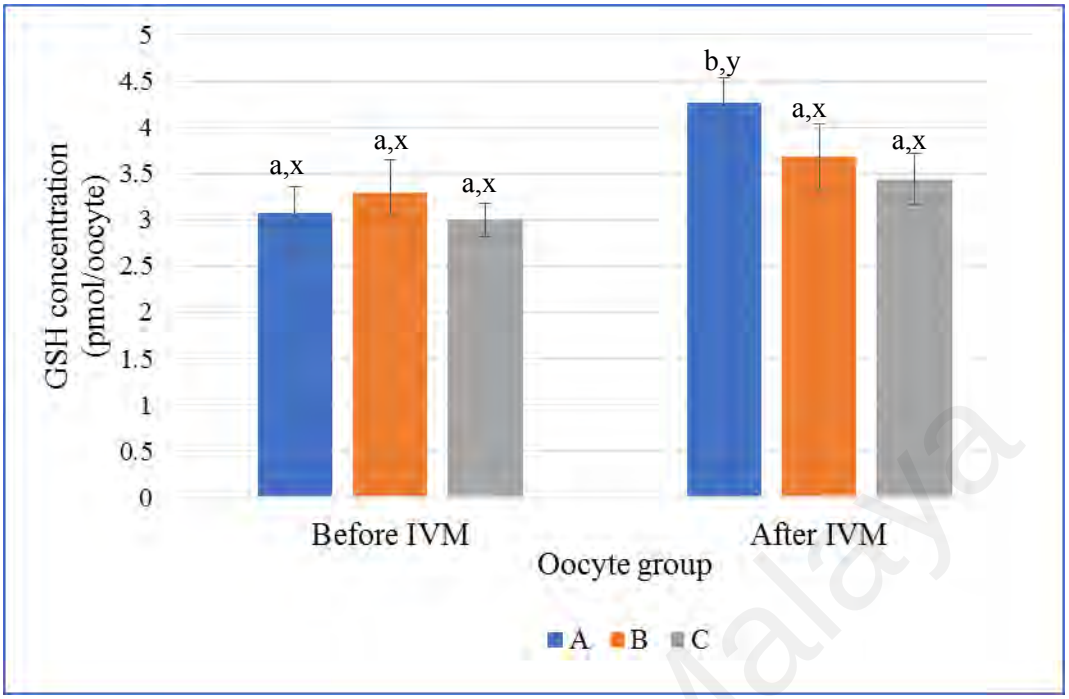

Figure 4.2: Mean GSH concentrations in different cumulus cells morphology of bovine oocytes before and after 24-hour IVM

4.1.3 Effect of Cumulus Cells on Nuclear Maturation of Bovine Oocytes

Table 4.3 and Figure 4.3 represent the meiotic resumption of different cumulus cells morphology of oocytes after 24-hour culture in IVM medium. The rates of oocytes reaching all meiotic stages i.e. zygotene/pachytene, metaphase I and metaphase II did not differ significantly between different groups of oocytes ($P>0.05$). The mean percentage of oocytes reaching zygotene/pachytene stage for Groups A, B and C were 17.54%, 18.66% and 15.64%, respectively. The rates of oocytes showing Metaphase I after 24-hour maturation in IVM medium were 33.94%, 29.84% and 35.38% in Group A, B and C, respectively.

No significant difference was found in the rates of resumption to metaphase II stage between different groups of oocytes matured in IVM medium for 24 hours ($P>0.05$). The mean percentage of Groups A, B and C oocytes reaching metaphase II stage were 44.19%, 42.33% and 39.61%, respectively. The rates of all the different groups of oocytes that achieved nuclear maturation (metaphase II) were slightly higher compared to metaphase I stage but the difference is only significant in Group B (metaphase 1 (29.84%); metaphase II (42.33%)) ($P<0.05$).

Table 4.3: Mean percentage of meiotic stages in different cumulus cells morphology of bovine oocyte after 24-hour IVM

Oocyte group	N	Meiotic stage (% ± SEM) (n)			
		Zygotene/ Pachytene	Metaphase I	Metaphase II	Unidentified
A	67	17.54 ± 1.22[a,y] (12)	33.94 ± 1.88[a,z] (22)	44.19 ± 1.45[a,z] (30)	4.34 ± 1.96[a,x] (3)
B	89	18.66 ± 1.57[a,x] (17)	29.84 ± 1.74[a,y] (27)	42.33 ± 1.00[a,z] (37)	9.18 ± 0.91[a,w] (8)
C	95	15.64 ± 1.60[a,y] (14)	35.38 ± 2.55[a,z] (34)	39.61 ± 3.48[a,z] (38)	9.37 ± 2.62[a,x] (9)

(A = oocyte with compact and dense cumulus cells; B = oocyte with compact but less dense cumulus cells; C = oocyte with little remnants of cumulus cells)
N = total number of oocytes examined (at least 4 replicates)
n = number of oocytes showing the corresponding meiotic stage
[a] Means in a column with superscripts were not significantly different (P>0.05)
[wxyz] Means in a row with different superscripts were significantly different (P<0.05)

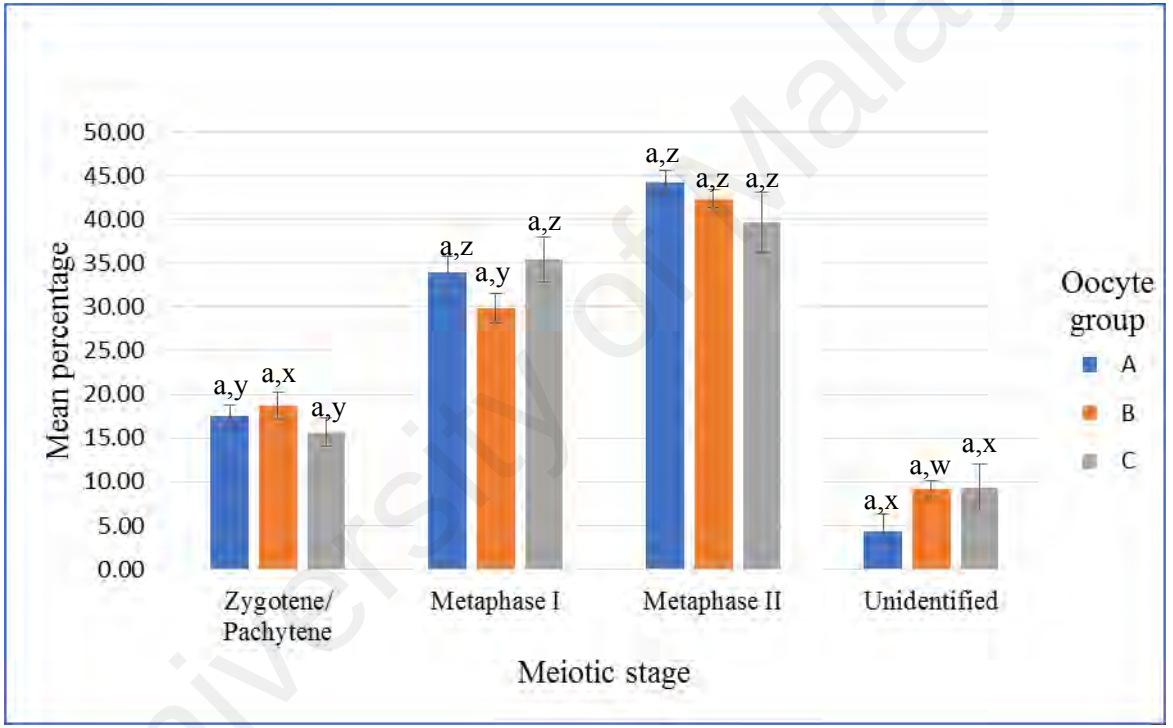

Figure 4.3: Mean percentage of meiotic stages in different cumulus cells morphology of bovine oocyte after 24-hour IVM

4.1.4 Effect of Cumulus Cells on Cleavage and Subsequent Embryonic Development after IVF

The rates of cleavage and subsequent embryonic development after IVF in different cumulus cells morphology of bovine oocytes matured in IVM medium after insemination are shown in Table 4.4 and Figure 4.4. The cleavage (2-cell) rates of Groups A, B and C oocytes after insemination did not differ significantly when matured in IVM medium for 24 hours. The highest cleavage rate was observed in Group A oocytes (72.25%), followed by Group C (67.95%) and Group B (60.00%). However, Group A oocytes showed a significant progression to 4-cell and 16-cell compared to Groups B and C ($P<0.05$). The mean percentage of 4-cell were 51.60%, 37.07% and 35.18%, and 16-cell were 38.39%, 22.66% and 26.21% in Groups A, B and C respectively. On the contrary, Group B oocytes showed significantly lower rates of oocytes progressed to morula (18.84%) than Groups A (22.57%) and C (24.74%). Nevertheless, no significant difference was found in the rates of embryo development to compact morula and blastocyst between the different groups of oocytes. Although not significant, Group A oocyte showed the highest percentage of progression up to blastocyst compared with Groups B and C. The rates of Group A oocytes managed to develop into compact morula and blastocyst were 13.22% and 10.61%, respectively. Group B oocytes managed to develop into compact morula stage with a mean percentage of 8.98% and blastocyst, 6.12%. Finally, Group C oocytes were able to reach compact morula and blastocyst with mean percentages of 9.40% and 7.61%, respectively.

Table 4.4: Mean percentage of cleavage and subsequent embryonic development after IVF in different cumulus cells morphology of bovine oocytes

Oocyte group	N	Embryo stage (Mean % ± SEM) (n)						
		2-cell	4-cell	8-cell	16-cell	morula	compact morula	blastocyst
A	55	72.25 ± 3.68[a] (39)	51.60 ± 6.41[b] (27)	43.46 ± 5.52[a] (23)	38.39 ± 4.84[b] (20)	22.57 ± 3.51[b] (12)	13.22 ± 1.43[a] (7)	10.61 ± 2.08[a] (6)
B	91	60.00 ± 3.70[a] (54)	37.07 ± 2.40[a] (33)	31.75 ± 3.75[a] (27)	22.66 ± 3.85[a] (20)	18.84 ± 2.14[a] (16)	8.98 ± 2.31[a] (8)	6.12 ± 0.91[a] (5)
C	66	67.95 ± 4.01[a] (44)	35.18 ± 0.65[a] (23)	28.48 ± 4.25[a] (18)	26.21 ± 3.36[ab] (17)	24.74 ± 2.13[b] (16)	9.40 ± 1.77[a] (6)	7.61 ± 0.70[a] (5)

(A = oocyte with compact and dense cumulus cells; B = oocyte with compact but less dense cumulus cells; C = oocyte with little remnants of cumulus cells). Developmental stages were examined daily from Day 2 to Day 9 (Day 1 = IVF)
N = Total number of oocytes examined (at least 4 replicates)
n = number of oocytes showing the corresponding stages
[a,b] means in a column with different superscripts were significantly different ($P<0.05$)

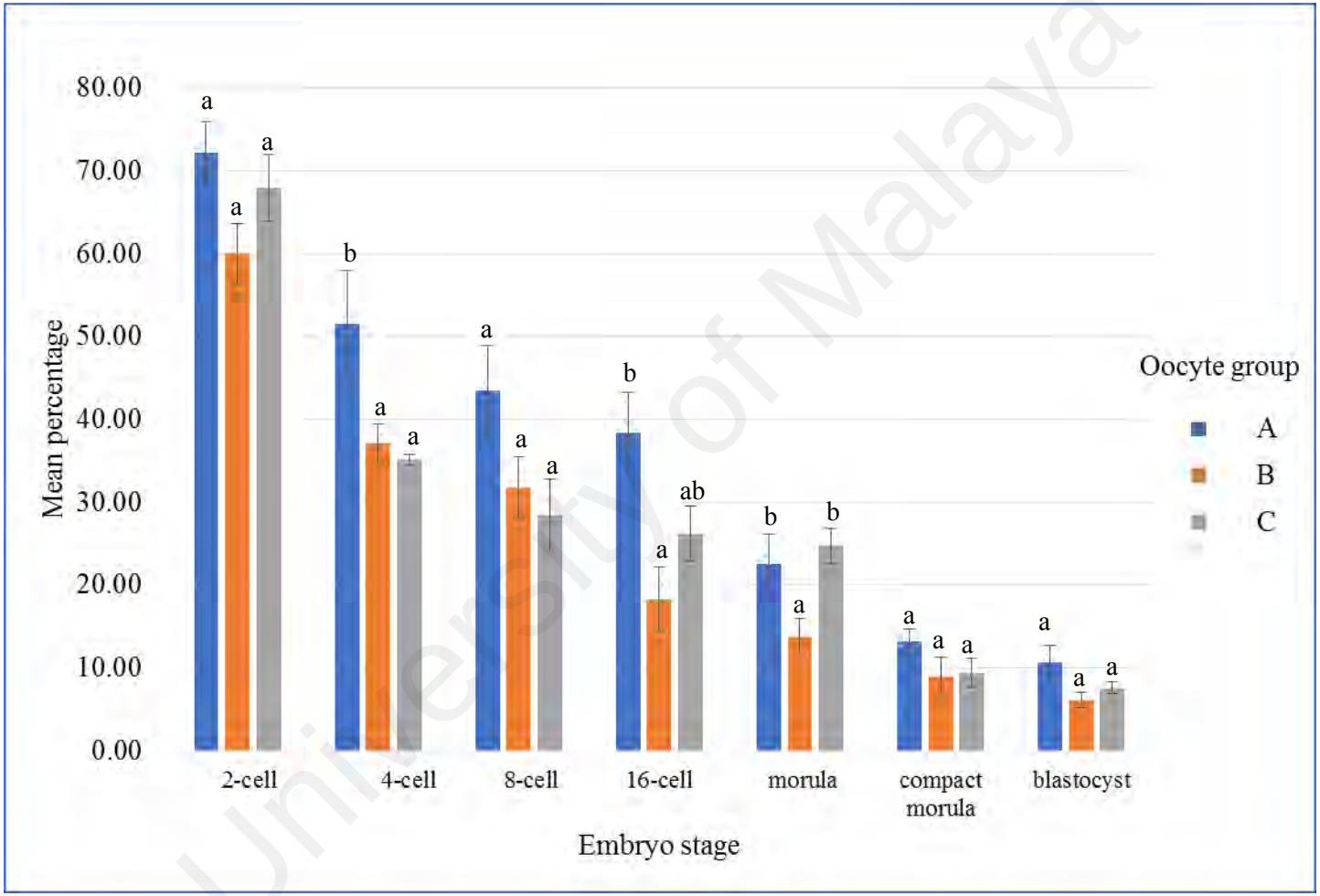

Figure 4.4: Mean percentage of cleavage and subsequent embryonic development after IVF in different cumulus cells morphology of bovine oocytes

4.2 EFFECT OF BME SUPPLEMENT DURING IVM ON OOCYTE AND EMBRYONIC DEVELOPMENTAL COMPETENCY (EXPERIMENT II)

4.2.1 Effect of BME Supplement during IVM on Early Apoptosis in Bovine Oocytes

Table 4.5 and Figure 4.5 represent the early apoptosis rates of different groups of bovine oocytes observed before and after 24-hour maturation in IVM medium supplemented with different concentrations of BME (0, 50, 100, 150 and 200 μM). In Group A oocytes, significant increase in the early apoptosis rates were shown after IVM compared with before IVM, regardless of BME concentration. The different concentrations of BME supplement during IVM did not exert any significant difference ($P>0.05$) to the rates of Group A oocytes showing early apoptosis. Percentage of oocyte showing early apoptosis before IVM was 10.20%, and after IVM for BME concentrations of 0, 50, 100, 150 and 200 μM were 28.40%, 37.60%, 46.60%, 44.75% and 42.80%, respectively.

No difference in the mean percentage of early apoptosis detected in Group B oocytes matured in medium without BME supplement i.e. Control (18.20%) compared to before maturation (19.00%). The occurrence rate of early apoptotic oocytes in Grade B after 24-hour maturation was found to increase significantly when BME, regardless of concentration was added to the IVM media ($P<0.05$). When BME was added (concentrations 50, 100, 150 and 200 μM) the rates of oocytes showing early apoptosis increased two-fold (44.40%, 38.40%, 47.75% and 43.00%, respectively) in comparison with control. The different concentrations of BME supplemented in the IVM media exerted no significant effect on the rate of apoptotic oocytes in Grade B.

Similar trend as Group B was displayed by Group C oocytes, whereby no differences in the rates of early apoptosis compared between before IVM (20.60%) and after IVM in medium without BME supplement i.e. Control (23.00%). However, the addition of BME into the IVM medium significantly increased the rates of early

apoptosis compared to before maturation ($P<0.05$). Early apoptosis rates in Group C oocytes after IVM supplemented with 50, 100, 150 and 200 μM BME were 43.00%, 40.50%, 39.00% and 40.00%, respectively.

Comparison between the different groups of oocytes within the same concentration of BME supplement during IVM showed that no significant difference was found between different oocyte groups ($P>0.05$) after 24-hour maturation in IVM medium supplemented with 50 μM BME (Table 4.5 and Figure 4.6). The early apoptosis rates in the oocytes were 37.60%, 44.40% and 43.00% in Groups A, B and C, respectively with the highest rate observed in Group B followed by Group C and A. Similar findings were found after maturation in IVM media added with 100 μM BME, whereby no significant difference in the early apoptosis rates was observed between the different groups of oocytes ($P>0.05$). The rates of early apoptosis in oocytes were 46.60%, 38.40% and 40.50% in Group A, B and C, respectively.

After 24-hour maturation in IVM media supplemented with 150 μM BME, the different oocyte groups did not appear to have any significant difference between them ($P>0.05$). The early apoptosis rates of the oocytes after IVM supplemented with 150 μM BME were 44.75%, 47.74% and 39.00% in Group A, B and C, respectively. Finally, it was found that no significant difference of the early apoptosis rates between different oocyte groups after maturation in IVM media supplemented with 200 μM BME ($P>0.05$). The early apoptosis rate observed among the oocytes were 42.80%, 43.00% and 40.00% in Groups A, B and C, respectively.

Table 4.5: Mean percentage of early apoptosis in different cumulus cells morphology of bovine oocytes before and after IVM with different concentrations of BME supplement

Oocyte group	N	Early apoptosis (Mean % ± SEM)				
		Before IVM	After 24-hour IVM + BME concentrations (μM) n(m)			
			0 (Control)	50	100	150	200
A	267	10.20 ± 2.82[a,x] 4(42)	28.40 ± 3.59[b,y] 14(46)	37.60 ± 7.91[a,y] 19(51)	46.60 ± 4.38[a,y] 17(40)	44.75 ± 7.60[a,y] 20(45)	42.80 ± 4.89[a,y] 18(43)
B	355	19.00 ± 2.28[b,x] 14(72)	18.20 ± 3.09[a,x] 12(63)	44.40 ± 4.47[a,y] 25(56)	38.40 ± 6.73[a,y] 20(53)	47.75 ± 5.47[a,y] 21(44)	43.00 ± 5.96[a,y] 29(67)
C	424	20.60 ± 2.36[b,x] 18(86)	23.00 ± 3.49[a,x] 16(71)	43.00 ± 5.37[a,y] 33(76)	40.50 ± 4.87[a,y] 23(57)	39.00 ± 6.38[a,y] 27(68)	40.00 ± 6.16[a,y] 26(66)

(A = oocyte with compact and dense cumulus cells; B = oocyte with compact but less dense cumulus cells; C = oocyte with little remnants of cumulus cells)
N = total number of oocytes examined (at least 4 replicates each experiment)
n = number of oocytes showing early apoptosis
m = number of oocytes examined for the experiment
[a,b] Means in a column with different superscripts were significantly different ($P<0.05$)
[x,y] Means in a row with different superscripts were significantly different ($P<0.05$)

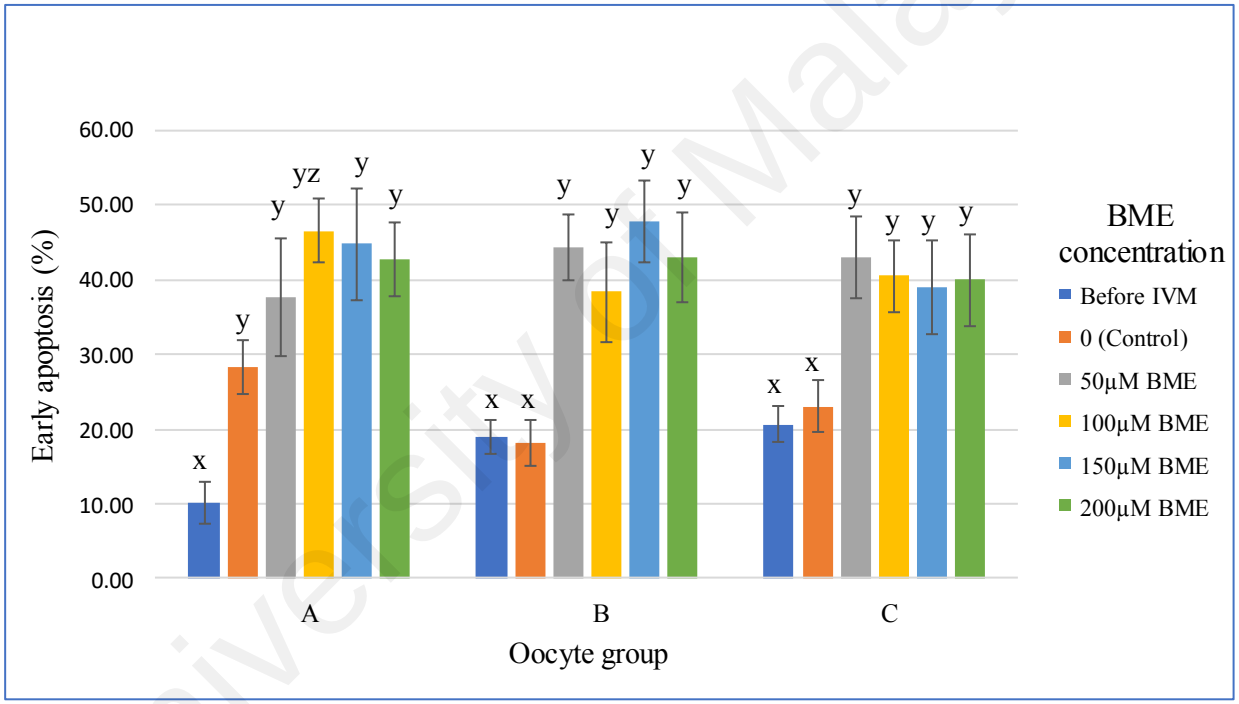

Figure 4.5: Mean percentage of early apoptosis before and after IVM with different concentrations of BME supplement (comparison within different cumulus cells morphology of bovine oocytes)

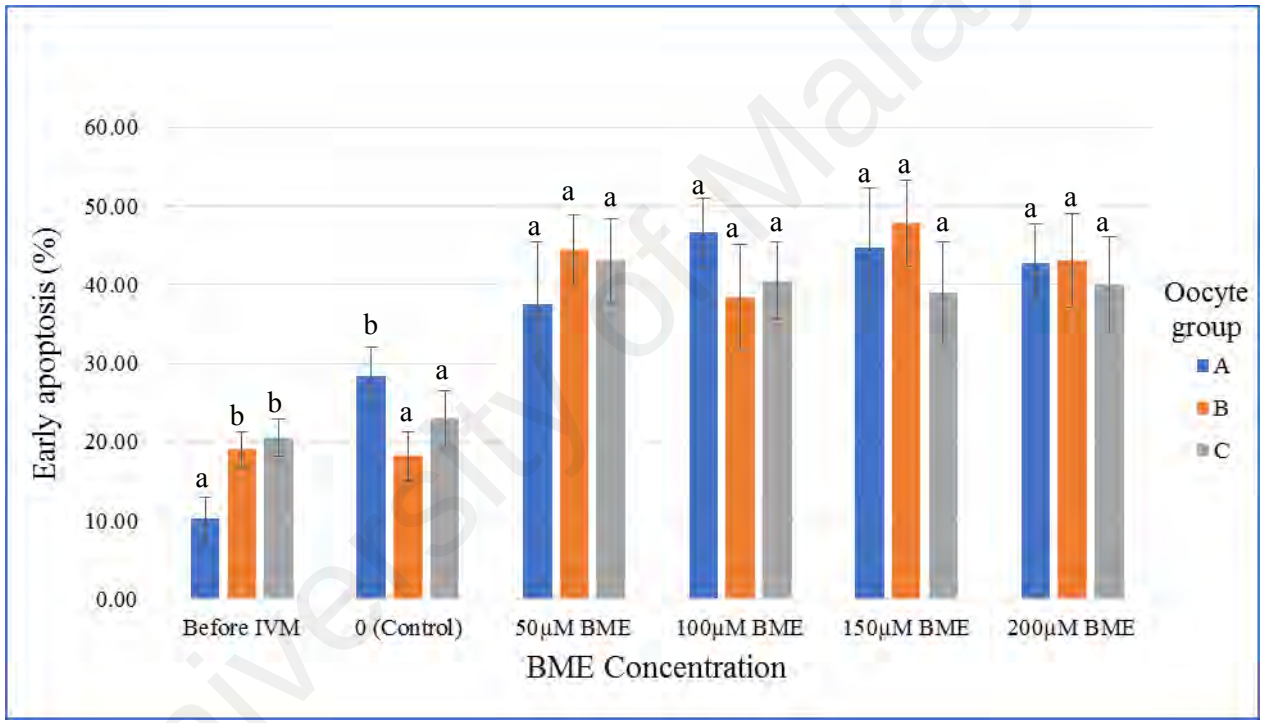

Figure 4.6: Mean percentage of early apoptosis before and after IVM with different concentrations of BME supplement (comparison between different cumulus cells morphology of bovine oocytes)

4.2.2 Effect of BME Supplement during IVM on Intracellular GSH in Bovine Oocytes

Table 4.6 and Figure 4.7 show the mean intracellular GSH concentrations in different oocyte groups matured in IVM medium with different concentrations of BME supplements. Generally, Group A oocytes GSH levels were increased significantly after IVM compared to before IVM regardless of BME concentration ($P<0.05$). The different concentrations of BME supplement in IVM media did not exert any significant difference in the GSH contents of Group A oocytes compared to Control. The mean GSH levels after IVM were 4.26, 4.65, 4.69, 4.80 and 5.07 pmol/oocyte for BME concentrations of 0, 50, 100, 150 and 200 μM, respectively.

Comparison between the mean GSH levels before and after IVM of Group B oocytes showed significant difference especially when the IVM medium was added with BME ($P<0.05$) as compared to without BME supplement (Control). A significant reading of mean GSH level (4.21 pmol/oocyte) was observed when Group B oocytes were matured in IVM medium supplemented with 50 μM BME ($P<0.05$) compared with other concentrations i.e. 100, 150 and 200 μM (4.19, 4.16 and 4.09 pmol/oocyte, respectively).

Group C oocytes showed significant increases in the intracellular GSH content after IVM compared to before IVM. The addition of BME supplements significantly affected the GSH contents of Group C oocytes compared to Control whereby GSH levels were increased ($P<0.05$). The mean GSH concentration of Group C oocytes matured in IVM media without BME supplement (Control) was 3.44 pmol/oocyte. The mean GSH levels in Group C oocytes matured in different BME concentrations 0, 50, 100, 150 and 200 μM were 4.15, 3.92, 4.14 and 4.30 pmol/oocyte, respectively.

In the comparison between different oocyte groups within the same concentrations of BME supplement during IVM, Group A oocytes showed significantly high readings of GSH concentrations in IVM medium (Control) and when added with BME concentrations of 100, 150 and 200 μM BME compared to Groups B and C. (Table 4.6 and Figure 4.8). The GSH levels of Groups A, B and C oocytes after 24-hour IVM with 100 μM BME were 4.69, 4.19 and 3.92 pmol/oocyte, respectively. *In vitro* maturation of the different oocyte groups with BME supplement of 150 μM showed GSH concentrations of 4.80, 4.16 and 4.14 pmol/ooyte in Groups A, B and C, respectively; and with BME supplement of 200 μM showed GSH concentrations of 5.07, 4.09 and 4.30 pmol/ooyte in Groups A, B and C, respectively.

Table 4.6: Mean GSH concentrations in different cumulus cells morphology of bovine oocytes before and after IVM with different concentrations of BME supplement.

Oocyte group	N	Mean GSH concentrations (pmol/oocyte ± SEM)					
		Before IVM	After 24-hour IVM + BME concentrations (μM)				
			0 (Control)	50	100	150	200
A	743	$3.08 \pm 0.28^{a,x}$	$4.26 \pm 0.28^{b,y}$	$4.65 \pm 0.65^{a,y}$	$4.69 \pm 0.39^{b,y}$	$4.80 \pm 0.37^{b,y}$	$5.07 \pm 0.42^{b,y}$
B	747	$3.30 \pm 0.26^{a,x}$	$3.68 \pm 0.35^{a,y}$	$4.21 \pm 0.27^{a,z}$	$4.19 \pm 0.20^{a,yz}$	$4.16 \pm 0.31^{a,yz}$	$4.09 \pm 0.16^{a,y}$
C	736	$3.00 \pm 0.18^{a,x}$	$3.44 \pm 0.28^{a,y}$	$4.15 \pm 0.21^{a,z}$	$3.92 \pm 0.39^{a,z}$	$4.14 \pm 0.28^{a,z}$	$4.30 \pm 0.39^{a,z}$

(A = oocyte with compact and dense cumulus cells; B = oocyte with compact but less dense cumulus cells; C = oocyte with little remnants of cumulus cells)
N = total number of oocytes used (minimum 30 oocytes per experiment, at least 4 replicates each experiment)
[a,b] Means in a column with different superscripts were significantly different ($P<0.05$)
[x,y,z] Means in a row with different superscripts were significantly different ($P<0.05$)

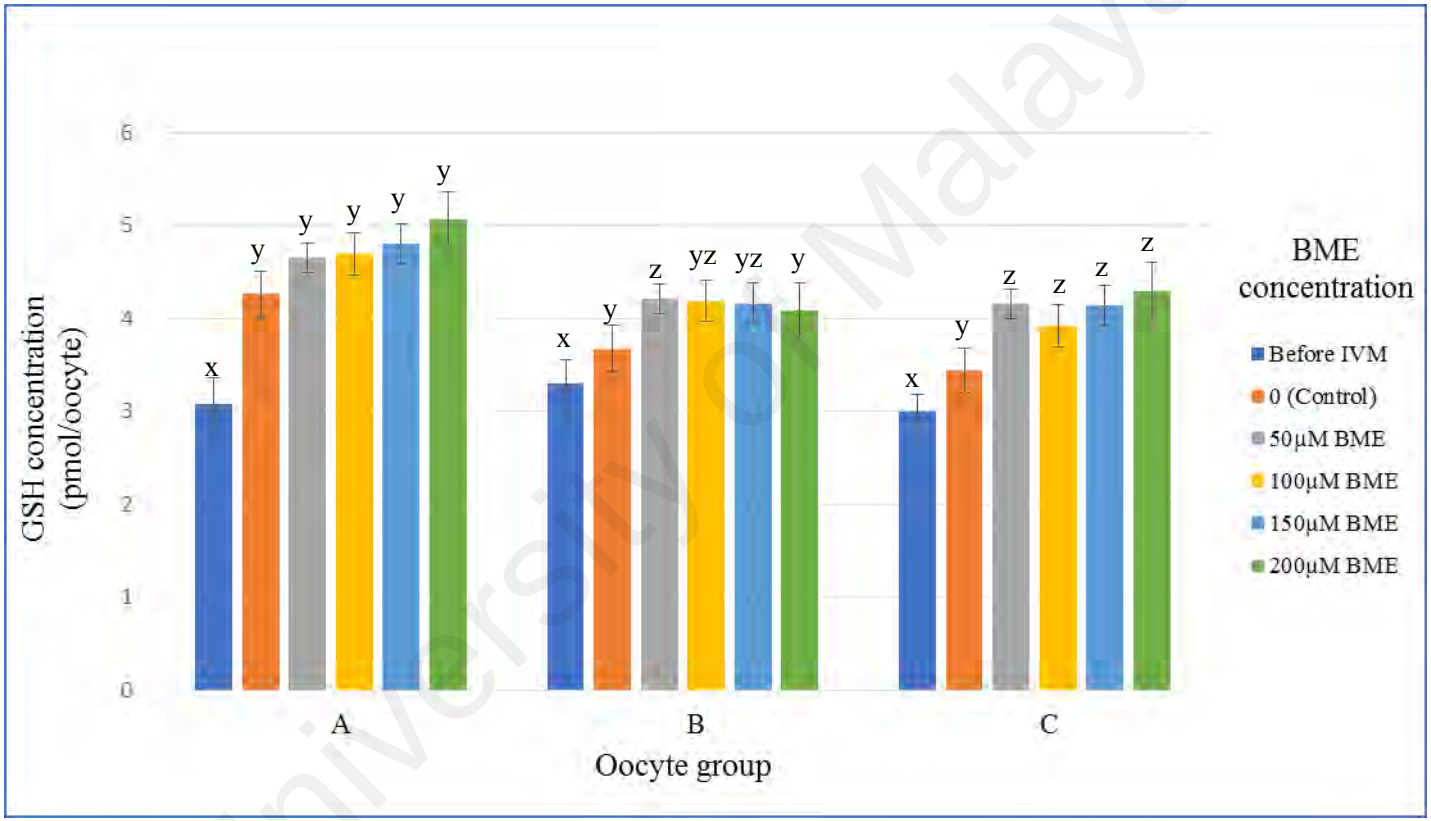

Figure 4.7: Mean GSH concentrations in different cumulus cells morphology of bovine oocytes before and after IVM with different concentrations of BME supplement (comparison within oocyte groups)

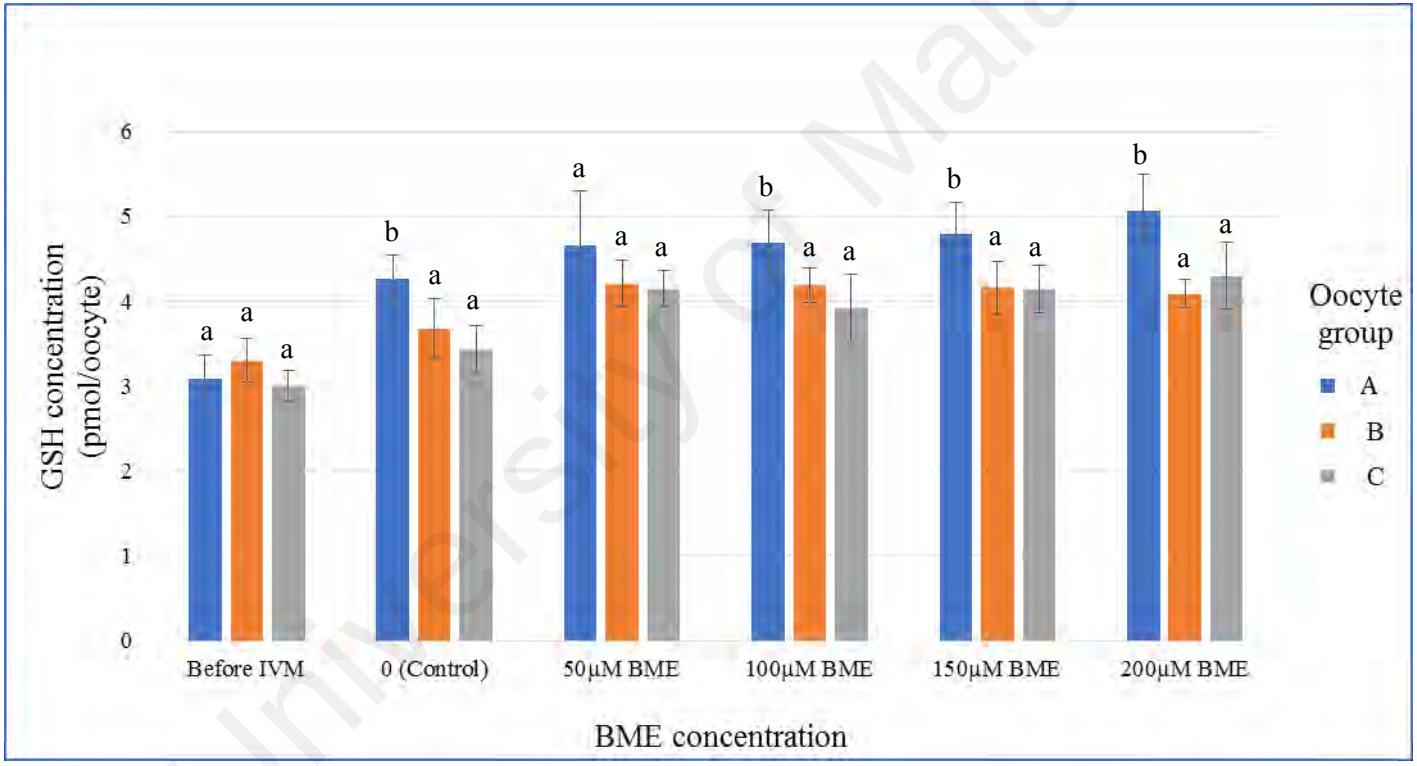

Figure 4.8: Mean GSH concentrations in different cumulus cells morphology of bovine oocytes before and after IVM with different concentrations of BME supplement (comparison between oocyte groups)

4.2.3 Effect of BME Supplement during IVM on Nuclear Maturation of Bovine Oocyte

4.2.3.1 Meiotic stages of Group A oocytes after 24-hour IVM in different concentrations of BME supplement

Table 4.7 and Figure 4.9 represents the rates of meiotic resumption in Group A oocytes matured for 24 hours in IVM medium alone (Control) and supplemented with different concentrations of BME (50, 100, 150 and 200 μM). In Group A oocytes, no significant difference was found between the different concentrations of BME supplement in the rates of meiotic resumption to stages zygotene/pachytene and metaphase I ($P>0.05$). The percentage of Group A oocytes reaching zygotene/pachytene stage were 17.54%, 9.87%, 11.17% 14.53% and 11.85% for BME concentrations of 0 (Control), 50, 100, 150 and 200 μM, respectively. The mean percentage of Group A oocytes reaching metaphase I were 33.94%, 24.78%, 33.07% 35.14% and 32.28% for BME supplement of 0, 50, 100, 150 and 200 μM, respectively. On the other hand, the rates of Group A oocyte achieving nuclear maturation i.e. Metaphase II stage showed a significant increase (60.85%) when matured in medium with 50 μM BME supplement compared with Control and other concentrations of BME supplement ($P<0.05$). The mean percentage of Group A oocytes reaching metaphase II for Control was 44.19% and other concentrations of BME were 51.00%, 43.06% and 49.57% (100, 150 and 200 μM, respectively).

Comparison between meiotic stages of Group A oocytes for the different BME supplement showed similar trend (Figure 4.10) whereby the oocytes were mainly found in metaphase II stage, followed by metaphase I and zygotene/pachytene stage for all concentrations of BME supplement.

Table 4.7: Mean percentage of meiotic stages in Group A oocytes after 24-hour maturation in different concentrations of BME supplement

BME concentration (µM)	**N**	**Meiotic stage (Mean % ± SEM) (n)**			
		Zygotene/ Pachytene	Metaphase I	Metaphase II	Unidentified
0 (Control)	67	17.54 ± 1.22^{a,y} (12)	33.94 ± 1.88^{a,z} (22)	44.19 ± 1.45^{a,z} (30)	4.34 ± 1.96^{a,x} (3)
50	79	9.87 ± 2.85^{a,x} (8)	24.78 ± 3.57^{a,y} (20)	60.85 ± 3.12^{b,z} (47)	4.50 ± 2.78^{a,w} (4)
100	86	11.17 ± 5.31^{a,y} (10)	33.07 ± 7.35^{a,z} (28)	51.00 ± 6.36^{a,z} (44)	4.86 ± 2.05^{a,y} (4)
150	82	14.53 ± 1.24^{a,y} (12)	35.14 ± 3.55^{a,z} (29)	43.06 ± 1.59^{a,z} (35)	7.27 ± 2.33^{a,x} (6)
200	71	11.85 ± 5.06^{a,y} (8)	32.28 ± 3.61^{a,z} (23)	49.57 ± 4.14^{a,z} (36)	6.30 ± 2.96^{a,y} (4)

N = Total number of oocytes (at least 4 replicates)
n = number of oocytes showing the corresponding stage
^{a,b} Means in a column with different superscripts were significantly different (P<0.05)
^{w,x,y,z} Means in a row with different superscripts were not significantly different (P<0.05)

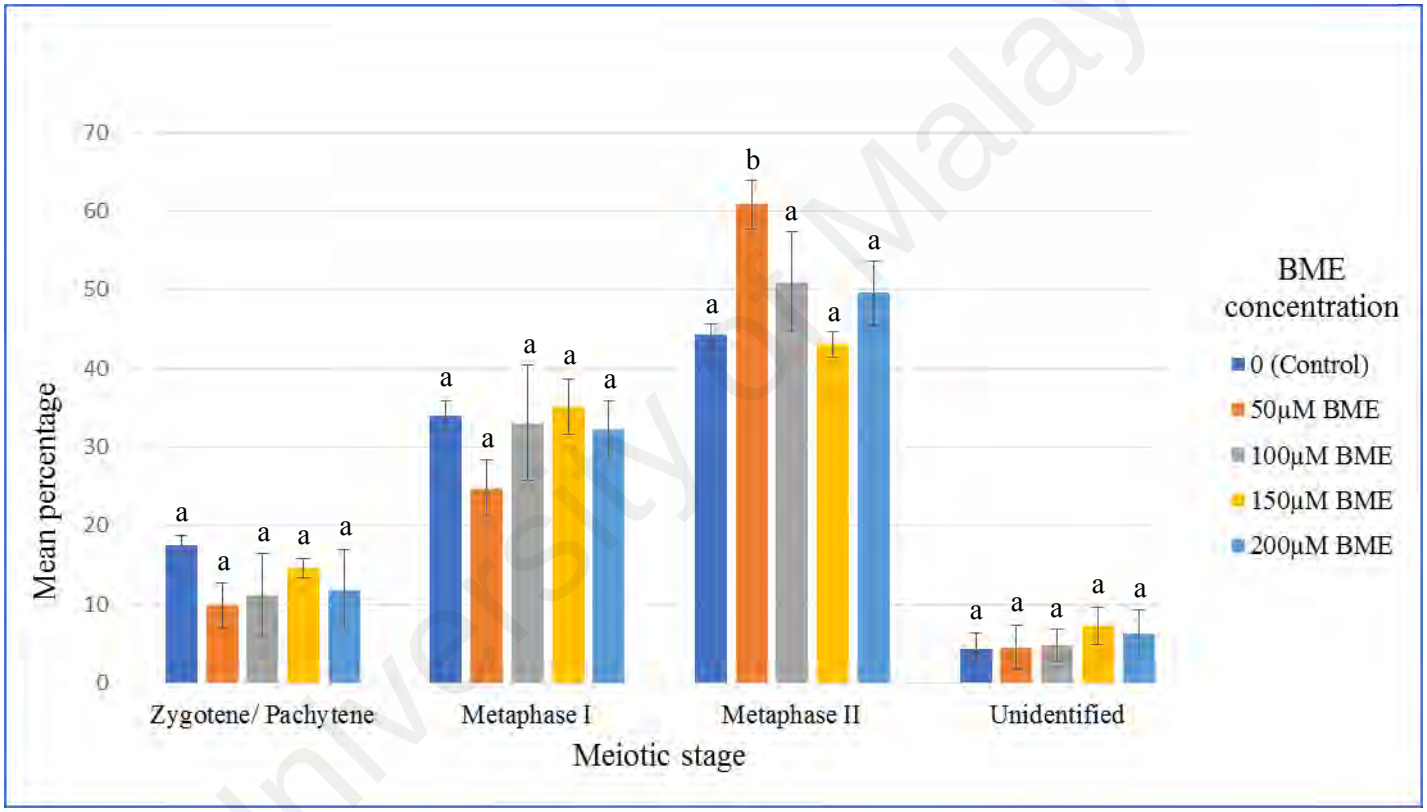

Figure 4.9: Mean percentage of meiotic stages in Group A oocytes after 24-hour maturation in different concentrations of BME supplement

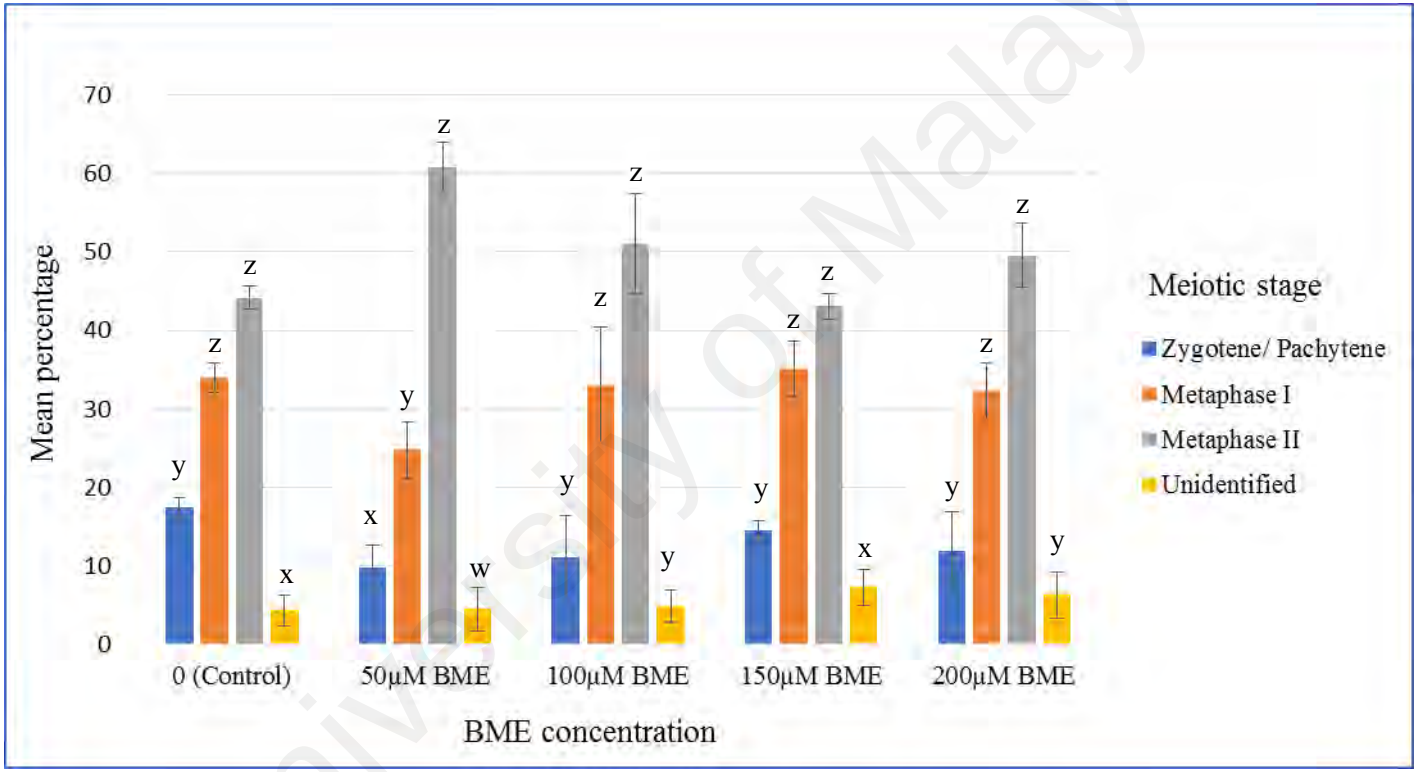

Figure 4.10: Mean percentage of meiotic stages in Group A oocytes after 24-hour maturation in different concentrations of BME supplement (comparison between stage)

4.2.3.2 Meiotic stages of Group B oocytes after 24-hour IVM in different concentrations of BME supplement

The rates of meiotic resumption in Group B oocytes matured for 24 hours in IVM medium (Control) and supplemented with different concentrations of BME (50, 100, 150 and 200 μM) are laid out in Table 4.8 and Figure 4.11. It was observed that the different concentrations of BME added to IVM medium did not exert any significant difference to the rates of Group B oocytes reaching all meiotic stages studied ($P>0.05$). The rates of meiotic resumption to stage zygotene/pachytene in Group B oocytes were 18.66%, 18.01%, 9.46%, 17.82% and 16.18% (BME concentrations: 0, 50, 100, 150 and 200 μM, respectively). The percentages of oocytes reaching metaphase I at different concentrations of BME supplement (0, 50, 100, 150 and 200 μM) were 29.84%, 23.22%, 32.40%, 31.33% and 32.08%, respectively. Group B oocytes reaching metaphase II were 42.33%, 52.67%, 50.49%, 43.72% and 47.66% when matured in IVM medium with 0, 50, 100, 150 and 200 μM BME supplement, respectively.

Similar trend was shown by the rates of meiotic progression in the comparison between meiotic stages for the different BME supplement (Figure 4.12). Group B oocytes were mainly found in metaphase II stage, followed by metaphase I and zygotene/pachytene stage for all concentrations of BME supplement during IVM.

Table 4.8: Mean percentage of meiotic stages in Group B oocytes after 24-hour maturation in different concentrations of BME supplement

BME concentration (µM)	**N**	**Meiotic stage (Mean % ± SEM) (n)**			
		Zygotene/ Pachytene	Metaphase I	Metaphase II	Unidentified
0 (Control)	89	18.66 ± 1.57[a,x] (17)	29.84 ± 1.74[a,y] (27)	42.33 ± 1.01[a,z] (37)	9.18 ± 0.91[a,w] (8)
50	97	18.01 ± 5.62[a,y] (18)	23.22 ± 7.21[a,y] (23)	52.67 ± 6.14[a,z] (50)	6.10 ± 3.53[a,y] (6)
100	92	9.46 ± 3.17[a,x] (9)	32.40 ± 1.42[a,y] (29)	50.49 ± 3.52[a,z] (47)	7.66 ± 1.71[a,x] (7)
150	85	17.82 ± 2.19[a,x] (15)	31.33 ± 2.24[a,y] (27)	43.72 ± 3.80[a,z] (37)	7.13 ± 2.17[a,w] (6)
200	99	16.18 ± 5.49[a,y] (16)	32.08 ± 6.00[a,z] (32)	47.66 ± 2.82[a,z] (47)	4.08 ± 2.04[a,x] (4)

N = Total number of oocytes (at least 4 replicates)
n = number of oocytes showing the corresponding stage
[a] Means in a column with superscripts were not significantly different (P>0.05)
[wxyz] Means in a row with different superscripts were significantly different (P<0.05)

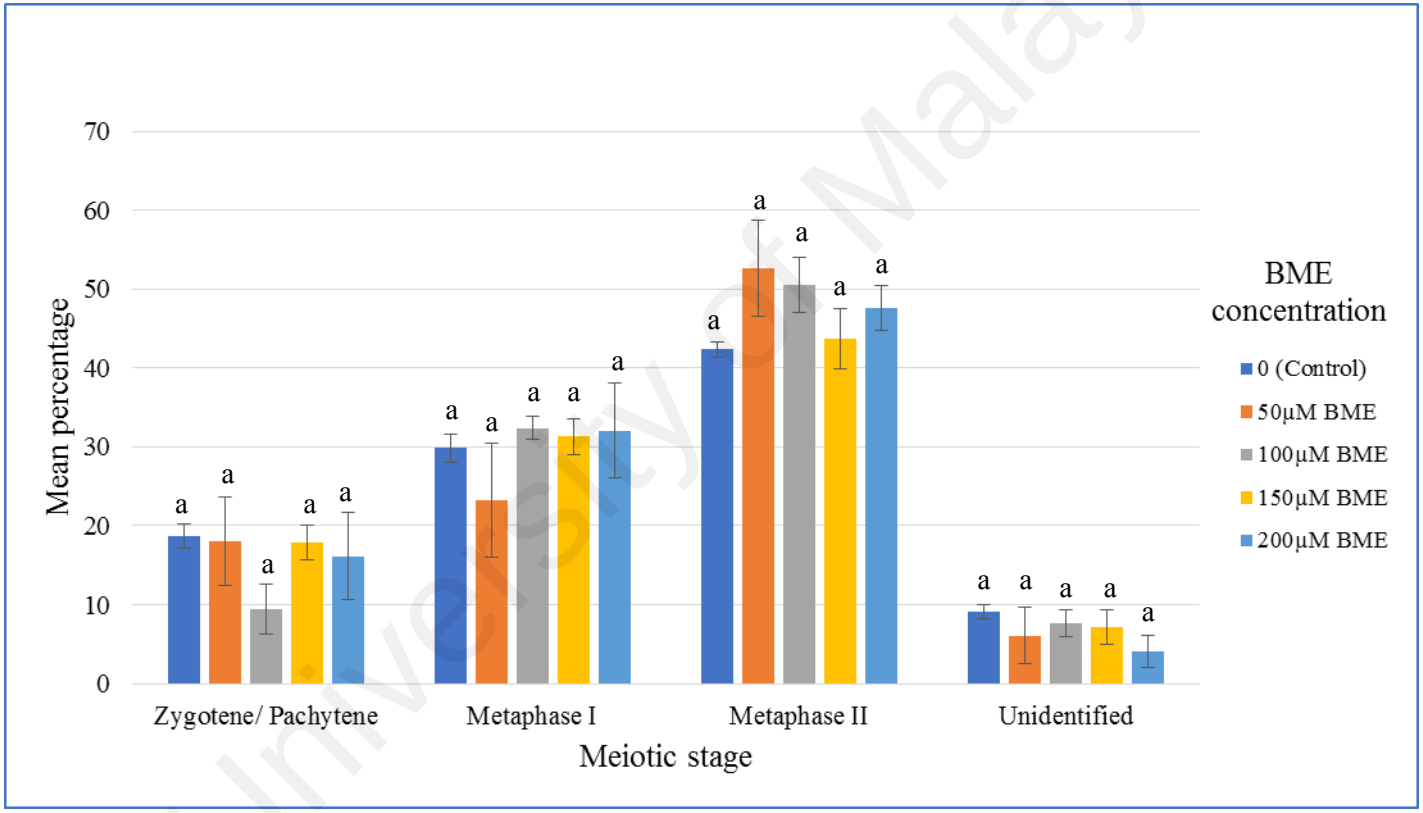

Figure 4.11: Mean percentage of meiotic stages in Group B oocytes after 24-hour maturation in different concentrations of BME supplement

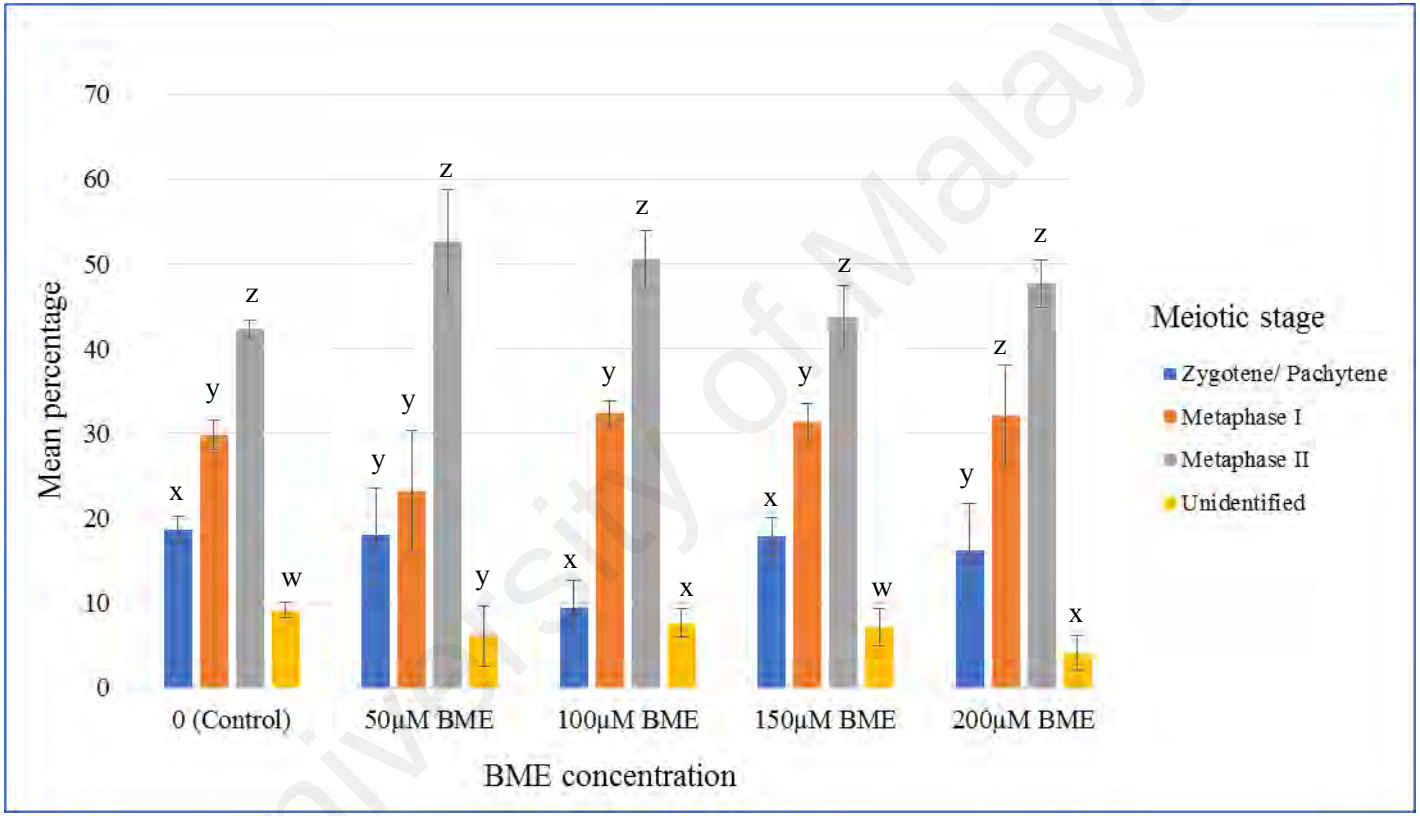

Figure 4.12: Mean percentage of meiotic stages in Group B oocytes after 24-hour maturation in different concentrations of BME supplement (comparison between stage)

4.2.3.3 Meiotic stages of Group C bovine after 24-hour IVM in different concentrations of BME supplement

Table 4.9 and Figure 4.13 displayed the rates of meiotic resumption in Group C oocytes matured for 24 hours in IVM medium (Control) and supplemented with different concentrations of BME (50, 100, 150 and 200 μM). The rates of Group C oocytes reaching all stages of meiosis did not differ between different concentrations of BME added to the IVM medium ($P>0.05$). The mean percentages of oocytes displaying zygotene/pachytene stage were 15.64%, 22.92%, 11.77%, 19.84% and 14.82% for BME concentrations of 0, 50, 100, 150 and 200 μM, respectively. The rates of oocytes found to be in metaphase I stage, with the addition of 0, 50, 100, 150 and 200 μM BME during IVM were 35.38%, 30.23%, 33.45%, 31.76% and 32.46%, respectively. The different concentrations of BME (0, 50, 100, 150 and 200 μM) added to IVM medium had no effect on the rate of Group C oocytes reaching nuclear maturation i.e. metaphase II stage, and the mean percentage were 39.61%, 34.29%, 43.28%, 39.49% and 45.47%, respectively ($P>0.05$).

Comparison between meiotic stages of Group C oocytes displayed similar trend for the different BME supplement (Table 4.9 and Figure 4.14) whereby the oocytes were mainly found in metaphase II stage, followed by metaphase I and zygotene/pachytene stage for all concentrations of BME supplement during IVM.

Table 4.9: Mean percentage of meiotic stages in Group C oocytes after 24-hour maturation in different concentrations of BME supplement

BME concentration (µM)	N	Meiotic stage (Mean % ± SEM) (n)			
		Zygotene/ Pachytene	Metaphase I	Metaphase II	Unidentified
0 (Control)	67	15.64 ± 1.60^{a,y} (14)	35.38 ± 2.55^{a,z} (34)	39.61 ± 3.48^{a,z} (38)	9.37 ± 2.62^{a,x} (9)
50	80	22.92 ± 2.90^{a,z} (18)	30.23 ± 2.90^{a,z} (24)	34.29 ± 4.55^{a,z} (28)	12.56 ± 4.75^{a,y} (10)
100	73	11.77 ± 3.50^{a,y} (8)	33.45 ± 5.05^{a,z} (25)	43.28 ± 1.81^{a,z} (32)	11.50 ± 4.42^{a,y} (8)
150	86	19.78 ± 4.77^{a,y} (17)	31.76 ± 1.52^{a,yz} (28)	39.49 ± 5.22^{a,z} (34)	8.98 ± 3.28^{a,y} (7)
200	74	14.82 ± 2.25^{a,x} (10)	32.46 ± 1.16^{a,y} (25)	45.47 ± 2.86^{a,z} (34)	7.25 ± 2.20^{a,w} (5)

N = Total number of oocytes (at least 4 replicates)
n = number of oocytes showing the corresponding stage
[a] Means in a column with superscripts were not significantly different (P>0.05)
[w,x,y,z] Means in a row with different superscripts were significantly different (P<0.05)

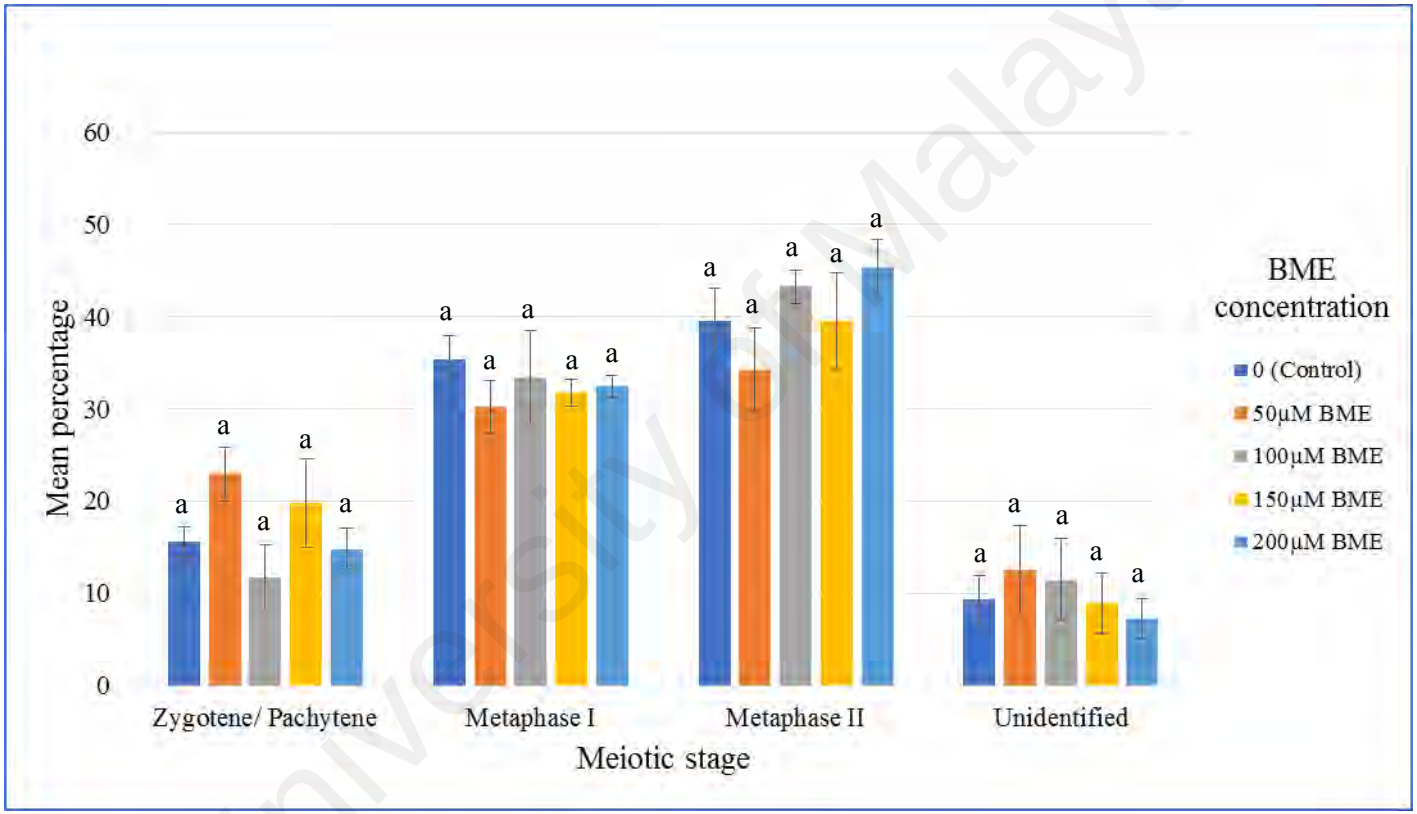

Figure 4.13: Mean percentage of meiotic stages in Group C oocytes after 24-hour maturation in different concentrations of BME supplement

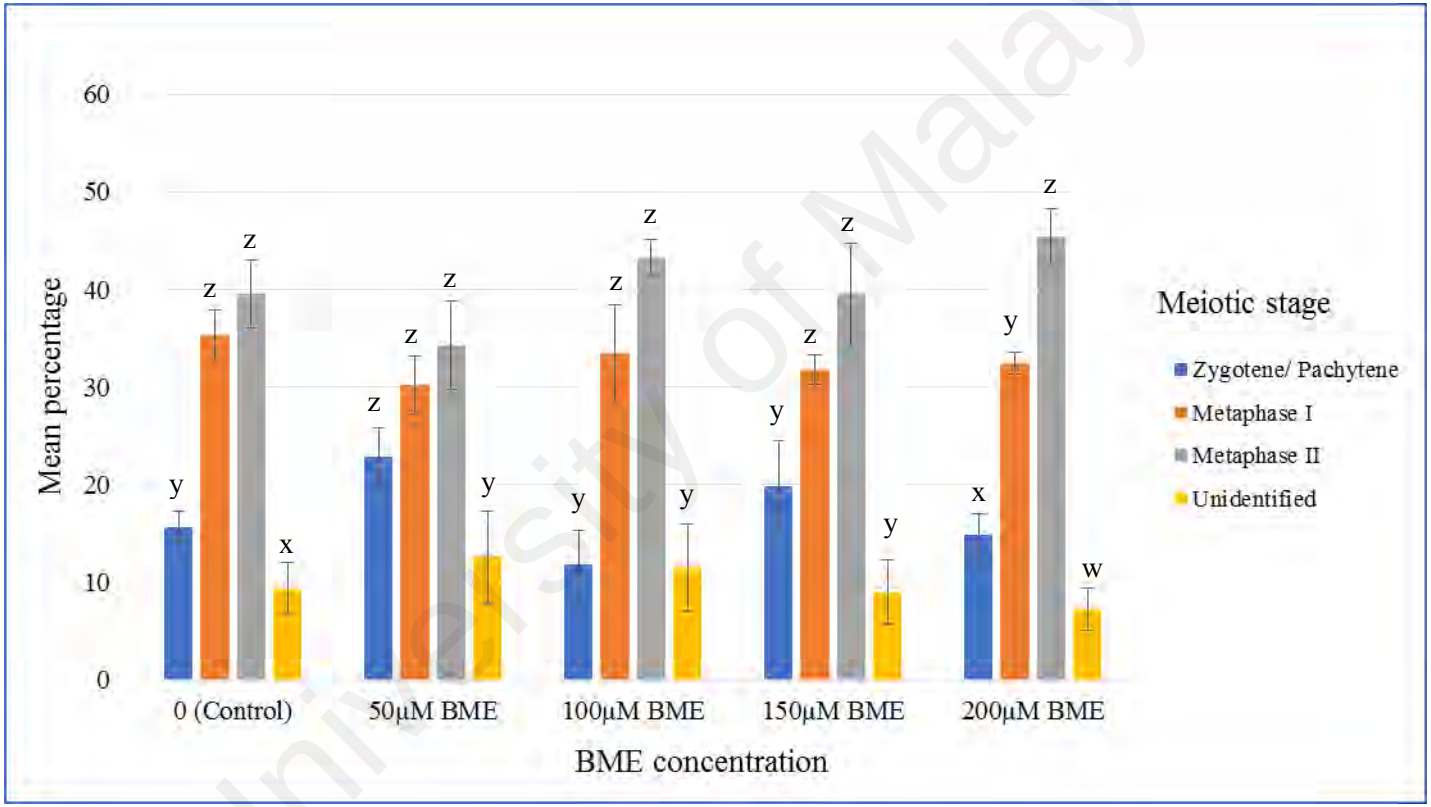

Figure 4.14: Mean percentage of meiotic stages in Group C oocytes after 24-hour maturation in different concentrations of BME supplement (comparison between stage)

4.2.4 Effect of BME Supplement during IVM on Cleavage and Subsequent Embryonic Development after *In vitro* Fertilisation (IVF)

4.2.4.1 Cleavage and subsequent embryonic development of Group A oocytes

The cleavage and subsequent embryo development rates of Group A oocytes matured for 24 hours in IVM medium without BME supplement (Control) and supplemented with different concentrations of BME (50, 100, 150 and 200 μM) are laid out in Table 4.10 and Figure 4.15. Generally, the supplementation of BME during IVM did not exert any difference to the cleavage (2-cell) and subsequent embryo development rates of Group A oocytes. The rates of embryo cleavage in Group A oocytes matured in BME concentrations 0 (Control), 50 and 100 μM were 72.25%, 79.33% and 75.59%, respectively. However, detrimental effect of BME supplement during IVM was found when Group A oocytes were matured in 150 μM BME supplement causing a significant reduction of cleavage rates to 53.23% ($P<0.05$). Similar trend was shown in Group A oocytes reaching 4-cell stage, whereby the addition of 150 and 200 μM BME caused significant reductions in the percentage i.e. 45.79% and 45.29% respectively, compared to 100 μM (64.15%). It was observed that the different concentrations of BME added to IVM medium did not exert any significant difference to the rates of Group A progression to 8-cell up to blastocyst. The range of Group A oocytes managed to reach 8-cell was between 36.80% and 44.93%, 16-cell between 22.35% and 38.39%, morula between 15.10% and 23.03%, compact morula between 9.89% and 13.59%, and blastocyst between 7.02% and 10.61%, for the different concentrations of BME supplement.

Table 4.10: Mean percentage of cleavage and subsequent embryonic development after IVF in Group A oocytes matured in different concentrations of BME supplement

BME conc. (µM)	N	Embryo Stage (Mean % ± SEM) (n)						
		2-cell	4-cell	8-cell	16-cell	morula	compact morula	blastocyst
0 (Control)	55	72.25 ± 3.68[b] (39)	51.60 ± 6.41[ab] (27)	43.46 ± 5.52[a] (23)	38.39 ± 4.84[b] (20)	22.57 ± 3.51[a] (12)	13.22 ± 1.43[a] (7)	10.61 ± 2.08[a] (6)
50	75	79.33 ± 4.76[b] (58)	51.23 ± 5.68[ab] (37)	40.02 ± 1.50[a] (30)	30.20 ± 1.98[ab] (23)	21.20 ± 2.84[a] (16)	14.07 ± 2.98[a] (13)	8.64 ± 1.49[a] (6)
100	70	75.59 ± 2.98[b] (52)	64.15 ± 5.15[b] (44)	44.93 ± 3.16[a] (31)	30.14 ± 2.48[ab] (21)	20.98 ± 2.14[a] (14)	12.17 ± 2.90[a] (8)	7.65 ± 1.65[a] (5)
150	76	53.23 ± 3.44[a] (39)	45.79 ± 5.80[a] (34)	36.80 ± 3.51[a] (28)	22.35 ± 1.85[a] (16)	15.10 ± 1.56[a] (11)	9.89 ± 2.28[a] (7)	7.02 ± 0.89[a] (5)
200	83	64.56 ± 6.04[ab] (53)	45.29 ± 4.72[a] (37)	43.73 ± 3.88[a] (36)	30.57 ± 2.42[ab] (25)	23.03 ± 1.92[a] (19)	13.59 ± 2.51[a] (11)	10.03 ± 1.96[a] (8)

Developmental stages were examined daily from Day 2 to Day 9 (Day 1 = IVF)
N = Total number of oocytes examined (at least 4 replicates)
n = number of oocytes showing the corresponding stages
[a,b,c] means in a column with different superscripts were significantly different ($P<0.05$)

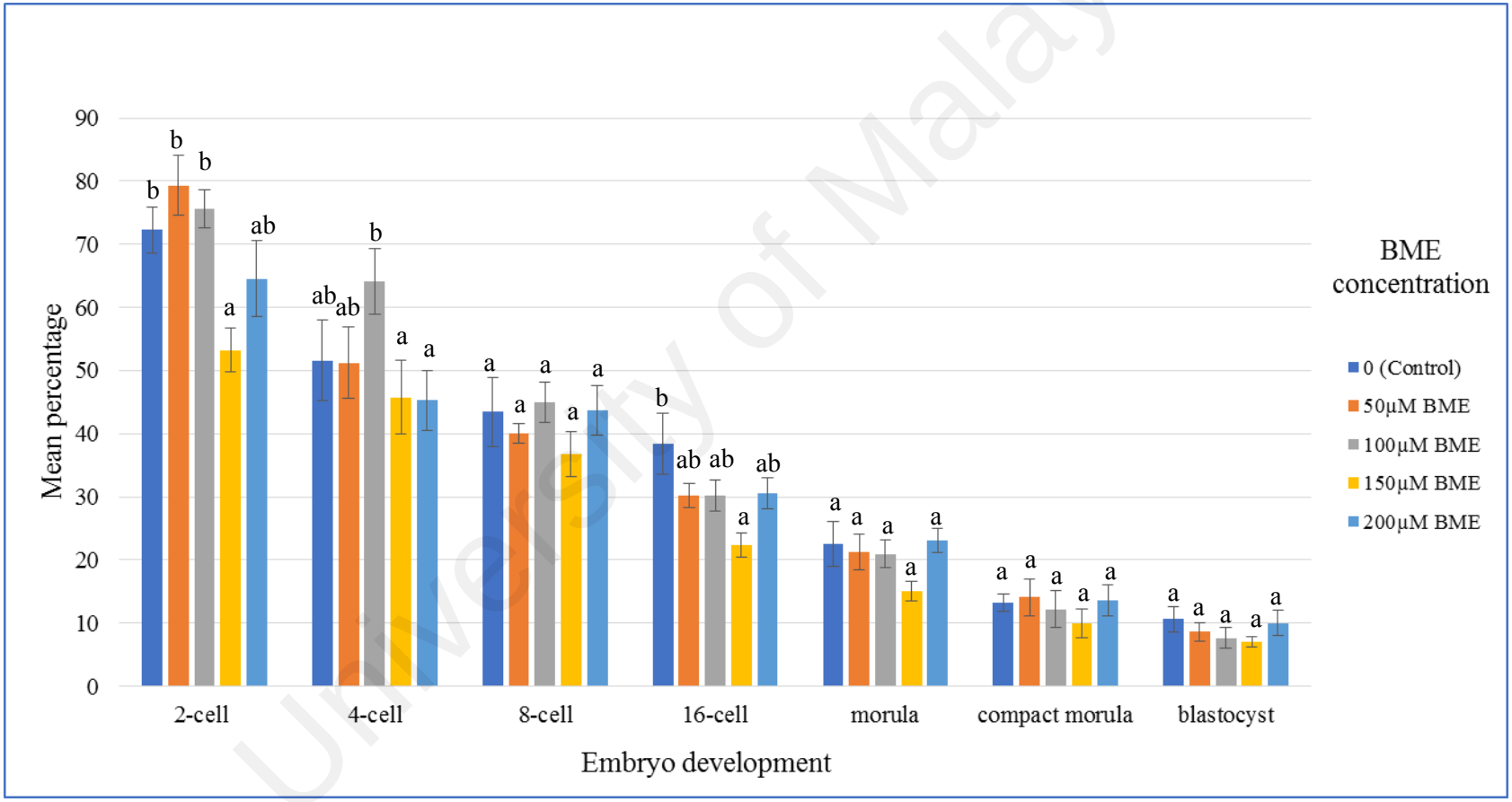

Figure 4.15: Mean percentage of cleavage and subsequent embryonic development after IVF in Group A oocytes matured in different concentrations of BME supplement

4.2.4.2 Cleavage and subsequent embryonic development of Group B oocytes

Table 4.11 and Figure 4.16 show the cleavage and subsequent embryo development rates of Group B oocytes matured for 24 hours in IVM medium without BME supplement (Control) and supplemented with different concentrations of BME (50, 100, 150 and 200 μM). The addition of BME in the maturation medium, generally did not exert any increase of the rates of cleavage and subsequent development of Group B oocytes. However, BME supplement of 100 μM and above significantly reduced the rates of cleavage (2-cell stage) (52.59%, 56.04% and 55.34% for 100, 150 and 200 μM BME, respectively) compared to Control (60.00%) and 50 μM BME (74.97%) ($P<0.05$). The progression of Group B oocytes to 16-cell stage were significantly different in Control and 50 μM BME (22.66% and 22.67%, respectively) compared to 200 μM BME (10.19%) ($P<0.05$). Significant difference was shown in morula of Group B oocytes between Control (18.18%) and 100 (8.76%) ($P<0.05$). The percentage of Group B oocytes reaching blastocyst was significantly high in 50 μM BME (6.98%) compared to 100, 150 and 200 μM BME (2.91%, 1.83% and 5.50%, respectively) ($P<0.05$). No difference was found between different concentrations of BME supplement and Control in the rates of oocytes progressed to 4-cell, 8-cell, morula and compact morula (range between 32.30% - 38.40%, 19.23% - 31.75%, 8.76% - 18.18% and 4.75% - 8.98%, respectively).

Table 4.11: Mean percentage of cleavage and subsequent embryonic development after IVF in Group B oocytes matured in different concentrations of BME supplement

BME conc. (µM)	**N**	**Embryo stage (Mean % ± SEM) (n)**						
		2-cell	4-cell	8-cell	16-cell	morula	compact morula	blastocyst
0 (Control)	91	60.00 ± 3.70^{ab} (54)	37.07 ± 2.40^{a} (33)	31.75 ± 3.75^{a} (27)	22.66 ± 3.85^{b} (20)	18.18 ± 2.14^{b} (16)	8.98 ± 2.31^{a} (8)	6.12 ± 0.91^{bc} (5)
50	88	74.97 ± 7.59^{b} (65)	33.60 ± 6.70^{a} (29)	28.14 ± 6.29^{a} (24)	22.67 ± 4.29^{b} (19)	15.30 ± 4.32^{ab} (13)	6.98 ± 1.90^{a} (6)	6.98 ± 1.90^{c} (6)
100	134	52.59 ± 5.62^{a} (69)	32.30 ± 4.50^{a} (43)	19.23 ± 1.09^{a} (25)	10.19 ± 1.60^{a} (13)	8.76 ± 1.25^{a} (12)	5.01 ± 0.79^{a} (6)	2.91 ± 0.80^{ab} (4)
150	193	56.04 ± 6.19^{a} (108)	32.86 ± 6.63^{a} (62)	22.88 ± 5.08^{a} (43)	16.05 ± 2.29^{ab} (30)	12.19 ± 2.89^{ab} (23)	4.75 ± 1.37^{a} (9)	1.83 ± 0.67^{a} (3)
200	109	55.34 ± 6.10^{a} (60)	38.40 ± 4.87^{a} (41)	27.59 ± 4.23^{a} (29)	18.19 ± 2.87^{ab} (19)	13.00 ± 1.00^{ab} (14)	6.62 ± 1.32^{a} (7)	5.50 ± 0.70^{b} (6)

Developmental stages were examined daily from Day 2 to Day 9 (Day 1 = IVF)
N = Total number of oocytes examined (at least 4 replicates)
n = number of oocytes showing the corresponding stages
^{a,b,c} means in a column with different superscripts were significantly different ($P< 0.05$)

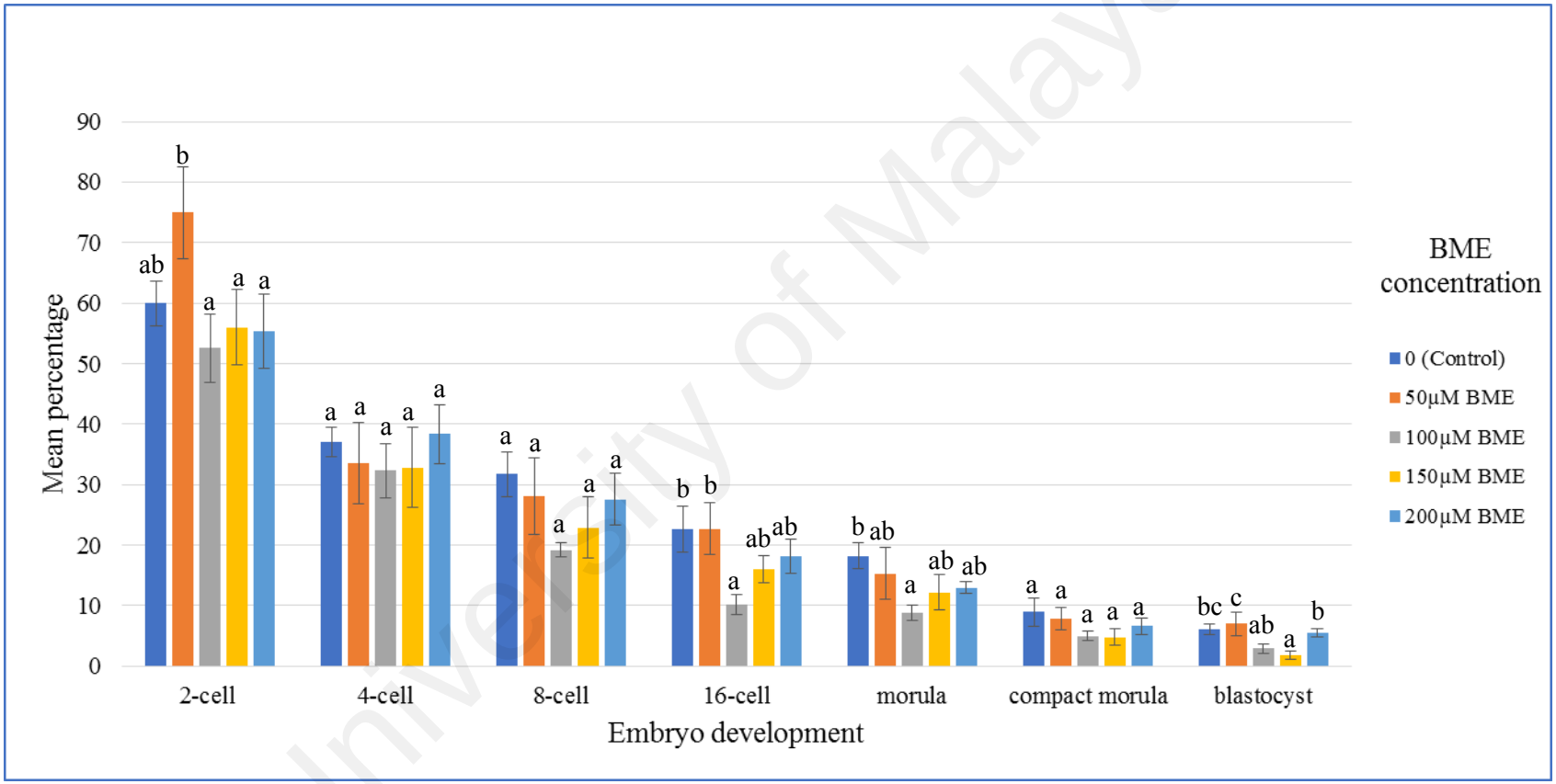

Figure 4.16: Mean percentage of cleavage and subsequent embryonic development after IVF in Group B oocytes matured in different concentrations of BME supplement

4.2.4.3 Cleavage and subsequent embryonic development of Group C oocytes

Table 4.12 and Figure 4.17 represent the rates of cleavage and subsequent embryonic development of Group C oocytes matured for 24 hours in IVM medium without BME supplement (Control) and supplemented with different concentrations of BME (50, 100, 150 and 200 μM). The cleavage rates of Group C oocytes matured in IVM medium without BME (Control) was 67.95% and were significantly reduced when supplemented with 50, 100, 150 and 200 μM BME (54.44%, 54.05%, 40.68% and 32.57%, respectively) ($P<0.05$). The progression of Group C oocytes to 4-cell stage was reduced significantly when 200 μM BME (23.23%) was supplemented in the IVM medium compared to 0, 50, and 100μM BME (35.18%, 36.72% and 38.12%, respectively) ($P<0.05$). Similar pattern was shown in the progression of Group C oocytes to 8-cell stage whereby the mean percentage for 200 μM BME supplement (13.45%) was significantly reduced compared to 0, 50, and 100 μM (28.48%, 26.12% and 25.71%, respectively) ($P<0.05$). Significantly high percentage of progression of Group C oocytes to 16-cell stage were shown when the oocytes were matured in Control IVM medium and supplemented with 100μM BME (26.21% and 22.69%, respectively) compared to 50, 150 and 200 μM BME (12.25%, 12.20% and 9.39%, respectively) ($P<0.05$).

Group C oocytes showed significant reduction in achieving morula when the IVM medium was supplemented with BME at all concentrations (50, 100, 150 and 200 μM; 11.14%, 12.63%, 8.65% and 8.19%, respectively) compared to Control (24.74%) ($P<0.05$). The highest mean percentage of Group C oocytes achieving compact morula was shown for 100 μM BME (10.81%) which differ significantly with Control and 50 μM BME (9.40% and 7.83%, respectively) ($P<0.05$). The addition of 150 and 200 μM BME in the IVM medium further reduced the mean percentage of Group C oocytes (4.95% and 4.09%, respectively) that progressed to compact morula, significantly

compared to Control and 50 μM BME (9.40% and 7.83%, respectively) ($P<0.05$). There were significant differences in the mean percentage between Group C oocytes achieving blastocyst for Control and 50 μM BME (7.61% and 7.83%, respectively) and oocytes matured in 100 - 200 μM BME (4.09% and 3.16%) ($P<0.05$).

Table 4.12: Mean percentage of cleavage and subsequent embryonic development after IVF in Group C oocytes matured in different concentrations of BME supplement

BME conc. (µM)	N	Embryo Stage (% ± SEM) (n)						
		2-cell	4-cell	8-cell	16-cell	morula	compact morula	blastocyst
0 (Control)	66	67.95 ± 4.01^{c} (44)	35.18 ± 0.65^{b} (23)	28.48 ± 4.25^{b} (18)	26.21 ± 3.36^{b} (17)	24.74 ± 2.13^{b} (16)	9.40± 1.77^{b} (6)	7.61 ± 0.70^{b} (5)
50	88	54.44 ± 6.08^{b} (47)	36.72 ± 3.00^{b} (32)	26.12 ± 4.92^{b} (33)	12.25 ± 1.503^{a} (11)	11.14 ± 1.02^{a} (10)	7.83 ± 0.85^{b} (6)	7.83 ± 0.85^{b} (6)
100	157	54.04 ± 3.54^{b} (83)	38.12 ± 4.12^{b} (59)	25.71 ± 3.8b^{b} (38)	22.69 ± 4.95^{b} (34)	12.63 ± 1.79^{a} (18)	10.81 ± 1.20^{c} (16)	4.09 ± 1.47^{a} (6)
150	143	40.68 ± 4.32^{ab} (57)	29.22 ± 3.76^{ab} (41)	17.74 ± 1.47^{ab} (24)	12.20 ± 1.89^{a} (16)	8.65 ± 1.17^{a} (12)	4.95 ± 0.27^{a} (7)	4.50 ± 0.39^{ab} (6)
200	105	32.57 ± 4.20^{a} (33)	23.23 ± 3.70^{a} (24)	13.45 ± 3.62^{a} (13)	9.39 ± 1.11^{a} (9)	8.19 ± 1.08^{a} (8)	4.09 ± 0.54^{a} (4)	3.16 ± 0.88^{a} (3)

Developmental stages were examined daily from Day 2 to Day 9 (Day 1 = IVF)
N = Total number of oocytes examined (at least 4 replicates)
n = number of oocytes showing the corresponding stages
^{a,b,c} means in a column with different superscripts were significantly different ($P< 0.05$)

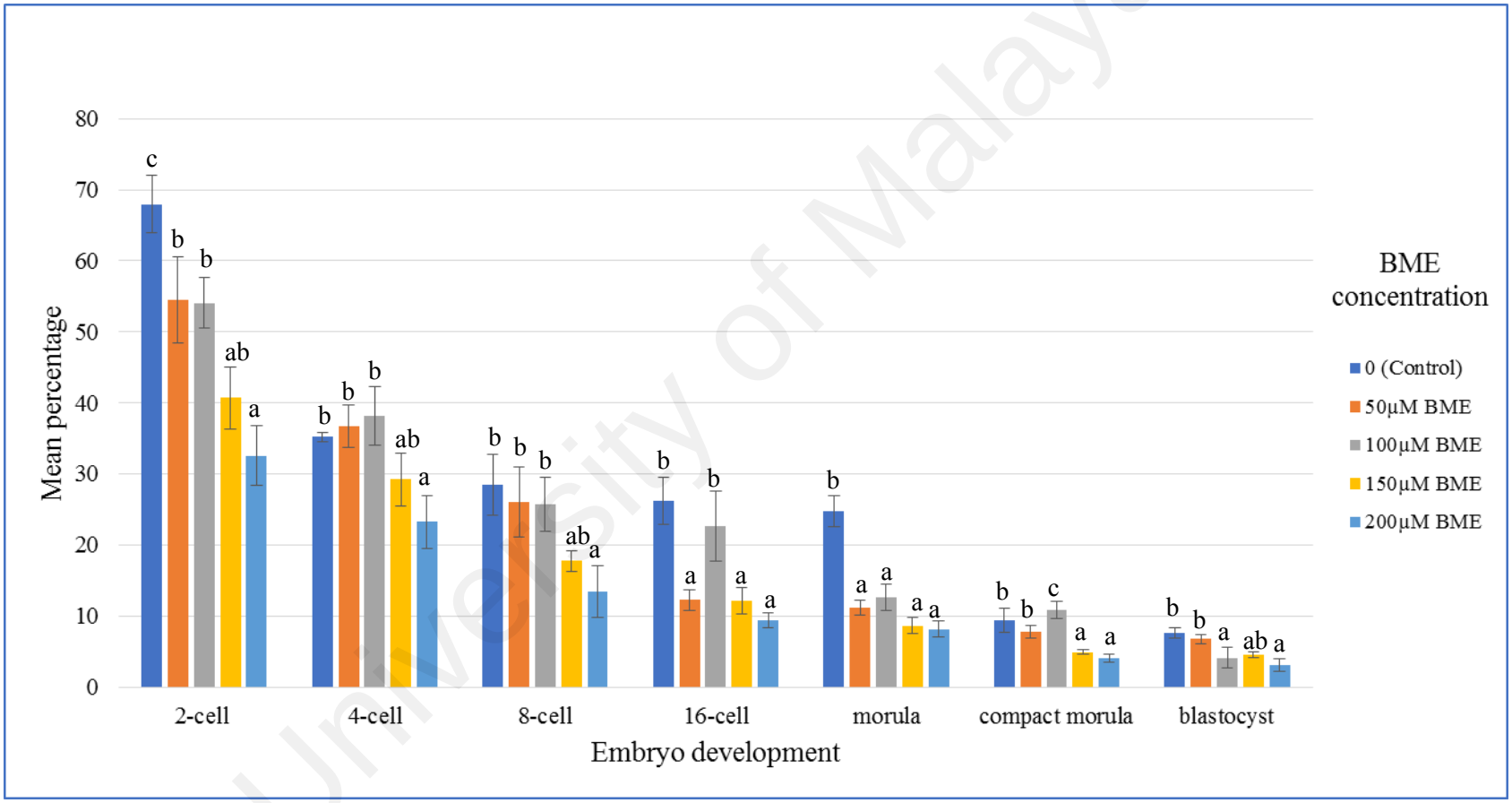

Figure 4.17: Mean percentage of cleavage and subsequent embryonic development after IVF in Group C oocytes matured in different concentrations of BME supplement

CHAPTER 5: DISCUSSION

5.1 EFFECTS OF CUMULUS CELLS ON OOCYTE AND EMBRYO DEVELOPMENTAL COMPETENCY (EXPERIMENT I)

5.1.1 Effect of Cumulus Cells on Early Apoptosis in Bovine Oocytes

To date, there were not many studies on apoptosis reported and the results were inconsistent. Previous studies have reported the occurrence of apoptosis in bovine ovarian follicles (Isobe & Yoshimura, 2007) and cumulus cells (Luciano *et al*., 2000). In the present study, it was found that early apoptosis as assessed by Annexin-V was detected in the different cumulus cells morphology of bovine oocytes (Groups A, B and C) before and after IVM (Table 4.1). These were in accordance to previous studies reporting the detection of apoptosis in both immature and mature oocytes (Matwee *et al*., 2000; Anguita *et al*., 2007; Li *et al*., 2009). Matwee *et al*. (2000) reported that apoptosis occurred both in mature and immature oocytes by using TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) staining. However, Yuan *et al*. (2005) detected no apoptotic oocytes before or after maturation using the same technique. In the present study, the use of Annexin-V staining detected phosphatydilserine exposure, a very early phenomenon during apoptosis, preceding nuclear condensation (late stage apoptosis) and breakdown of the intracellular cytoskeletal and nuclear matrix constituents (Oberhammer *et al*., 1994). This technique has been performed in oocytes in previous studies because it was believed to be an earlier marker of apoptosis than TUNEL which detected DNA fragmentation resulting from apoptotic signaling cascades (Anguita *et al*., 2007; Lobascio *et al*., 2007; de Felici *et al*., 2008).

Healthy oocytes are characterized by compact cumulus composed of several layers of cells and a homogeneous cytoplasm, and these oocytes are selected for *in vitro* maturation and *in vitro* fertilisation. Classification of immature oocytes according to the

number of layers of cumulus cells left around the oocytes following aspiration, the thicker the number of layers of cumulus cells (more than 5 layers), the better were the chances for development (Lonergan *et al*., 1994). The results of the present study found that before 24-hour IVM, Group A oocytes which was considered as good quality oocytes (having compact and dense cumulus layers) showed a significantly low rate of early apoptosis occurrence compared to Groups B (compact and less dense cumulus cells layers) and C (thin or little remnants of cumulus cell layers and expanded) oocytes (Table 4.1). Previous reports have indicated that oocytes with a compact cumulus cells seemed to originate from healthy follicles or from those with only the initial signs of atresia, whereas oocytes with incomplete and/or expanded cumulus originated from late atretic follicles (Leibfried & First, 1979; Lonergen *et al.*, 1992; Madison *et al.*, 1992; Brackett & Zuelke, 1993). In the ovary, apoptosis is responsible for follicular atresia which occurs continuously from birth until the complete depletion of the follicle pool (Hirshfield, 1991; Hsueh *et al*., 1994; Kaipia & Hsueh, 1997), and over half the follicles present in an ovary at any given moment are at different stages of atresia (Kruip & Dieleman, 1982). Therefore, the present findings indicated that Group A oocytes which displayed low occurrence of early apoptosis (Table 4.1) might possibly be originated from healthy follicles or from those with only the initial signs of atresia, since apoptosis was known to be responsible for follicular atresia.

The present study also showed that immature Groups B and C oocytes displayed higher incidences of early apoptosis compared with Group A (Table 4.1). These results agreed with Matwee *et al.* (2000), who reported that apoptosis was related to the process of degeneration in bovine immature oocytes. Most oocytes used for *in vitro* embryo production were retrieved from slaughtered animals; therefore, these oocytes (Groups B and C) may likely to have arisen from atretic follicles. This suggested that these oocytes may be at earlier stages of apoptosis before DNA breakdown since they are the last

compartment to be affected by apoptosis during atresia of antral follicle (de Wit *et al*., 2000).

Comparison between the different oocyte groups after 24-hour IVM in the present study found significantly high incidence of early apoptosis in Group A oocytes than Groups B and C (Table 4.1). This was in accordance to previous reports whereby *in vitro* conditions have been shown to promote apoptosis (Gjørret *et al*., 2003; Knijn *et al*., 2003; Warzych *et al*., 2007). Since the early apoptosis occurrence in Group A was significantly low compared to Groups B and C oocytes before IVM (Table 4.1), it was suggested that probably IVM induced apoptosis in Group A oocytes. This finding also agreed with previous reports indicating the negative effect of culture *in vitro* in the incidence of apoptosis in oocytes (Takase *et al.*, 1995; Fujino *et al.*, 1996; Van Blerkom & Davis, 1998; Matwee *et al.*, 2000). On the contrary, early apoptosis was associated with improved developmental potential in bovine oocytes, i.e. cytoplasmic maturation (Li *et al.*, 2009). It was proposed that to promote final maturation, i.e. cytoplasmic maturation, oocytes were required to go through some changes in the ultrastructure which is similar to the changes occurring during oocyte degeneration (Sirard, 1989; Assey *et al.*, 1994). It was suggested in previous studies that the occurrence of early apoptosis in oocytes did not mean that they must develop into late apoptosis because apoptosis is a sequential, but reversible process of cell death, with ultimate committance being made before cells enter the latter stages of regulation (Morita & Tilly, 1999; Anguita *et al.*, 2007; Jaroudi & SenGupta, 2007). Therefore, the increased incidence of early apoptosis in Group A oocytes after IVM, might indicate cytoplasmic maturation. However, the results contradicted to previous reports on goat (Anguita *et al.*, 2009) and cattle (Anguita *et al.*, 2007) that observed a reduction of early apoptosis occurrence after maturation.

The rates of early apoptosis detected in Groups B and C oocytes were similar before and after 24-hour IVM, indicating that early apoptosis occurrence in Groups B and C oocytes were not affected by maturation process (Table 4.1). A previous study by Yuan *et al.* (2005), reported that no apoptotic oocyte was detected in the samples of oocytes studied. There were discrepancies in previous reports on early apoptosis occurrences that might be due the differences in media composition and among species (Warzych *et al*., 2007; Wasielak & Bogacki, 2007). Moreover, the oocytes used in the present study were retrieved from ovaries of slaughtered females with unknown history, source and origin which probably affect the variation among studies as reported by Gordon (1994) on animal related factors (age and breed of donor, stage of oestrous cycle). It was suggested that further refined studies to be carried out on how these different groups of oocytes would individually behave when cultured in different types of media and the specific medium for a specific oocyte group could be determined.

5.1.2 Effect of Cumulus Cells on Intracellular GSH in Bovine Oocytes

Intact cumulus cells were known to produce glutathione (GSH) during IVM and this GSH could subsequently be accumulated in the ooplasm of the oocytes (Yoshida *et al*., 1993). Glutathione is a major free thiol compound with important biological functions like protection of the oocytes against oxidative stress (Meister, 1983; Tatemoto *et al*., 2001), and the levels of intracellular GSH and ROS were reported to influence IVM and oocyte developmental competence (Luberda, 2005; Jiao *et al.*, 2013; Mukherjee *et al.*, 2014). In the present study, immature oocytes showed no significant difference in the GSH contents between the different groups (Groups A, B and C) (Table 4.2). However, after 24 hours maturation in IVM medium, the GSH contents of Group A oocytes were significantly increased when compared with Groups B and C. This indicated that GSH was synthesised during *in vitro* maturation in Group A oocytes

which could be related to the compactness and density of cumulus cell layers surrounding the oocytes. The finding was in accordance with previous reports that GSH was synthesised during oocyte *in vitro* maturation in mouse (Calvin *et al.*, 1986), hamster (Perreault *et al.*, 1988), pig (Yoshida *et al.*, 1993), cattle (Miyamura *et al.*, 1995) and buffalo (Gasparrini *et al.*, 2006).

The increase in GSH concentrations during IVM of cattle oocytes was important in subsequent embryo development to blastocyst stage (Miyamura *et al.*, 1995; de Matos *et al.*, 1995; 1996; Furnus *et al.*, 1998). The presence of high concentrations of GSH in Group A oocytes after IVM (Table 4.2) agreed with previous report that high GSH levels served as reservoir that would protect embryo against oxidative damage before genomic activation and *de novo* GSH synthesis (Deleuze & Goudet, 2010). During IVM, intact cumulus cells produced GSH, an antioxidant that performs strong basal reactive oxygen species (ROS) scavenging activity, and subsequently accumulated in the ooplasm of the oocytes (Yoshida *et al.,* 1993; Johnson & Nasr-Esfahani, 1994; Gardiner *et al.*, 1998; Dröge, 2002). It could possibly react with many ROS and act as a cofactor for glutathione peroxidase that catalysed the reduction of toxic H_2O_2 and hydroperoxides (Bilodeau *et al*., 2001). Therefore, the present study indicated that the presence of cumulus cells is important in maintaining the synthesis of GSH during IVM. On the other hand, Rocha-Frigoni *et al.* (2016) demonstrated depletion in the amounts of intracellular GSH in mature bovine oocytes compared to immature oocytes. It was suggested that IVC conditions (i.e. the media) resulted in higher mobilisation and utilisation of intracellular GSH to neutralise the high and continuous amounts of ROS produced by the cells or even in the culture medium (Cetica *et al.*, 2001; Curnow *et al.*, 2008; Livingstone *et al.*, 2009).

In addition, the results of the present study also showed that after IVM, the GSH concentrations in Group A oocytes were significantly higher than Groups B and C

oocytes (Table 4.2). This indicated that after IVM, oocytes which were presumed to be good quality oocytes having compact and dense cumulus cell layers (Group A) demonstrated a high content of GSH compared with oocytes having less dense cumulus cells (Group B) and thin cumulus cells (Group C) (Table 4.2). The findings supported the role for the cumulus cells in the production of GSH which have been reported in previous studies, indicating that GSH production is stimulated in oocytes surrounded by cumulus cells, but not in denuded oocytes (Takahashi *et al*., 1993; de Matos *et al*., 1997; Nagai, 2001). In another report, it was suggested that impaired GSH synthesis may be dependent on the amount of cumulus cells surrounding the oocytes (Merton *et al*., 2013). Furthermore, the findings of the present study that GSH levels in Groups B and C oocytes were not increased during IVM (Table 4.2), also agreed with the report showing that only intact COC could utilise the substrates present in the TCM-199 medium for GSH synthesis during IVM (de Matos *et al*., 1997). As reported previously that oocytes maturing *in vitro* without cumulus cells have, especially, low intracellular GSH (Zhou *et al*., 2010), the results of the present study suggested that cumulus cell layers may possibly play a role in enhancing the synthesis and storage of GSH in bovine oocytes, and that cumulus cells are necessary during IVM of bovine oocytes.

5.1.3 Effect of Oocyte Cumulus Cells on Nuclear Maturation of Bovine Oocytes

Cumulus cells have been known to be not only important for oocyte maturation and fertilisation but also affect the development of embryo to the blastocyst stage. Therefore the presence of an intact complement of cumulus cell layers surrounding the oocyte and a homogenous appearing cytoplasm determine the ability of immature oocyte to go through maturation and embryonic development (Janowski, 2012). On the contrary, the results of the present study found no difference in the rates of oocytes achieving different stages of meiosis between the three oocyte groups (Table 4.3). The

percentage of nuclear maturation (MII) in Group A oocytes observed in the present study, i.e. 44.2% was found to be lower compared to 79.0% as reported by Habsah Bidin (2006), which used similar oocyte classification and culture medium (TCM 199 + oestradiol + steer serum), and to other studies which ranged from 75% to 93% maturation rates in similar group of oocytes (Xu *et al.*, 1986; Shioya *et al.*, 1988; Suss *et al.*, 1988; Kim & Park, 1990; Laurincik *et al.*, 1992; Fukui *et al.*, 2000; Khurana & Niemann, 2000; Kelly *et al.*, 2007). On the other hand, the maturation rates of Group B oocytes (42.3%) were comparable to other studies which ranged from 27.8% to 68.8% in similar oocyte group (Xu *et al.*, 1986; Laurincik *et al.*, 1992), but still lower compared to 70.0% reported by Habsah Bidin (2006). The maturation rates (MII) of Group C oocytes (39.6%) in the study were also observed to be consistent with other studies which ranged from 40% to 58% in similar group of oocytes (Liebfried-Rutledge & First, 1979; Yoshida & Kanagawa, 1983; Shioya *et al.*, 1988; Kim & Park, 1990; Habsah Bidin, 2006). On the contrary, there were studies that demonstrated higher rates of maturation in similar group of oocytes, i.e. 84.7% and 71.0% (Kim & Park, 1990; Khurana & Niemann, 2000). Although similar oocyte classification and maturation medium were used, the discrepancies between the present study and reported by Habsah Bidin (2006) might be due to the different settings of laboratory i.e. environmental factors such as the air quality. The air quality referring to the level of pollutants namely, particulate matter and volatile organic compounds which have been reported to adversely affect the gametes (maturation and morphology) and embryos in early stages of development (Vásquez Cubillos & De los Santos Molina, 2017).

The mean percentages of all oocytes in the study found to be arrested at MI were similar and no significant difference was found between Groups A, B and C (Table 4.3). However, these rates were observed to be relatively higher (29.80% - 35.40%) compared to the figures reported by Habsah Bidin (2006) (14.30% - 26.90%). In the

present study, IVM was carried out for a duration of 24 hours as proposed by Habsah Bidin (2006) and Nor Fadillah Awang (2013). These studies were in accordance to a report that duration of 24 hours of IVM gave better blastocyst-reaching results compared with 18 hours IVM (Monaghan *et al*., 1993). Since the finding of the study showed lower MI rates in the oocytes compared to other reports, it could be that the oocytes probably require a longer IVM duration of more than 24 hours to achieve nuclear maturation for the current laboratory settings (air quality) (Vásquez Cubillos & De los Santos Molina, 2017). Proper maturation is important for full developmental competence of embryo development, the formation of abnormal chromatin, oocyte aging and impaired development may occur due to inappropriate timing of maturation (Marston & Chang, 1964; Yanagimachi, 1994; Dominko & First, 1997; Hunter & Greve, 1997). Nevertheless, oocyte cytoplasmic maturation could be improved by temporary maintenance of oocytes in meiotic arrest immediately before IVM culture (pre-IVM) (Gilchrist & Thompson, 2007) since the growing oocytes accumulated compounds essential for maturation, fertilisation and embryogenesis during folliculogenesis (Eppig, 1996; Marteil *et al.,* 2009). Therefore, the low maturation rates shown in the present study might be due to inadequate cytoplasmic maturation (absence of crucial oocyte cytoplasmic components) as proposed by previous report (Huang *et al.,* 2013). However, further detailed studies need to be carried out in order to substantiate this phenomenon.

5.1.4 Effect of Cumulus Cells on Cleavage and Subsequent Embryonic Development after *In vitro* Fertilisation (IVF)

No difference was found in the cleavage (2-cell) rates between the different oocyte groups in the present study (Table 4.4). The cleavage rates (60.0% - 72.25%) were comparable to the figures reported by Habsah Bidin (2006) (65.8% - 71.7%) and Nor Fadillah Awang (2013) (24.0% - 78.3%), and in accordance to other reports

(Shioya *et al*., 1988; Zuelke & Brackett, 1990; Ward *et al*., 2000; Miller *et al*., 2003). However, the male and female pronucleus formation was impaired and higher incidence of polyspermy was observed in these reports.

On the other hand, the results of the present study were not in accordance to the report that the presence of cumulus cells surrounding oocytes influenced embryo development, whereby the cleavage rates were significantly decreased when cumulus cells were removed prior to *in vitro* fertilisation (Fatehi *et al*., 2002). Previous study also reported that oocytes maturing *in vitro* without cumulus cells were of low developmental potential since oocytes maturing *in vitro* without cumulus cells caused zona hardening, resulting in decreased fertilisation rate (Schroeder & Eppig, 1984). The structure of the zona pellucida was maintained by disulfide bonds, which were thought to be the cause of zona hardening (Iwamoto *et al.*, 1999). It was reported that GSH enhanced oocyte fertility by reducing the number of disulfide bonds in the zona pellucida (Takeo *et al.*, 2015), and the role for the cumulus cells in the production of GSH have been indicated in many reports (Takahashi *et al*., 1993; de Matos *et al*., 1997; Nagai, 2001; Merton *et al*., 2013). Although cleavage rates were not different between experimental groups, development rates to 4-cell and 16-cell stage were significantly higher in Group A than Group B and C (Table 4.4). Since the present findings did not agree with previous reports, therefore the possible factors that might affect the rates of cleavage in bovine oocytes in the present study need to be substantiated through further refined studies.

Nevertheless, the present study found that the developmental rates of Group A oocytes to 4-cell and 16-cell stages were significantly higher compared with Groups B and C (Table 4.4). The results of the present study were in accordance to previous reports that cumulus cells play a role in GSH synthesis during IVM (Mori *et al*., 2000), and the increase in GSH concentrations during IVM of cattle oocytes is important in

subsequent embryo development to blastocyst stage (Miyamura *et al.*, 1995; de Matos *et al.*, 1995; 1996; Furnus *et al.*, 1998). The presence of high levels of GSH in oocytes at the end of maturation served as reservoir that would protect embryo against oxidative damage before genomic activation and *de novo* GSH synthesis (Deleuze & Goudet, 2010). This was supported by the earlier finding of the present study that GSH concentration was increased after IVM in Group A oocytes (Table 4.2). It is suggested that the compact and dense cumulus cells of Group A oocytes increased the GSH level during IVM that supported early embryonic development up to 16-cell stage. In the present study, it is suggested that Groups B and C oocytes might be affected by ROS resulting in loss of developmental potential due to insufficient concentration of intracellular GSH to support growth. This finding agreed with the reports that the presence of compact and dense cumulus cells increased GSH synthesis during IVM forming a reservoir pool which reduced oxidative stress during early stage embryos and hence increasing the rates compared to oocytes with lesser cumulus cells (Calvin *et al*., 1986; Telford, *et al*., 1990; Eppig, 1996; Knappen *et al*., 1999; de Matos & Furnus, 2000; Fujii *et al*., 2005).

The present study also showed that there was no significant difference found between the different oocyte groups in the rates of embryo that developed to morula up to blastocyst (Table 4.4). Therefore, the present study suggested that although Group A oocytes having compact and dense cumulus cells may have increased the GSH level during IVM, the GSH synthesised may not be enough to support embryo development up to the blastocyst stage. Based on the present findings, apparently there is a need for a means of intervention such as adding extracellular supplement (e.g. antioxidant) that could protect the embryos from oxidative stress which could be implemented during IVM of oocytes or during IVC of embryos in order to improve the developmental competency.

5.2 EFFECTS OF BME SUPPLEMENT DURING IVM ON OOCYTE AND EMBRYO DEVELOPMENTAL COMPETENCY (EXPERIMENT II)

5.2.1 Effect of BME Supplement during IVM on Early Apoptosis of Bovine Oocytes

The promoting effects of low molecular-weight thiol compounds, such as BME during *in vitro* embryo production have been indicated in many studies. BME maintain the redox state of cells and protect them against the harmful effects of oxidative injuries (Camaño *et al*., 1996; 1998; Geshi *et al*., 1999; Takahashi *et al*., 2002; Mori *et al*., 2006). Oxidative injuries include membrane lipids peroxidation, oxidation of amino acids and nucleic acids, apoptosis and necrosis (Halliwell *et al*., 1992). Therefore, addition of BME into the maturation media is expected to reduce oxidative stress and hence the rate of apoptosis in the developing oocyte.

In the present study, when BME was added into the IVM medium, the early apoptosis rates in Group A oocytes were found to be increased after maturation regardless whether BME was added or not (Table 4.5). However, the rates of early apoptosis in Groups B and C observed after IVM increased significantly compared to before IVM, and the rates did not differ significantly between the different concentrations of BME supplement in all groups of oocytes. These findings suggested that the addition of BME during IVM increased the early apoptosis rates in Groups B and C, but not in Group A oocytes. The results of the present study disagreed with the reports indicating that BME protect cells against harmful effects of oxidative injuries including apoptosis (Halliwell *et al*., 1992; Camaño *et al*., 1996; 1998; Geshi *et al*., 1999; Takahashi *et al*., 2002; Mori *et al*., 2006).

As been discussed earlier, although previous report indicated that apoptosis was related to the process of degeneration in bovine immature oocytes (Matwee *et al.,* 2000), another report suggested that early apoptosis was associated with improved developmental potential in bovine oocytes (Li *et al.*, 2009). Previous studies proposed

that to promote final maturation, i.e. cytoplasmic maturation, oocytes needed to go through some changes in the ultrastructure which is similar to the changes occurring during oocyte degeneration (Sirard, 1989; Assey *et al.*, 1994). The occurrence of early apoptosis in oocytes did not necessarily develop into late apoptosis, which decreases oocytes developmental competence, since apoptosis is a sequential, but reversible, process of cell death, with ultimate committance being made before cells enter the latter stages of regulation (Morita & Tilly, 1999; Anguita *et al.*, 2007; Jaroudi & SenGupta, 2007). Therefore, the high rates of early apoptosis in all oocytes in the study did not necessarily indicate low developmental competence. Nevertheless, a report on late apoptosis detected using TUNEL demonstrated that IVM medium supplementation (FBS, PVP40) did not affect the incidence of apoptosis (Warzych *et al*., 2007). To our knowledge, no other study has investigated the effect of BME supplement in IVM media on early apoptosis occurrence in bovine oocytes. Therefore, it is recommended that further investigations should be conducted to elucidate the understanding of the roles of BME on the early apoptosis in different groups of bovine oocytes.

5.2.2 Effect of BME Supplement during IVM on Intracellular GSH in Bovine Oocytes

GSH is a tripeptide thiol synthesised by glutamic acid, cysteine, and glycine in the pathway of the γ-glutamyl cycle (Meister & Tate, 1976), which is known to have important roles for keeping intracellular redox state for protecting the cells against oxidative stress (Pastore *et al*., 2003). Oxidative stress caused serious DNA damage in bovine embryos and is associated with a low rate of embryo development (Takahashi *et al*., 2000). Therefore, maintenance of the intracellular redox state by increasing the GSH level is an important factor for embryo development.

The present study demonstrated that the GSH content in Group A oocytes were increased significantly after maturation in IVM medium without BME (Control) and

with BME supplement (Table 4.6). Therefore, the addition of BME in the IVM medium did not exert any effect on the GSH levels of Group A oocytes. On the other hand, IVM with BME supplement produced significant increase in Groups B and C oocyte GSH levels compared with Control, and the increase in BME supplement exerted no significant difference in GSH concentration (Table 4.6). The results observed in Groups B and C in the present study agreed with the reports that the addition of cysteamine, cysteine, and BME to maturation medium increased GSH synthesis in bovine oocytes during *in vitro* maturation (de Matos *et al*., 1996; 1997). In the process of GSH synthesis, cysteine is known to be a rate-limiting factor (Meister & Tate, 1976) and GSH synthesis is highly dependent on the availability of cysteine outside of the cells (Ishii *et al*., 1981; Rathburn & Murray, 1991). However, cysteine in the medium is easily oxidised even under usual culture conditions, forming cystine, a dimer of cysteine, causing the oxidative stress (Toohey, 1975; Sagara *et al*., 1993). As a consequence, the growth of cells is inhibited because of the low utility of cystine causing the depletion of GSH and growth inhibition (Gmünder *et al*., 1991). The presence of BME protects cysteine from oxidation into cystine and increases its entry into the cell, which would trigger GSH synthesis (Takahashi *et al*., 2002). In the present study, significant increases in GSH levels observed in Groups B and C oocytes when BME was added to IVM medium compared with IVM medium (Control) (Table 4.6), indicated that BME is probably required for lower quality oocytes to reduce oxidative stress to the oocytes. Therefore, the present study suggested that IVM medium supplemented with low molecular weight thiol compounds, such as BME, increased GSH content in oocytes with less dense or thin layers of cumulus cells.

The results displayed by Group A oocytes in the present study suggested that good quality oocytes could increase the GSH levels during IVM without the need to add BME in the IVM medium (Table 4.6). Therefore, the findings of the present study

supported the reports showing that only intact COC could utilise the substrates present in the TCM-199 medium for GSH synthesis during IVM (de Matos *et al*., 1997). As reported previously that oocytes maturing *in vitro* without cumulus cells have, especially, low intracellular GSH (Zhou *et al*., 2010), the results of the present study, suggested that cumulus cell layers may possibly play a role in enhancing the synthesis and storage of GSH in bovine oocytes, and that cumulus cells are necessary during IVM of bovine oocytes. In a nutshell, the present study suggested that addition of BME in IVM medium may have no effect on the synthesis of GSH in "good quality' oocytes having compact and dense cumulus cells as in Group A, but may be useful in enhancing the GSH synthesis in "low quality' oocytes such as Group B having compact and less dense cumulus cells and Group C, having thin layers of cumulus cells, thus increasing the efficacy of IVM of these oocytes.

5.2.3 Effect of BME Supplement during IVM on Nuclear Maturation of Bovine Oocytes

Studies on oocyte maturation have shown that modifications of a culture system for the *in vitro* maturation (IVM) of oocytes could improve cytoplasmic maturation (Cayo-colca *et al*., 2011; Hu *et al*., 2011). A high level of GSH production had been shown to improve cytoplasmic maturation of oocytes, which may result in a higher success rate of embryonic development (De Matos & Furnus, 2000; Takahashi *et al*., 2002; Rodriguez-Gonzalez *et al*., 2003). Previous reports have also shown that addition of thiol containing precursors of GSH such as cysteine (CySH), cysteamine, β-mercaptoethanol or use of a cysteine-rich medium (TCM 199 or Waymouth MB 75211) increased GSH content of oocytes after maturation (de Matos *et al*., 2002). In the present study, nuclear maturation rates up to metaphase II stage was found to be significantly high among Group A oocytes with compact and dense cumulus cells, when treated with 50 μM BME supplement (60.85%) during IVM compared with other BME

concentrations (100, 150 and 200 μM; metaphase II 51.00%, 43.06%, 49.57%, respectively) (Table 4.7). Thus, the present study indicated that BME at concentration 50 μM, probably stimulated the intracellular GSH level of bovine oocytes during IVM as reported by de Matos and Furnus (2000), hence increasing the maturation rate of Group A oocytes.

However, the present study also showed that GSH levels in Group A oocytes were increased after IVM, with or without BME supplement (Table 4.6). Hence, it is not possible to relate the increase in the maturation rates of Group A oocytes with the addition of 50 μM BME and the increase in GSH levels. There could be a possibility that in the present study, BME in the IVM medium promoted nuclear maturation of oocytes with compact and dense cumulus cells (Group A), by triggering some unknown factors and mechanisms. This would require further study to elucidate the possible role of BME in the present study.

The results of the present study showed that the addition of BME during IVM of Groups B and C oocytes did not produce any effect on the maturation rates Table 4.8 and 4.9). This is in accordance to a previous study that nuclear maturation rates up to the MII stage did not increase in any groups treated with BME (0, 10, 50, 100, 500 μM) ($p>0.05$) (Tsuzuki *et al.,* 2005). Although in the earlier findings of the present study, it was found that GSH levels were increased in these groups of oocytes with the addition of BME during IVM, the maturation rates were not increased (Table 4.6). This disagreed with reports that showed high level of GSH production improved cytoplasmic maturation of oocytes (De Matos & Furnus, 2000; Takahashi *et al*., 2002; Rodriguez-Gonzalez *et al*., 2003). It is suggested that the addition of BME stimulated the synthesis of GSH in low quality oocytes such as Groups B (compact and less dense cumulus cells) and C (thin or little remnants of cumulus cells), but had no effect on the maturation rates up to MII stage.

5.2.4 Effect of BME Supplement during IVM on Cleavage and Subsequent Embryonic Development after IVF

It was reported that addition of thiol compounds such as BME and cysteamine during bovine IVM caused the high intracellular GSH level and decrease hydrogen peroxide levels, improving bovine embryo development and quality, producing more embryos reaching the blastocyst stage than embryos matured in unsupplemented medium (de Matos *et al.,* 1995; 1996; de Matos & Furnus, 2000). In the present study, it was found that the cleavage and embryonic development to 4-cell stage rates in Group A oocytes were significantly higher in Control (0 μM), and when 50 and 100 μM BME were added in IVM medium compared to other concentrations (Table 4.10). Generally, the addition of BME during *in vitro* maturation of bovine oocytes had no effect on the rates of embryonic development to 8-cell up to blastocyst for Group A oocytes. It was noteworthy that when higher concentrations of BME (150 μM and 200 μM) were added to the IVM medium, the rates of cleavage and subsequent embryonic development to 4-cell stage were reduced significantly. Therefore, the present study indicated that the addition of BME did not exert any effect on the cleavage and 4-cell stage rates of Group A oocytes, and BME concentrations of more than 100 μM reduced these rates. The present study suggested that higher concentrations of BME supplement during IVM may possibly exerted a detrimental effect on cleavage and early embryo development rates of bovine oocytes.

Furthermore, the embryonic development rates of Groups B (Table 4.11) and C (Table 4.12) were also found to be not affected by the addition of BME during the IVM of the oocytes. Earlier finding of the present study showed that GSH level were increased during IVM of these oocytes (Groups B and C) (Table 4.6). These findings were contradictory to previous reports, suggesting that the increase in GSH concentrations during IVM of cattle improved subsequent embryo development to blastocyst stage (Miyamura *et al*., 1995; de Matos *et al*., 1996; Furnus *et al*., 1998).

Thus, in the present study, it may be suggested that although the addition of thiol compound such as BME may have increased the intracellular levels of GSH during maturation, it did not necessarily enhance the subsequent embryo development. Therefore, it is proposed for future study that BME to be supplemented during IVC of embryos to protect them from oxidative stress and consequently, improved bovine embryo development competency.

5.3 GENERAL DISCUSSION

The development of IVEP technology was established in accelerating the genetic improvement of herds. The IVEP process involved harvesting of bovine oocytes from donor's ovaries of slaughtered animals, which served as the most common economical source of oocytes that allows for large scale and economical production of bovine embryos. Consequently, the oocytes retrieved from slaughterhouse ovaries might be of highly variable quality and quantity, resulting in reduced developmental competency of the *in vitro* produced embryos. Oocyte retrieval was followed by IVM, IVF and IVC, i.e. the development of the fertilised oocyte to the blastocyst. To establish a viable pregnancy that will deliver a normal offspring from the embryos obtained through IVEP, each of these developmental steps should be completed successfully. However, it is generally known that IVEP procedure reduced the viability and competency of *in vitro* bovine embryos.

In Malaysia, the development of IVEP technology for practical applications in the livestock industry have been hampered by the low efficiency and high cost of IVEP research (Ariff *et al*., 2015*)*. A major limitation for conducting IVEP research was the limited availability of cattle ovaries, and poor recovery of the number and good quality immature oocytes. Furthermore, the percentage of presumably good quality oocytes with compact and dense cumulus cells was only about 20% to 30% of the total number

of oocytes retrieved each collection (Habsah Bidin, 2006; Nor Fadillah Awang, 2013). Since the oocytes which were considered as low quality make up the large portion of oocytes retrieved in developing countries such as Malaysia, therefore the salvation of these oocytes through the optimisation of the technical and biological factors influencing the successful outcome of IVEP could probably cater for the low number of immature oocytes available obtained from these slaughtered bovine species.

One of the most important and critical stages for successful IVEP is oocyte maturation, and *in vitro* maturation conditions were normally simpler and contain limited materials compared to *in vivo* maturation which may affect performance of oocyte maturation. In addition to that, cumulus cells factor also contributed to IVM competency of oocytes. The necessity of intact cumulus cells surrounding the oocytes during IVM have been explained by Yoshida *et al.* (1993), whereby these cells were shown to produce glutathione (GSH) during IVM, and this GSH can subsequently be accumulated in the ooplasm of the oocytes (Calvin *et al.,* 1986; Perreault *et al.,* 1988; Miyamura *et al.,* 1995; Gasparrini *et al.,* 2006). GSH was responsible for protection against oxidative stress, and a potential marker of oocyte cytoplasmic maturation and developmental competence (Meister, 1983; Deneke & Fanburg, 1989; Eppig, 1996; Merton *et al.,* 2013). The present findings were in consistent to these reports, whereby only Group A oocytes with compact and dense cumulus cell layers was found to synthesise GSH during IVM, and the GSH levels were significantly higher compared with Groups B and C oocytes after maturation. Therefore, the importance of intact cumulus cells during IVM in the production of GSH was displayed in the present study.

Most previous studies reported on late apoptosis (DNA fragmentation detected using TUNEL staining) but very few on the early apoptosis (phosphatydilserine (PS) exposure) detected using Annexin-V assay (Yuan *et al.,* 2005; Anguita *et al.,* 2007; Lobascio *et al.,* 2007; de Felici *et al.,* 2008; Li *et al.,* 2009). Annexin-V staining has

been performed in oocytes because it was believed to be an earlier marker of apoptosis than TUNEL which detected DNA fragmentation resulting from apoptotic signaling cascades (Anguita *et al*., 2007; Lobascio *et al*., 2007; de Felici *et al.*, 2008). In terms of early apoptosis occurrence, the correlation between early apoptosis occurrence in immature oocytes and the compactness of surrounding cumulus cells of good quality oocytes such as Group A was observed in the present study, whereby the occurrence of early apoptosis in immature Group A oocytes were lower compared with Groups B and C. This suggested that Group A oocytes may have originated from healthy follicles, whereas Groups B and C oocytes having less dense and thin cumulus cells were retrieved from late atretic follicles. Therefore, the presence of compact cumulus cells surrounding Group A oocytes may have exerted a positive effect by maintaining a low rate of apoptosis occurrence in the follicles at the time of retrieval. On the contrary, early apoptosis rates were found to be increased after IVM in Group A oocytes even though GSH was synthesised during maturation. Although this indicated negative effect of *in vitro* culture on oocytes, one possible reason was that the ultracellular changes involved in apoptosis were similar to the changes in the cytoplasmic maturation. Therefore, the early apoptosis detected in Group A oocytes did not necessarily mean that these oocytes were subjected to oxidative stress and will develop into late apoptosis.

It also appeared in the present study that Groups B and C oocytes maintained the rates of early apoptosis after IVM, thus indicating that IVM may have no influence on the early apoptosis rates in these oocytes. Earlier reports had indicated that cumulus cells were essential during IVM for cumulus expansion, meiotic resumption and cytoplasmic maturation of oocytes (Warriach & Chohan, 2004; Kelly *et al.,* 2007; Davachi *et al.,* 2012), and *in vitro* maturation without the presence of cumulus cells found to be detrimental to oocyte maturation (Schroeder & Eppig, 1984; Vanderhyden

& Armstrong, 1989; Chian *et al*., 1994; Zhang *et al*., 1995; Wongsrikeao *et al*., 2005). In contrast to the above findings, the present study showed that there was no difference in the meiotic competence assessed by their ability to achieve nuclear maturation (metaphase II stage) between Groups A (oocytes with compact and dense surrounding cumulus cells), Group B (oocytes with compact but less dense cumulus cells) and Group C (oocytes with thin or little remnants of cumulus cells). Another factor to be considered was that more oocytes were found to be arrested at meiotic stages prior to MII. Based on these observations, it was suggested that the oocytes to be subjected to a longer IVM duration than 24 hours to achieve nuclear maturation in future study. The duration of IVM of 24 hours was applied in the present study as proposed in previous similar studies (Habsah Bidin, 2006; Nor Fadillah Awang, 2013). The variations might also be due to the differences in numerous genetic and environmental factors such as the breeds, the tropical climates, culture regime and the air quality (Vásquez Cubillos & De los Santos Molina, 2017) in the different laboratory settings.

It is known that intact cumulus cells produce GSH during IVM, which is necessary for viability in both oocytes and early embryos (Yoshida, 1993; Eppig, 1996; Knappen *et al.,* 1999; de Matos & Furnus, 2000; Mori *et al.,* 2000; Fujii *et al.,* 2005), and has beneficial effects on subsequent embryo development (Calvin *et al.,* 1986; de Matos *et al.,* 1995; 1996; Miyamura *et al.,* 1995; Furnus *et al.,* 1998; Zhou *et al.,* 2010). In terms of embryo development, the cleavage rates of all oocyte groups in the present study were similar regardless of the compactness of the cumulus cells layer, but Group A oocytes were found to have significantly higher rates of early embryo development, i.e. 4-cell and 16-cell stage compared with other oocyte groups. Earlier finding of the present study demonstrated an increase in GSH levels in Group A oocyte during maturation. This indicated that although Group A oocytes (compact and dense cumulus

cells) may have increased GSH level during IVM, it was only enough to support early embryo development to 16-cell stage but not up to the blastocyst stage.

In addition, studies have reported that the *de novo* synthesis of GSH begins to increase at the 9-16 cell stage embryos (Van Langendonckt *et al.,* 1998). The findings of the present study that early bovine embryo development rates were higher in Group A oocytes indicated that the presence of compact and dense cumulus cells may have increased GSH synthesis during IVM, which reduced oxidative stress during early stage embryos, and hence increasing early developmental rates compared to oocytes with lesser cumulus cells. However, *de novo* synthesis of GSH that may have increase at the 9-16 cell stage embryos was insufficient to support subsequent development of the embryos up to blastocyst stage. An important factor needs to be considered is that oocytes are removed of the cumulus cells prior to *in vitro* fertilisation (IVF) (a normal procedure in IVF) which actually compromised fertilisation and embryo development (Zhang *et al.,* 1995; Fatehi *et al.,* 2002). Therefore, the results of the present study supported the report, suggesting that the oocytes maturing *in vitro* with less cumulus cells have low intracellular GSH, as found in Groups B and C in the present study. Thus, such oocytes were affected by reactive oxygen species (ROS), and hence lose developmental potential.

Low molecular weight thiol compounds such as BME have been shown to have promoting effects during *in vitro* embryo production by maintaining the redox state of cells and protect them against the harmful effects of oxidative injuries (Caamano *et al.,* 1996; 1998; Geshi *et al.,* 1999; Takahashi *et al.,* 2002; Mori *et al.,* 2006). Generally, the occurrences of early apoptosis and GSH levels were increased in Group A oocytes after maturation, regardless of whether BME was supplied or not. The difference in concentrations of BME also produced no significant difference. This indicated that BME did not promote GSH synthesis and exerted no effect in the rates of early

apoptosis in Group A oocytes. However, oocyte competency in terms of progression to metaphase II was only shown by Group A oocytes matured in IVM medium supplemented with 50 μM BME. In terms of embryo development, progression of Group A oocytes to blastocyst was not influenced by the addition of BME during IVM, but BME concentration of more than 100 μM were detrimental to embryo development. It is worthy to note that there may be a possible negative effect due to high levels of BME, whereby an excess of thiol or redactor compounds may be deleterious to subsequent embryo development and that the redox equilibrium is important for oocyte maturation, and altering the thiol-redox status induced cell cycle arrest or cell death (Liu & Yang, 1999; Guérin, *et al.,* 2001). Since the early apoptosis, GSH synthesis, oocyte competency to achieve nuclear maturation (except for 50 μM BME supplement) and subsequent embryo development were not affected by BME addition in IVM medium, therefore, the increase in GSH synthesis in Group A oocytes may be attributable to the surrounding cumulus cells supporting the importance of cumulus cells as a criterion of good quality oocytes.

Among inferior oocytes, i.e. Group B with compact but less dense cumulus cell layers, and Group C with thin or little remnants of cumulus cell layers, BME supplementation in the IVM medium was found to induce the synthesis of GSH and early apoptosis occurrence. However, BME addition in the IVM medium had no influence on the meiotic competence and embryo development of these bovine oocytes. This indicated that BME addition in IVM medium promoted the intracellular GSH levels and early apoptosis incidence of low quality bovine oocytes (Groups B and C) during IVM. Nevertheless, the increase in GSH content in these groups of oocytes was not shown to induce the meiotic competence and embryo development. Therefore, it could be suggested that although BME induced an increase in GSH synthesis, it may not be adequate to reduce oxidative stress displayed by the increase in early apoptosis, and

to promote nuclear maturation and embryo development in these oocytes. Accordingly, as reported in many previous studies, adequate glutathione (GSH) is necessary for viability in both oocytes and early embryos (Eppig, 1996; Knappen *et al.,* 1999; de Matos & Furnus, 2000; Fujii *et al.,* 2005).

In summary, based on the results of the present study, cumulus cells were shown to be an important criterion in the selection of good quality oocytes for IVEP. Next, the addition of 50 μM BME during *in vitro* maturation of bovine oocytes had significant effect on the good quality oocytes. Among low quality oocytes, BME supplement increased the early apoptosis incidences and GSH synthesis but exerted no effect to the maturation and embryo development.

5.3.1 Significant Findings

There were four significant findings achieved in this research which include:

a) The incidence of early apoptosis in Group A oocytes (compact and dense cumulus cell) before *in vitro* maturation was significantly lower compared to Groups B (compact and less dense cumulus cells) and C (thin or little remnants of cumulus cells).

b) The intracellular GSH concentration of Group A oocyte was significantly increased after 24-hour maturation culture.

c) BME supplement in IVM medium increased the early apoptosis rates in Group B and C oocytes after IVM.

d) BME concentration of 50 μM added into the IVM medium promoted nuclear maturation of Group A oocytes, suggesting BME supplement may support subsequent oocyte and embryo developmental competence.

5.3.2 Constraints of the Studies and Future Directions

The present study provided a baseline data or information on bovine *in vitro* embryo production research, as it is important to understand and look at how it could assist IVEP research, especially in Malaysia, where variable oocytes were obtained from donors with diverse backgrounds. Research in the west region often use good quality oocytes since they were easily available and abundant, therefore the rapid progress of bovine IVEP research could be established and efficient protocol could be developed. The diversity of cows might be attributable to different climates, i.e. cool and dry in the temperate region, whereas it was hot and humid in the tropical region such as Malaysia in the Southeast Asia region. Compared to developed temperate western region, the oocyte source of eastern tropical region was relatively scarce and of unknown history of livestock animals and the low-quality oocytes outnumbered the good quality ones. Hence, there was a need to salvage these low-quality oocytes in order to cater for bovine embryos from IVEP for bovine production and industry in the developing countries. Although the present study only provided a baseline information, it might possibly be used to help alleviate and increase the efficiency of IVEP research, and after further detailed research under the local settings, it is hoped that reproductive technologies could be an integral component of farm management practices in bovine industry for the livelihood and well-being of people in the developing countries.

The main constraints encountered and subsequent solutions carried out along the journey of the research are listed as follows:

a) Due to the shortage in the numbers of cows available for slaughtering, there was inconsistent numbers of oocyte yield per replicate since the breed, age, health, and physiological status of the cows could not be determined and standardised throughout the study. However, the present study was able to obtain the ovaries from various possible slaughterhouses in the nearby states such Shah Alam Abbatoir, Banting Abbatoir and Senawang Abbatoir. The oocytes retrieved from these ovaries obtained from these slaughterhouses were pooled for various experiments conducted in this research without jeopardizing the outcomes of the experimental results.

b) Longer time was taken to complete and meet the milestone of each experiment due to disruption of oocyte supply. Furthermore, the tedious and delicate protocols of IVEP needs laboratory skills and longer 'learning curve' particularly the current author did not have prior exposure to IVEP protocols. However, through perseverance, interest and patience, the author overcame these challenges and standardised the protocol for the various experiments in this research.

c) Another challenge was the logistics of this research, whereby the abbatoir, research laboratory in MARDI and University of Malaya were relatively far apart, thus affecting the time and space for the author to coordinate personally and to conduct the experiments efficiently. However, the author managed to design the experiments accordingly by taking considerations on this constraint and subsequently completed this research as scheduled.

d) In some cases, the equipment used were not operational, thus resulting in the delay of the scheduled experiments. After the repair, the experiments were continued even though the timing were a bit affected.

e) The delivery of chemicals was sometimes unexpectedly delayed, consequently affecting the scheduled experiments. However, the author adjusted the experimental schedules and subsequently carried out the experiments.

The findings of the present study formed a basis of understanding for more detailed studies in the future to improve *in vitro* bovine embryo production research, and hence lead to recommendation for the practical application of the reproductive technology in the farm management for the livestock industry. Although there are still scientific and technical loop holes in the present study, the findings could provide a window of opportunity to be extended for future studies in the area of bovine IVEP as suggested below:

a) *Optimisation of different regime for different oocyte groups*. Group A with compact and dense cumulus cells surrounding the oocytes seemed to be 'in its own league', for it displayed factors such as early apoptosis rates, GSH levels, oocyte competency in terms of maturation and embryo development which were contradictory to the other oocyte groups, i.e. Groups B and C. Further refined studies could determine how these different groups would individually behave in different types of medium and protocols of bovine IVEP.

b) *Optimisation of IVM duration for individual groups of oocytes*. More oocytes were observed to be in the meiosis stage prior to metaphase II in the present study. Individual oocyte groups' optimisation of the complete regime and

protocol may provide a more accurate IVEP procedure for the different oocyte groups in order to maximise the use of all oocytes retrieved.

c) *Determination of reactive oxygen species concentration such as* H_2O_2. The levels of ROS in oocytes could be assessed to be used as an indicator of oocyte quality. The correlation of extracellular anti-oxidant supplement and the levels of ROS in different stages of IVEP can be determined to support the importance of a particular anti-oxidant.

d) *Supplementation of BME in other steps of the IVEP procedure.* The lower quality oocytes may be salvaged through refined studies on a molecular basis by looking at the whole series of protocols from the slaughterhouse follows through the IVEP procedure. Addition of BME at different stages especially during IVC of the embryo and use of different medium would be useful in providing more data. The development before and after IVM, and during morula and blastocyst can be traced. This information can provide more information for a stable procedure to be recommended to be used in the application for the industry e.g. embryo transfer or other applications, such as embryonic stem cell in order to obtain good quality embryo. Next, the role of BME can be studied further on IVF embryo, ICSI embryo and cloned embryo to look at the possibilities of BME factor aid in the competency of lower quality oocytes for a sustainable development.

CHAPTER 6: CONCLUSION

Oocyte quality and the oocyte/embryo culture microenvironment were two of the many important factors in *in vitro* embryo production which were the considered factors in the present study. The compactness of the cumulus cells surrounding the oocytes were used to classify the bovine oocytes retrieved from the slaughterhouse namely, Group A oocytes with compact and dense cumulus cells (considered as 'good quality' oocytes); Group B with compact but less dense cumulus cells; and Group C with thin layers of cumulus cells. Improvement of culture microenvironment was achieved by supplementing the IVM medium with BME, a thiol compound known to enhance oocyte competency. In the present study, the effects of these two factors were evaluated by determining the i) early apoptosis rates; ii) intracellular GSH levels; iii) nuclear maturation rates; and iv) cleavage and subsequent embryo development rates after IVF. Based on the findings of the study, it could be concluded that:

6.1 EFFECTS OF CUMULUS CELLS ON OOCYTE AND EMBRYO DEVELOPMENTAL COMPETENCY (EXPERIMENT I)

a) Immature Group A oocytes (compact and dense cumulus cells) showed significantly low early apoptosis rates, contradictory to Groups B (compact but less dense cumulus cells) and C (thin or little remnant of cumulus cells) before IVM. Culture of oocytes during in vitro maturation caused a significant increase of early apoptosis occurrence in Group A oocytes but no effect to Groups B and C. Therefore, cumulus cells compactness affected early apoptosis rates of immature bovine oocytes, before and after in vitro maturation.

b) The intracellular GSH concentrations were increased significantly in Group A oocytes compared with Groups B and C which maintained the concentrations,

after *in vitro* maturation. Therefore, intracellular GSH was synthesised during maturation only in good quality bovine oocytes (Group A) having compact and dense cumulus cells.

c) The compactness of cumulus cell layers exerted no effect on the bovine oocyte maturation rate after *in vitro* maturation (IVM) since no significant difference was observed in the maturation rates of Groups A, B and C.

d) Good quality bovine oocytes, i.e. Group A displayed significantly high rates of cleavage and embryo development to 16-cell compared with Groups B and C. Therefore, compactness of cumulus cells played an important role in promoting cleavage and subsequent early embryo development after *in vitro* fertilisation.

6.2. EFFECTS OF B-MERCAPTOETHANOL (BME) SUPPLEMENT DURING IVM ON OOCYTE AND EMBRYO DEVELOPMENTAL COMPETENCY (EXPERIMENT II)

a) The addition of BME in IVM medium had no effect on the early apoptosis rates in Group A oocytes. However, the early apoptosis rates in Group B and C were increased when BME was added to IVM medium.

b) Groups B and C intracellular GSH levels were significantly increased when BME was added in IVM medium, but not in Group A oocytes. No significant difference in intracellular GSH levels was found between the different concentrations of BME supplement in all oocyte groups studied.

c) The addition of BME in IVM medium at concentration 50 μM increased Group A maturation rate after *in vitro* maturation.

d) The addition of BME in IVM medium at concentration of more than 100 μM posed a detrimental effect on cleavage and subsequent embryo development to 4-cell stage, without affecting later stages of embryo development in Group A oocytes after *in vitro* fertilisation.

In a nutshell, BME addition into the IVM medium did not exert any effect on the above factors related to oocyte and embryo developmental competency of good quality oocytes, except for nuclear maturation (metaphase II) of Group A which was induced by addition of 50 μM BME supplement in the IVM medium. It is worthy to note that BME increased the GSH synthesis and early apoptosis in Groups B and C oocytes. As suggested earlier, based on the findings of the present study, whereby Group A oocytes, considered as good quality oocytes, having a compact and dense cumulus cells displayed a particular characteristic compared to Groups B and C. In conclusion, specific *in vitro* embryo production protocols and procedures should be established for the different oocyte groups, clarifying the understanding of the molecular basis and mechanism of oocyte maturation and embryo development *in vivo* and *in vitro*.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications:

1. **Nor Azlina Abd Aziz**, Nurul Atikah Osman, Habsah Bidin, Wan Khadijah Embong & Noor Hashida Hashim. (2014). Influence of early apoptosis incidence on *in vitro* maturation of bovine oocyte. *APCBEE Procedia 8*, 272-276.

Papers presented:

1. Habsah, B. Nurul Atikah, O. **Nor Azlina, A. A**., & Musaddin, K. (2012). Effects of calcium ionophore A23187 on oocyte activation in the production of bovine embryos using ICSI procedure. *Proceedings of the 33rd Malaysian Society of Animal Production Annual Conference, Langkawi, Malaysia* (pp. 148-149). Langkawi, Malaysia.

2. Habsah, B., **Nor Azlina, A. A**., & Musaddin, K. (2010). Enhancing *in vitro* bovine embryo development success rate through cysteamine and β-mercaptoethanol supplementation. *Proceedings of the 7th Annual Conference on Asian Reproductive Biotechnology Society, Kuala Lumpur, Malaysia* (pp. 76). Kuala Lumpur, Malaysia.

3. Habsah, B., Wan Somarny, W. M. Z., **Nor Azlina, A. A**., & Musaddin, K. (2010). Timed cleavage of bovine zygote determines sex of embryo. *Proceedings of the 31st Malaysian Society of Animal Production Annual Conference, Kota Bharu, Kelantan, Malaysia* (pp. 148-149). Kota Bharu, Kelantan, Malaysia.

4. **Nor Azlina, A.A**., Habsah, B., & Musaddin, K. (2009). Effects of β-mercaptoethanol on the maturation rate of bovine oocytes. *Proceedings of the 2nd International Conference on Sustainable Animal Agriculture for Developing Countries, Kuala Lumpur, Malaysia* (pp. 221-222). Kuala Lumpur, Malaysia.

5. Habsah, B., **Nor Azlina, A. A.**, Maisarah, T. A., & Musaddin, K. (2009). Effects of β-mercaptoethanol on the subsequent development of cattle oocytes under *in vitro* conditions. *Proceedings of the 30th Malaysian Society of Animal Production Annual Conference, Kota Kinabalu, Sabah, Malaysia* (pp. 142-143). Kota Kinabalu, Sabah, Malaysia.

6. Habsah, B., Intan Suliana, S., **Nor Azlina, A. A**., Maisarah, T. A., Musaddin, K., & Mohd Rafi, I. (2008). Effects of cysteamine on the maturation rate of cattle oocytes. *Proceedings of the 29th Malaysian Society of Animal Production Annual Conference, Penang, Malaysia* (pp. 76-77). Penang, Malaysia.

APPENDIX A

List of Equipment/instrument

Equipment/Instrument	Manufacturer
Autoclave	Hirayama Hiclave, Japan
Centrifuge	Medilite Thermo IEC, USA
CO2 Incubator	GmbH, Tuttlingen, USA
Digital balance	Mettler, Toledo, Switzerland
Dissecting microscope	Olympus, Japan
Fluorescent microscope	Carl Zeiss, Inc., Germany
Inverted microscope	Olympus, Japan
Laminar flow chamber	Gelman Sciences, Australia
Liquid nitrogen tanks	MVE, USA
Micropipette dispenser	Appendorf, Humburg
Microplate reader	Fisher Schientific, USA
Oven	Memmert GmbH, Germany
pH meter	Hanna Instruments, Singapore
Stage warmer	Tokai Hit , Japan Linkam Scientific, UK
Stereomicroscope	Olympus, Japan
Ultrapure water purification system (Milli-Q PF Plus)	Millipore Corporation, USA
Vortex Mixer	LMS Co ltd., Tokyo, Japan
Water bath	Memmert, Germany

APPENDIX B

List of labware and disposables

Labware and Disposables	Manufacturer
Aluminium foil	Richmond Consumer Products, Richmond, Virginia, USA
Autoclavable disposable bag	Megalab Supplies, Malaysia
Beakers	Iwaki, Pyrex, India
Conical centrifuge tubes	Becon Dcikinson Labware, New Jersey, USA
Conical flasks	Boeco, Germany
Cover slips	Hirschmann® Laborgerete, Germany
Culture Petri dishes	Nunc, Roskilde, Denmark
Disposable glass Pasteur pipettes	John Poulten Ltd., Essex, England
Disposable gloves	Protex, Malaysia
Disposable hand tissues	Scott, Kimberly-Clark, Malaysia
Disposable plastic pipettes	LP Italiana, Milano, Italy
Falcon® polystyrene round-bottom test-tube, snap cap	Becton, Dickinson, New Jersey, USA
Glass bottle	Schott Duran, UK
Lens tissues	Kimwipes, Kimberly-Clark, Canada
Measuring cylinders	Iwaki, Pyrex, India
Microcentrifuge tubes	Elkay, Costelloe
Micropipette	Wiretrol II, Drummond Scientific Co. Broomall, Pennsylvania, USA
Micropipette tips (white, yellow, blue)	Axygen Scientific, USA
Microscope slides	Shanghai, China
	Corning, New York, USA
Millipore^{TM} membrane filters	Merck, Germany
Multi-well plates	Nuclon, Denmark

Labware and Disposables	Manufacturer
Parafilm	Pechiney, Plastic Packaging,
Scalpel (Size 4)	Benz Stainless
Serological pipettes	Beckon Dickinson, New Jersey, USA
Surgical blades (Size 21)	Benz Stainless
Syringes	Terumo, Japan
Syringe needles	Terumo, Japan

APPENDIX C

List of Chemicals and Reagents

Chemicals and Reagents	Catalogue no.	Manufacturer
70% ethanol	-	Prepared from absolute ethanol
7X®-PF nontoxic detergent	-	FlowLab™, Sydney, Australia
Absolute ethanol (C_2H_2OH)	1322219	HmbG Chemicals, Germany
Absolute methanol (CH_3OH)	-	RandM Chemicals, Essex, UK
Basal Media Eagle (BME) amino acid solution (50x)	B6766	Sigma-Aldrich Co. St Louis, USA
Bovine serum albumin, BSA	A-9418	Sigma-Aldrich Co. St Louis, USA
Calcium chloride dehydrate ($CaCI_2.2H_2O$)	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, U=England
Dulbecco`s phosphate buffered saline (DPBS, Dulbecco tablet A tablet)	BR0014G	Oxoid, UK
Folligon (PMSG, 1000 IU)	-	Intervet International, Holland

Chemicals and Reagents	Catalogue no.	Manufacturer
Gentamicin sulphate	G1264	Sigma-Aldrich Co. St Louis, USA
Glycerol, anhydrous ($CH_3H_4O_2$)	0411-00	R.A.M Chemicals, Essex, UK
Giemsa stain	GS500	Sigma-Aldrich Co. St Louis, USA
Heparin, sodium salt	H0777	Sigma-Aldrich Co. St Louis, USA
HEPES sodium salt	H7006	Sigma-Aldrich Co. St Louis, USA
Hepes-buffered medium 199 (TCM199)	M7528	Sigma-Aldrich Co. St Louis, USA
Hoechat 33342	B2261	Sigma-Aldrich Co. St Louis, USA
Hyaluronidase (Type IV-S from bovine testers)	H4272	Sigma-Aldrich Co. St Louis, USA
Hydrochloric acid (HCI)	-	HmbG Chemicals, Germany
Hydrocfluoric acid (HFI)	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 nitrogern	-	MOX Gases Bhd, PJ, Selangor, Malaysia
Magnesium chloride hexahydrate ($MgCI_2.6H_2O$)	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

Chemicals and Reagents	Catalogue no.	Manufacturer
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 (KCI)	P-5404	Sigma-Aldrich Co. St Louis, USA
Potassium 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 ($C_3H_3O_3Na$)	P3622	Sigma-Aldrich Co. St Louis, USA
Silicone oil	S-5761	Sigma-Aldrich Co. St Louis, USA
Sodium bicarbonate ($NaHCO_3$)	S-5886	Sigma-Aldrich Co. St Louis, USA
Sodium chloride (NaCI)	L4263	Sigma-Aldrich Co. St Louis, USA
Sodium phosphate monobasic ($NaH_2PO_4.H_2O$)	S5011	Sigma-Aldrich Co. St Louis, USA
Steer serum (SS, heat inactivated)	-	Prepared `in house'

Chemicals and Reagents	Catalogue no.	Manufacturer
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 Chemic GmbH, Germany

APPENDIX D

ANOVA Tables

ANOVA

Early apoptosis

Grade		Sum of Squares	df	Mean Square	F	Sig.
A	Between Groups	.743	5	.149	7.705	.000
	Within Groups	.444	23	.019		
	Total	1.187	28			
B	Between Groups	.504	5	.101	5.462	.002
	Within Groups	.424	23	.018		
	Total	.928	28			
C	Between Groups	.238	5	.048	4.283	.008
	Within Groups	.222	20	.011		
	Total	.460	25			

ANOVA

Early apoptosis

Concentration		Sum of Squares	df	Mean Square	F	Sig.
Immature	Between Groups	.097	2	.048	4.166	.040
	Within Groups	.151	13	.012		
	Total	.248	15			
Control	Between Groups	.038	2	.019	2.539	.124
	Within Groups	.082	11	.007		
	Total	.120	13			
50	Between Groups	.008	2	.004	.211	.813
	Within Groups	.216	11	.020		
	Total	.224	13			
100	Between Groups	.019	2	.010	.641	.545
	Within Groups	.165	11	.015		
	Total	.185	13			
150	Between Groups	.020	2	.010	.502	.620
	Within Groups	.201	10	.020		
	Total	.221	12			
200	Between Groups	.023	2	.012	.418	.669
	Within Groups	.275	10	.028		
	Total	.298	12			

ANOVA

GSH

Grade		Sum of Squares	df	Mean Square	F	Sig.
A	Between Groups	10.058	5	2.012	9.038	.000
	Within Groups	4.006	18	.223		
	Total	14.064	23			
B	Between Groups	2.701	5	.540	5.161	.004
	Within Groups	1.884	18	.105		
	Total	4.585	23			
C	Between Groups	5.123	5	1.025	13.543	.000
	Within Groups	1.362	18	.076		
	Total	6.484	23			

ANOVA

GSH

Conc		Sum of Squares	df	Mean Square	F	Sig.
immature	Between Groups	.202	2	.101	.498	.623
	Within Groups	1.822	9	.202		
	Total	2.024	11			
control	Between Groups	1.441	2	.720	6.391	.019
	Within Groups	1.014	9	.113		
	Total	2.455	11			
50	Between Groups	.583	2	.291	1.612	.252
	Within Groups	1.626	9	.181		
	Total	2.209	11			
100	Between Groups	1.207	2	.604	6.997	.015
	Within Groups	.776	9	.086		
	Total	1.984	11			
150	Between Groups	1.145	2	.572	5.464	.028
	Within Groups	.943	9	.105		
	Total	2.087	11			
200	Between Groups	2.158	2	1.079	9.067	.007
	Within Groups	1.071	9	.119		
	Total	3.228	11			

ANOVA

Meiotic resumption

Grade			Sum of Squares	df	Mean Square	F	Sig.
A	ZP	Between Groups	.128	4	.032	1.091	.384
		Within Groups	.676	23	.029		
		Total	.804	27			
	MI	Between Groups	.044	4	.011	.959	.448
		Within Groups	.263	23	.011		
		Total	.307	27			
	MII	Between Groups	.109	4	.027	3.671	.019
		Within Groups	.171	23	.007		
		Total	.281	27			
	UNID	Between Groups	.026	4	.006	.205	.933
		Within Groups	.729	23	.032		
		Total	.755	27			
B	ZP	Between Groups	.131	4	.033	.774	.552
		Within Groups	1.102	26	.042		
		Total	1.233	30			
	MI	Between Groups	.149	4	.037	1.182	.342
		Within Groups	.818	26	.031		
		Total	.967	30			
	MII	Between Groups	.046	4	.011	.969	.441
		Within Groups	.307	26	.012		
		Total	.353	30			
	UNID	Between Groups	.109	4	.027	1.017	.417
		Within Groups	.696	26	.027		
		Total	.804	30			
C	ZP	Between Groups	.136	4	.034	1.502	.227
		Within Groups	.655	29	.023		
		Total	.790	33			

	MI	Between Groups	.011	4	.003	.395	.810
		Within Groups	.197	29	.007		
		Total	.208	33			
	MII	Between Groups	.053	4	.013	1.259	.309
		Within Groups	.307	29	.011		
		Total	.360	33			
	UNID	Between Groups	.027	4	.007	.178	.948
		Within Groups	1.118	29	.039		
		Total	1.146	33			

ANOVA

Meiotic resumption

Concentration			Sum of Squares	df	Mean Square	F	Sig.
Control	ZP	Between Groups	.005	2	.003	1.072	.369
		Within Groups	.033	14	.002		
		Total	.038	16			
	MI	Between Groups	.009	2	.004	1.280	.309
		Within Groups	.048	14	.003		
		Total	.057	16			
	MII	Between Groups	.007	2	.004	.858	.445
		Within Groups	.061	14	.004		
		Total	.069	16			
	UNID	Between Groups	.070	2	.035	1.441	.270
		Within Groups	.341	14	.024		
		Total	.411	16			
50	ZP	Between Groups	.124	2	.062	1.572	.240
		Within Groups	.592	15	.039		
		Total	.716	17			
	MI	Between Groups	.075	2	.038	.849	.447
		Within Groups	.666	15	.044		
		Total	.741	17			
	MII	Between Groups	.224	2	.112	6.367	.010
		Within Groups	.264	15	.018		
		Total	.488	17			
	UNID	Between Groups	.109	2	.054	1.278	.307
		Within Groups	.639	15	.043		
		Total	.748	17			
100	ZP	Between Groups	.006	2	.003	.058	.944
		Within Groups	.727	15	.048		
		Total	.733	17			
	MI	Between Groups	.000	2	.000	.006	.994
		Within Groups	.244	15	.016		
		Total	.244	17			
	MII	Between Groups	.024	2	.012	1.319	.297
		Within Groups	.136	15	.009		
		Total	.160	17			
	UNID	Between Groups	.038	2	.019	.572	.576
		Within Groups	.492	15	.033		
		Total	.530	17			
150	ZP	Between Groups	.006	2	.003	.165	.850
		Within Groups	.324	17	.019		
		Total	.330	19			
	MI	Between Groups	.006	2	.003	.600	.560

		Within Groups	.080	17	.005		
		Total	.086	19			
	MII	Between Groups	.009	2	.004	.365	.699
		Within Groups	.202	17	.012		
		Total	.211	19			
	UNID	Between Groups	.003	2	.002	.051	.950
		Within Groups	.539	17	.032		
		Total	.542	19			
200	ZP	Between Groups	.036	2	.018	.409	.671
		Within Groups	.757	17	.045		
		Total	.793	19			
	MI	Between Groups	.001	2	.000	.023	.977
		Within Groups	.241	17	.014		
		Total	.242	19			
	MII	Between Groups	.006	2	.003	.388	.684
		Within Groups	.122	17	.007		
		Total	.128	19			
	UNID	Between Groups	.032	2	.016	.511	.609
		Within Groups	.532	17	.031		
		Total	.564	19			

ANOVA

Embryo development

Group			Sum of Squares	df	Mean Square	F	Sig.
A	2-cell	Between Groups	.298	4	.075	4.943	.007
		Within Groups	.287	19	.015		
		Total	.585	23			
	4-cell	Between Groups	.123	4	.031	1.746	.182
		Within Groups	.336	19	.018		
		Total	.459	23			
	8-cell	Between Groups	.022	4	.006	.764	.562
		Within Groups	.139	19	.007		
		Total	.161	23			
	16-cell	Between Groups	.077	4	.019	4.052	.015
		Within Groups	.090	19	.005		
		Total	.168	23			
	morula	Between Groups	.033	4	.008	1.695	.193
		Within Groups	.092	19	.005		
		Total	.125	23			
	cmorula	Between Groups	.014	4	.003	.512	.727
		Within Groups	.128	19	.007		
		Total	.141	23			
	blastocyst	Between Groups	.013	4	.003	.799	.540
		Within Groups	.076	19	.004		
		Total	.088	23			
B	2-cell	Between Groups	.206	4	.052	2.612	.068
		Within Groups	.375	19	.020		
		Total	.581	23			
	4-cell	Between Groups	.018	4	.005	.301	.874
		Within Groups	.285	19	.015		
		Total	.303	23			
	8-cell	Between Groups	.061	4	.015	1.283	.312
		Within Groups	.227	19	.012		

		Total	.288	23			
	16-cell	Between Groups	.097	4	.024	3.112	.040
		Within Groups	.149	19	.008		
		Total	.246	23			
	morula	Between Groups	.054	4	.013	1.920	.148
		Within Groups	.132	19	.007		
		Total	.186	23			
	cmorula	Between Groups	.022	4	.006	1.196	.345
		Within Groups	.089	19	.005		
		Total	.111	23			
	blastocyst	Between Groups	.073	4	.018	4.070	.015
		Within Groups	.085	19	.004		
		Total	.158	23			
C	2-cell	Between Groups	.323	4	.081	7.922	.001
		Within Groups	.173	17	.010		
		Total	.496	21			
	4-cell	Between Groups	.082	4	.020	3.408	.032
		Within Groups	.102	17	.006		
		Total	.183	21			
	8-cell	Between Groups	.106	4	.026	2.609	.072
		Within Groups	.172	17	.010		
		Total	.278	21			
	16-cell	Between Groups	.160	4	.040	5.903	.004
		Within Groups	.115	17	.007		
		Total	.275	21			
	morula	Between Groups	.141	4	.035	16.029	.000
		Within Groups	.037	17	.002		
		Total	.178	21			
	cmorula	Between Groups	.048	4	.012	12.166	.000
		Within Groups	.017	17	.001		
		Total	.064	21			
	blastocyst	Between Groups	.056	4	.014	3.330	.035
		Within Groups	.072	17	.004		
		Total	.128	21			

ANOVA

Embryo development

Concentration			Sum of Squares	df	Mean Square	F	Sig.
Control	2-cell	Between Groups	.045	2	.023	2.733	.109
		Within Groups	.091	11	.008		
		Total	.136	13			
	4-cell	Between Groups	.080	2	.040	4.435	.039
		Within Groups	.100	11	.009		
		Total	.180	13			
	8-cell	Between Groups	.064	2	.032	2.867	.100
		Within Groups	.123	11	.011		
		Total	.188	13			
	16-cell	Between Groups	.083	2	.042	4.327	.041
		Within Groups	.106	11	.010		
		Total	.189	13			
	morula	Between Groups	.015	2	.008	1.359	.297
		Within Groups	.061	11	.006		

		Total	.076	13			
	cmorula	Between Groups	.023	2	.011	2.783	.105
		Within Groups	.045	11	.004		
		Total	.068	13			
	blastocyst	Between Groups	.017	2	.008	2.252	.151
		Within Groups	.041	11	.004		
		Total	.058	13			
50	2-cell	Between Groups	.225	2	.112	4.281	.040
		Within Groups	.315	12	.026		
		Total	.540	14			
	4-cell	Between Groups	.099	2	.049	3.007	.087
		Within Groups	.197	12	.016		
		Total	.296	14			
	8-cell	Between Groups	.072	2	.036	2.489	.125
		Within Groups	.173	12	.014		
		Total	.245	14			
	16-cell	Between Groups	.129	2	.065	10.514	.002
		Within Groups	.074	12	.006		
		Total	.203	14			
	morula	Between Groups	.048	2	.024	2.725	.106
		Within Groups	.105	12	.009		
		Total	.153	14			
	cmorula	Between Groups	.031	2	.016	2.948	.091
		Within Groups	.064	12	.005		
		Total	.095	14			
	blastocyst	Between Groups	.004	2	.002	.597	.566
		Within Groups	.039	12	.003		
		Total	.043	14			
100	2-cell	Between Groups	.161	2	.081	9.102	.005
		Within Groups	.098	11	.009		
		Total	.259	13			
	4-cell	Between Groups	.265	2	.133	12.454	.001
		Within Groups	.117	11	.011		
		Total	.383	13			
	8-cell	Between Groups	.184	2	.092	18.060	.000
		Within Groups	.056	11	.005		
		Total	.240	13			
	16-cell	Between Groups	.158	2	.079	8.851	.005
		Within Groups	.098	11	.009		
		Total	.256	13			
	morula	Between Groups	.070	2	.035	11.500	.002
		Within Groups	.034	11	.003		
		Total	.104	13			
	cmorula	Between Groups	.045	2	.022	6.526	.014
		Within Groups	.038	11	.003		
		Total	.082	13			
	blastocyst	Between Groups	.036	2	.018	2.142	.164
		Within Groups	.093	11	.008		
		Total	.129	13			
150	2-cell	Between Groups	.051	2	.025	1.865	.205
		Within Groups	.136	10	.014		
		Total	.187	12			
	4-cell	Between Groups	.053	2	.027	1.433	.284
		Within Groups	.185	10	.019		
		Total	.238	12			
	8-cell	Between Groups	.115	2	.058	7.192	.012
		Within Groups	.080	10	.008		

		Total	.195	12			
	16-cell	Between Groups	.043	2	.021	6.868	.013
		Within Groups	.031	10	.003		
		Total	.074	12			
	morula	Between Groups	.023	2	.011	2.654	.119
		Within Groups	.043	10	.004		
		Total	.065	12			
	cmorula	Between Groups	.028	2	.014	3.376	.076
		Within Groups	.041	10	.004		
		Total	.069	12			
	blastocyst	Between Groups	.050	2	.025	9.494	.005
		Within Groups	.026	10	.003		
		Total	.076	12			
200	2-cell	Between Groups	.258	2	.129	7.252	.010
		Within Groups	.196	11	.018		
		Total	.453	13			
	4-cell	Between Groups	.131	2	.065	5.837	.019
		Within Groups	.123	11	.011		
		Total	.254	13			
	8-cell	Between Groups	.279	2	.140	14.681	.001
		Within Groups	.105	11	.010		
		Total	.384	13			
	16-cell	Between Groups	.170	2	.085	20.619	.000
		Within Groups	.045	11	.004		
		Total	.216	13			
	morula	Between Groups	.103	2	.052	29.329	.000
		Within Groups	.019	11	.002		
		Total	.123	13			
	cmorula	Between Groups	.068	2	.034	8.180	.007
		Within Groups	.046	11	.004		
		Total	.114	13			
	blastocyst	Between Groups	.046	2	.023	7.530	.009
		Within Groups	.034	11	.003		
		Total	.080	13			