Chapter 5

5.0 DISCUSSION

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5.1 EFFECT OF OOCYTE GRADING AND INSEMINATION DURATION ON CLEAVAGE RATE OF EMBRYOS OBTAINED FROM *IN VITRO* FERTILISATION IN BOVINE (EXPERIMENT 1)

The percent distribution of bovine oocytes obtained in this experiment were 49.09, 33.75 and 17.06%, for Grades A, B and C, respectively. Habsah (2006) acquired 31.28, 39.81 and 28.91% for Grades A, B and B'. The oocyte grading of the present study was the newly improved oocyte classification in ABEL laboratory (modified from Rahman, 2008) compared to Habsah (2006) as the former gave clearer, detailed and refined method of oocyte classification. The grades of oocyte in the present study were apparently developmentally competent as shown in the maturation, fertilisation and cleavage rates of this experiment.

In this experiment, the maturation rates generally decreased as the grades of the oocytes declined. Due to the grading of oocytes was based on cumulus cell layers and cytoplasm uniformity, it was suggested that the cumulus cell layers played an important role in oocyte maturation and acquisition of developmental competence in oocytes (Shioya *et al*., 1988; Younis *et al*., 1989, 1991a; Mochizuki *et al*., 1991; Zhang *et al*., 1995). By referring to Mingoti *et al.* (2002), cumulus cells of cumulus-enclosed oocytes secreted progesterone in culture systems during *in vitro* maturation. Removal of cumulus cells from the oocytes prior to *in vitro* maturation adversely affects the maturation, fertilisation and embryo development in bovine (Leibfried-Rutldge *et al*., 1989; Zhang *et al*., 1995). With regard to insemination durations used in this experiment, there was no significant difference in maturation rate between 8 to 14 hours and 18 to

24 hours. Therefore, it is suggested that the insemination duration for bovine *in vitro* fertilisation could be within 8 to 24 hours.

According to Li *et al.* (2009), cumulus oocyte complexes were also divided into 4 groups according to the appearance of their cumulus and cytoplasm: GI cumulus oocyte complexes, with granular cytoplasm and more than 5 layers of cumulus cells; GII cumulus oocyte complexes, with homogeneous cytoplasm and more than 5 layers of cumulus cells; GIII cumulus oocyte complexes, with granular cytoplasm and between 3 and 5 layers of cumulus cells; and GIV cumulus oocyte complexes, with homogeneous cytoplasm and 3 to 5 layers of cumulus cells.

Bilodeau-Goeseels and Panich (2002) divided the cumulus oocyte complexes (COCs) into 6 classes based on their cumulus investment and on the texture of the ooplasm. Embryos originating from oocytes of class I which showed no signs of atresia and originating from oocytes with more than 5 layers of cumulus cells with slight expansion of the cumulus and/or granulation in the ooplasm (class II) developed to the blastocyst stage with the rates of 13.90 and 13.70%, respectively. Oocytes with fewer than 5 layers of cumulus cells and homogeneous ooplasm (class III) had lower cleavage (63.10%) than oocytes with more than 5 layers of cumulus cells (77.20 and 83.60% for classes I and II, respectively); however, it was noted that the development to the blastocyst stage was similar (12.70%). More advanced atresia such as the presence of granulations in oocytes with less than 5 layers of cumulus cells (class IV), the absence of cumulus (class V), or the presence of expanded cumulus with dark clumps (class VI) reduced cleavage (57.40, 35.90 and 56.30% for classes IV-VI, respectively) and blastocyst formation (3.50, 0.50 and 1.90% for classes IV-VI, respectively).

Habsah *et al.* (2005) indicated that the resumption of the meiotic process in bovine oocytes is influenced by the characteristics and quality of cumulus oocytes complexes. Bovine oocytes invested with compact cumulus cells had greater success of

reaching maturation than those with loose or no cumulus cells (Xu *et al*., 1986; Suss *et al*., 1988). Dalhausen *et al*. (1981) found that oocytes devoid of cumulus cells investment did not mature *in vitro*. Hence, the selection criteria for complexes with compact cumulus cells are sufficient in indicating the ability to complete the first meiotic division *in vitro*. Dekel *et al.* (1984) showed that the nutrients required for maturating an oocyte were transported into the ooplasm through the cumulus cells. It has also been shown that gap junctions present between the oocyte and cumulus cells allowed the reciprocal metabolic cooperation (Gilula *et al*., 1978; Mori *et al*., 2000). This coupling played an important role in the control of resumption of meiosis by the oocyte (Eppig *et al*., 1983).

The maturation rate of Grade A oocytes obtained in this experiment was 49.09% which was lower compared to Li *et al.* (2009) who obtained 71.20% of maturation rate with Grade I oocytes. This phenomenon may be due to the different categorisation method in oocytes used in this experiment and Li *et al.* (2009). Besides, the low percentage of mean maturation rate (52.88%) in this experiment may be due to the reason that oocytes used in this experiment were mostly poor quality as in Malaysia, the sources of ovaries are limited and subsequently the number of oocytes is limited. Thus, slaughterhouse ovaries are used to salvage available oocytes for *in vitro* embryo production. Most of the female cows slaughtered in Malaysia are quite old and this contributed to the poor quality oocytes.

The average of combined fertilisation rate (50.00%) of competent oocytes in the present experiment was slightly lower than those reported by Kafi *et al.* (2002), Way and Killian (2006) as well as Xiang *et al.* (2008) who obtained 73.30, 75.11 and 81.60%, respectively. There were differences in the fertilisation percentages and this may be due to all grades of oocytes were used in this experiment; however, only good oocytes were used in other researches. The fertilisation rates were significantly highest for Grades A

and mixed grade compared to Grade C which were in agreement with some other research groups showing that the *in vitro* fertilisation in the presence of cumulus cells increased the fertilisation rate (Bavister, 1982; Siddiquey and Cohen, 1982; Fraser, 1985; Itagaki and Toyoda, 1991; Kikuchi *et al*., 1993; Nandi *et al*., 1998; Kafi *et al*., 2002; Way and Killian, 2006; Xiang *et al*., 2008). Cumulus cells are involved in the interaction between male and female gametes, guiding sperm towards the oocytes (Schroeder and Eppig, 1984; Hunter, 1988), inducing capacitation (Crozet, 1984; Cox *et al*., 1993), acrosome reaction (Fukui, 1990; Chian *et al*., 1995), maintaining sperm motility and viability (Fukui, 1990; Ijaz *et al.*, 1994), preventing zona pellucida from precocious hardening (Downs *et al*., 1986; Mattioli *et al*., 1988; Katska *et al*., 1989) and enhancing sperm penetration and *in vitro* fertilisation (Fukui, 1990; Magier *et al*., 1990; Cox *et al*., 1993; Legendre and Stewart-Savage, 1993). Besides, Tanghe *et al*. (2003) has provided evidences which were: (i) the fertilising ability of penetrating sperm is facilitated by cumulus cells by producing a complex mixture of cumulus secretions since both cumulus conditioned medium and a cumulus monolayer partially restored the decrease in fertilisation rate of cumulus-denuded oocytes, (ii) direct, gap junctional communication between the oocyte and corona cells is needed to optimally support fertilisation, (c) the cumulus oophorus plays a role as sperm trap, (d) sperm upon interaction with the cumulus oophorus encounter a higher redox state stimulating sperm penetration and (e) atmospheric oxygen concentrations are needed for the cumulus cells and/or fertilising sperm to create this redox environment. However, Ball *et al.* (1983), Cox (1991) as well as Behalova and Greve (1993) showed that the presence of cumulus cells surrounding the oocytes is not necessary for successful *in vitro* fertilisation in cattle as they obtained almost equal rates of sperm penetration during *in vitro* fertilisation of cumulus-free and cumulus-enclosed oocytes; whereas Hawk *et al*. (1992) observed a negative effect of cumulus cells on sperm penetration. On the other hand,

Younis and Brackett (1991b), Cox *et al*. (1993), Zhang *et al*. (1995) and Tanghe *et al*. (2003) reported a positive effect of cumulus cells on the *in vitro* fertilisation of bovine oocytes.

Previous reports showing that the presence of cumulus cells facilitates fertilisation of bovine oocytes leading to a higher penetration rate (Cox *et al*., 1993; Chian *et al*., 1995, 1996), higher cleavage rate (Fukui, 1990; Younis and Brackett, 1991b; Liu *et al*., 1995; Zhang *et al*., 1995) and a higher yield of blastocysts (Liu *et al*., 1995; Zhang *et al*., 1995). However, in the present experiment, the cleavage rates for all embryo developmental stages were significantly higher in mixed grade, except morula. The number of morula obtained from each grades was low and thus may be the reason of the insignificant difference. The higher number of oocytes in mixed grade oocytes may contribute the significant difference as it stands a higher chance in getting the cleavage with sufficient number of oocytes. Nevertheless, in other studies, it was found that removal of cumulus cells did not affect the penetration rate (Ball *et al*., 1983; Cox, 1991; Behalova and Greve, 1993) but impair the male and female pronucleus formation (Ball *et al*., 1983) and cause higher polyspermy incidence (Behalova and Greve, 1993). Cox *et al*. (1993) reported that the positive effect of cumulus cells on fertilisation is expressed only when the cells are in contact with the zona pellucida. In contrast, Fatehi *et al*. (2002) found that the presence of loose cumulus cells partially restored the effect of denudation prior to *in vitro* fertilisation and this suggests that both the presence of cumulus cells and cell-oocyte contact contribute to a proper fertilisation process. The partial restoration of the fertilisation rate might be due to the fact that the mechanical procedure of cumulus cell removal has harmed part of the oocytes which results in the lower male pronuclear formation (Ball *et al*., 1983).

Oxygen may contribute to the difference in maturation, fertilisation and cleavage rates between present study and other research groups since oxygen is directly involved

in the generation of reactive oxygen species (ROS) (Tanghe *et al*., 2003). The carbon dioxide incubator system used in this experiment was lacking in oxygen control. There were several research groups have studied the importance of oxygen tension during *in vitro* maturation (Pinyopummintr and Bavister, 1995; Hashimoto *et al*., 2000) and embryo development in cattle (Tervit *et al*., 1972; Thompson *et al*., 1990; Fukui *et al*., 1991; Khurana and Niemann, 2000). Takashi and Kanagawa (1998) suggested that bovine oocytes are fertilised at the same rate under 5 and 20% oxygen, however, according to Tanghe *et al*. (2003), it was clearly indicates that lowering the external oxygen concentration decreases the fertilisation rate. It was believed that cumulus cells and/or fertilising sperm need atmospheric oxygen concentrations to generate sufficient reactive oxygen species in the vicinity of the oocyte and to maximally support fertilisation. This was in agreement with Pinyopummintr and Bavister (1995) who showed low oxygen is detrimental for *in vitro* fertilisation of bovine cumulus-enclosed oocytes.

By using various high ionic strength (HIS) fertilisation media such as Brackett and Oliphant's medium (BO) and modified Tyrode's medium (MTM), a short incubation period (6 hours) of bovine oocytes with frozen-thawed sperm were usually applied (Brackett and Oliphant, 1975; Fukuda *et al*., 1990; Brackett and Zuelke, 1993). Brakett and Oliphant (1975) recommended removing oocytes as soon as possible from suspensions of dead or dying sperm in Brackett and Oliphant's medium. Extending *in vitro* fertilisation periods produces high levels of reactive oxygen species (ROS), generated by dying sperm in fertilisation drops, would result in zona hardening and subsequently affect an embryo's developmental viability (Kattera and Chen, 2003). Thus, the media currently utilised, for both the washing of frozen-thawed sperm for *in vitro* fertilisation is yet not entirely satisfactory, although much improvement has been achieved in recent years. *In vitro* fertilisation medium used play a crucial role in

separating frozen-thawed motile sperm from their cryoprotectants and providing conditions meeting the appropriate physiological requirements for metabolic activities of both sperm and oocyte during *in vitro* fertilisation incubation. Simple media with high ionic strength, such as Brackett and Oliphant's medium (Brackett and Oliphant, 1975), has been widely utilised for both washing frozen-thawed semen, and subsequent *in vitro* fertilisation. According to Fukuda *et al.* (1990), there were only a few components contained in this medium and oocyte aging may be accelerated which can be a factor in causing the fertilisation by multiple sperm (polyspermy) and this reduced embryonic development if used for a prolonged (18 hours) *in vitro* fertilisation interval.

By using Brackett and Oliphant's medium, the mean cleavage rate of 87.80 and 1.72% of morula were obtained in this experiment. This was slightly different from the findings of several research groups where Jaakma *et al*. (1997) and Prentice *et al*. (2011) obtained mean percentage of 69.10% cleaved zygotes as well as 93.00% cleavage rate and 31.00% blastocyst rate, respectively for 22 hours co-incubation period. Yang *et al*. (2008) and Li *et al*. (2009) obtained 36.00% cleavage rate and 4.80% blastocyst as well as 84.10% cleavage rate and 16.40% hatched blastocyst rates, respectively, by using Brackett and Oliphant's medium for 6 hours insemination duration. However, Feng *et al*. (2007) used 5 hours insemination duration with Brackett and Oliphant's medium obtained 82.70% cleavage rate and 19.30% hatched blastocyst.

Nedambale *et al.* (2006) has shown the percentages of polyspermy with different *in vitro* fertilisation media and *in vitro* fertilisation duration which were 7, 6, 1, 1, 11 and 3% for Brackett and Oliphant's medium with 6 hours, modified Tyrode's medium (MTM) with 6 hours, modified medium 199 (IVF-M199) with 6 hours, modified KSOM (m-KSOM) with 6 hours, Brackett and Oliphant's medium with 18 hours and modified medium 199 with 18 hours, respectively. Extended sperm-oocyte incubation periods increased the incidence of polyspermic fertilisation and decreased the blastocyst

formation rate (Long *et al*., 1993; Rehman *et al*., 1994), although the magnitude of these effects may vary from one bull to another (Parrish *et al*., 1986; Sumantri *et al*., 1997). This is in agreement with the results obtained in this experiment whereby the insemination duration of 8 to 14 hours showed significantly higher fertilisation rate than the insemination duration of 18 to 24 hours.

No blastocyst was obtained in this experiment and this could be explained by the findings of Nedambale *et al.* (2006) that the development up to the blastocyst stage was somewhat dependent upon the type of medium used for preparing frozen-thawed sperm and used for its incubation with oocytes *in vitro*. For the post-fertilisation development of cleaved embryos, factors such as the duration of the first cell cycle (Yadav *et al*., 1993; Plante and King, 1994; Van Soom *et al*., 1997), chromosome complement of the zygote (Yadav *et al*., 1993; King *et al*., 1995; Kawarsky *et al*., 1996) and the culture conditions (Gutierrez-Adan *et al*., 1999) are involved. According to Plante and King (1994) and Van Soom *et al*. (1997), embryos with normal chromosome complement that complete their first cell cycle within 30 hours post-insemination (hpi) have the greatest chance of survival to morula stage by day 5 post-insemination and to blastocyst stage by day 7 post-insemination.

The fertilisation rate and the quality of the embryos produced are also thought to be influenced by the length of time allowed for oocyte maturation and gamete interaction (Gianaroli *et al*., 1996a; Dominko and First, 1997a). Gamete interaction process allows for the penetration of oocyte by sperm, pronuclear formation and the subsequent cleavage. The efficacy of this process depends on the vitality and capacity of the sperm as well as on the maturational status of the oocyte at the time they encounter sperm.

5.2 EFFECT OF OOCYTE GRADING AND INSEMINATION DURATION ON CLEAVAGE RATE OF EMBRYOS OBTAINED FROM *IN VITRO* FERTILISATION IN CAPRINE (EXPERIMENT 2)

In this experiment, the percent distribution of oocytes were 37.78, 37.50 and 44.72% for Grades A, B and C, respectively. These were comparable with Chan *et al*. (2008) with 42.83, 23.71 and 33.47% for Grades A, B and C, respectively. Grade A oocytes obtained by Chan *et al*. (2008) were higher than that in this experiment may be due to the different hormones used to stimulate the experimental goats. The hormone used in Chan *et al*. (2008) was Ovagen^{TM} (oFSH) whereas in this experiment, Folligon (pregnant mare's serum gonadotrophin) was used for laparoscopic oocyte pick-up goats and some oocytes source was from the abattoir. Katska-Ksiazkiewicz *et al.* (2007) used brilliant cresyl blue stain to evaluate the quality of oocytes whereby positive staining results indicating the good quality oocytes. The authors obtained the mean distribution of 18.80, 2.40 and 0.80% for brilliant cresyl blue (+), brilliant cresyl blue (-) and degenerated oocytes, respectively (brilliant cresyl blue test was used to evaluate the activity of the glucose-6-phosphate dehydrogenase of the oocytes) by using slaughtered goats treated with follicle stimulating hormone.

Generally, the maturation rates of Grade A oocytes were higher than that of other grades of oocytes. The more layers of cumulus cells around the oocytes was considered as more competent oocytes. Therefore, the maturation rate of competent oocytes usually was higher. Due to this reason, Katska-Ksiazkiewicz *et al.* (2007) also selected developmentally competent oocytes by a combination of cumulus oocyte complexes morphology (based on the thickness and compactness of the cumulus investment and the homogeneity of the ooplasm) and staining with brilliant cresyl blue methods. The mean maturation rate obtained in the present experiment was 61.26%. This was higher compared to Rho *et al*. (2001) with 27.00% and was comparable with Pawshe *et al.*

(1996), Mogas *et al*. (1997a, b) and Cognié *et al*. (2003) with 62.6, 72.4 and 64.1, and 79.00%, respectively. The differences could be explained by several factors used for their experiments such as different maturation medium used, hormones and the source of ovaries.

Several reports showed that gonadotrophins are the primary regulators of nuclear maturation in mammalian oocyte *in vitro*. Higher maturation and fertilisation rates were achieved by oocytes that matured *in vitro* in the presence of gonadotrophins and oestradiol compared to those matured without hormones showed (Saeki *et al*., 1990; Totey *et al*., 1993). This was because gonadotrophins alter the metabolism of the cumulus cells and induce resumption of meiosis in the oocytes (Salustri and Siracusa, 1983) by interrupting the mode of inhibitory substances through the gap junctions (Ball *et al*., 1983). Follicular stimulating hormone and oestradiol were used in the *in vitro* maturation medium in their experiments as follicular stimulating hormone was found to enhance early embryo development rather than meiotic maturation (Eyestone and Boer, 1993); and oestradiol improves the completion of maturational changes, including the synthesis of presumed male pronucleus growth factor (MPGF; Thibault *et al*., 1975; Moor, 1978).

According to Rose and Bavister (1992), *in vitro* maturation medium could also greatly influence both fertilisation and embryo development. Serum was supplemented in the *in vitro* maturation medium of most mammalian studies. In the present study, oocytes matured in medium supplemented with foetal bovine serum (10%). Younis *et al*. (1989) showed that oestrus serum has a beneficial effect on maturation and subsequent development. On the other hand, there was report showing that foetal calf serum has the most favourable effect on *in vitro* maturation and subsequent development (Totey *et al*., 1993). However, some research groups observed that there was no significant difference

between foetal calf serum and oestrus serum in the culture medium (Fukui and Ono, 1989; Sanbuissho and Threlfall, 1990; Wiemer *et al*., 1991).

Pawshe *et al*. (1996) observed that gonadotrophins and oestradiol caused synergistic enhancement of nuclear maturation, depending on the type of serum supplement used in the maturation medium as they found out that the addition of gonadotrophins and oestradiol to the medium (Ham's F-12 or M-199) supplemented with oestrus goat serum (10%) did not increase the maturation rate above that of the control medium but the addition of foetal calf serum (10%) did.

For fertilisation rate in this experiment, oocytes with better grades generally obtained significantly higher rate than those lower grade of oocyte. Moreover, the fertilisation rates of insemination durations of 8 to 14 hours and 18 to 24 hours were also observed, and it was found out that the fertilisation rates of insemination durations of 8 to 14 hours (39.82%) was higher than the fertilisation rates of insemination durations of 18 to 24 hours (33.59%). However, the mean fertilisation rates of both insemination durations (37.05%) was lower than Younis *et al*. (1991c) who aspirated the oocytes from > 7 mm follicles of hyperstimulated donors (56.7%) and De Smedt *et al*. (1992) who used FSH-stimulated adult goats (71.4%).

The co-incubation of oocytes with sperm for 17 hours was originally established for practical reasons and corresponds to the time for observation of pronuclei. From the observation of Cognié *et al*. (2003) in goat *in vitro* fertilisation system, there was only 10% of oocytes were fertilised following 1 hour co-incubation with sperm, but the cleavage rates (3 vs. 17 hours co-incubation) were 71 vs. 78% and blastocyst rates (3 vs. 17 hours co-incubation) were 50 vs. 53%, respectively. The 6 hours delay in gamete interaction indicated that this duration is necessary for sperm to cross the cumulus or that differences in sperm capacitation kinetics exist between *in vitro* fertilisation systems.

The fertilisation rate may also be influenced by the methods of preparing the sperm for *in vitro* fertilisation. Rho *et al*. (2001) has shown that the proportion of sperm isolated, of the initial concentrations, by Percoll density-gradient method (33.90%) was higher compared to swim-up (7.70%) and glass-wool (18.20%) procedures, respectively. Thus, Percoll density-gradient centrifugation appeared to be approximately 4 times more efficient than swim-up and approximately 2 times better than glass-wool filtration for recovering sperm from frozen-thawed semen. Besides, Percoll treatment resulted in higher numbers of viable sperm than were yielded by the swim-up and glass-wool filtration procedures. Accelerated development of embryos obtained by *in vitro* fertilisation with Percoll-treated sperm was also evidenced at later stages of culture. This was proven by blastocysts obtained from *in vitro* fertilisation with such sperm had significantly more cells than when glass-wool- or swim-up-treated sperm were used. This suggested that Percoll treatment of the sperm might facilitate the acrosome reaction and hence lead to more rapid fertilisation.

The present study showed that Grade A oocytes had developed up to morula stage and higher cleavage rate compared to other grades of oocytes. This result indicated that cumulus cells are crucial in promoting normal cytoplasmic maturation of oocyte necessary for fertilisation, cleavage and subsequent development of *in vitro* fertilised embryos. There was no blastocyst obtained in the present study. This may be due to the culture system used was differ from other research groups where co-culture system was used. Teotia *et al*. (2001) used granulose cell co-culture and obtained 35.20% of morulae whereas Izquierdo *et al*. (1999) obtained 10.00% of blastocysts by co-culturing embryos with oviduct epithelial cells and Mogas *et al*. (1997a) managed to obtain 8.30% of blastocysts by co-culturing embryos with granulose cells.

In addition, factors that confer oocytes developmental competence include stage of the oestrous cycle (Hagemann *et al*., 1999), hormonal patterns (Kruip and Dieleman,

1982), composition of follicular fluid (Chang *et al*., 2002; Chiu *et al*., 2002; Anifandis *et al*., 2005; Wunder *et al*., 2005), follicular diameter (Lonergan *et al*., 1994; Blondin and Sirard, 1995; Crozet *et al*., 1995; Hagemann *et al*., 1999) and follicular atresia (Blondin and Sirard, 1995). However, it is difficult assess these variables to select the most competent oocytes from the ovaries of slaughtered female animals for *in vitro* embryo production. In addition, when the oocyte recovery involving the method of ovary slicing, a large number of oocytes are recovered but regardless of the diameter of the follicles or their extent of atresia. Consequently, the oocyte population recovered is very heterogeneous, and thus must be subjected to a strict selection procedure to identify the most competent oocytes.

Tanghe *et al*. (2002) showed that the close contact between cumulus cells and oocyte through gap junctions allows the bidirectional interchange of molecules. Hence, any factor that could affect cumulus cells, for example, an apoptotic state, might also reflect a lower oocyte quality and, as a consequence, *in vitro* embryo production may be impaired. In general, it is accepted that cumulus oocyte complexes that come from non-atretic follicles show compact cumulus cell layers and an homogeneous oocyte cytoplasm (De Wit *et al*., 2000) as well as showing higher developmental competence (Lee *et al*., 2001; Zeuner *et al*., 2003; Corn *et al*., 2005; Yuan *et al*., 2005).

Apoptosis in the oocyte is able to affect embryo quality as the presence of molecules that regulate the apoptotic mechanism in the maternal messenger ribonucleic acid (mRNA) stored in the oocyte (Jurisicova *et al*., 1998; Exley *et al*., 1999; Metcalfe *et al*., 2004). This messenger ribonucleic acid is used to maintain oocyte maturation, fertilisation and embryo development until the embryonic genome is activated (Bachvarova, 1992; Gandolfi and Gandolfi, 2001). According to Han *et al*. (2005), the maternal genotype, through apoptosis, is also playing a significant role in cell fragmentation of the embryo. Apoptosis in the blastocyst is a feature of normal

development (Hardy, 1997) even though a high incidence of apoptosis in the blastocyst will compromise embryonic development and possibly lead to abnormalities in the foetus (Brison and Schultz, 1997).

5.3 EFFECT OF OOCYTE GRADING IN BOVINE AND CAPRINE ON PRODUCTION OF PARTHENOGENETIC EMBRYOS (EXPERIMENT 3)

In this experiment, the cleavage and blastocyst rates obtained for bovine and caprine parthenogenetic activations were 63.90 vs. 0.15% and 26.77 vs. 0.12%, respectively. The bovine cleavage rate was comparable but the blastocyst rate was lower than Wang *et al*. (2008) who used the combination of ionomycin with 6-dimethylaminopyridine (69.20% cleavage rate and 28.00% blastocyst rate) and the combination of ethanol with cycloheximide (CHX) (70.20% cleavage rate and 25.50% blastocyst rate). For caprine oocytes, both cleavage and blastocyst rates were lower compared to Ongeri *et al*. (2001), who obtained 57.80 and 28.50%, respectively. Under optimal conditions, 20.00 to 30.00% of porcine blastocyst rates were obtained (Zhu *et al*., 2002; Im *et al*., 2003; Iwamoto *et al*., 2005). Che *et al*. (2007) obtained 81.60 vs. 66.60% cleavage rate and 43.70 vs. 20.30% blastocyst rate using ionomycin 15 µM for 5 minutes followed by 7.5 mg/ml cytochalasin B (CB) and 10 mg/ml cyclohexemide in porcine zygote medium (PZM-3) (Yoshioka *et al*., 2002), and 20 mM strontium chloride for 4 hours, respectively. By applying the same chemicals (calcium ionophore and 6-dimethylaminopyridine) used in this experiment, Khatir *et al*. (2009) achieved 25.00% of blastocyst rate from dromedary oocytes by using 4 hours of activation duration.

The slightly lower cleavage rates obtained in this experiment compared to other research groups may be due to different agents, activation durations and different species used. Besides, bovine and caprine oocytes may be more sensitive and the low number of good quality oocytes used may also contribute to the lower cleavage rate and

blastocyst rate. *In vitro* matured porcine oocytes have been activated with cycloheximide and 6-dimethylaminopyridine or combined treatment with a calcium ionophore and 6-dimethylaminopyridine (Jilek *et al*., 2001; Yi and Park, 2005). However, according to Jilek *et al*. (2001), 6-dimethylaminopyridine had a detrimental effect on the further development of activated porcine oocytes. Ionomycin (ION) and electrical pulses are other agents that are able to induce oocyte activation through calcium mobilisation from intracellular stores (Machaty and Prather, 1998; Alberio *et al*., 2001). The proper modulation of electrical field pulses could result in $(Ca^{2+})_i$ spikes that resemble those induced after fertilisation (Ozil and Huneau, 2001; Ozil *et al*., 2005) which improved the developmental performance of parthenogenetic embryos. However, Mitalipov *et al*. (1999) reported that the percentage of young rabbit oocytes treated with inositol 1, 4, 5-triphosphate (IP3) followed by 6-dimethylaminopurine (dDMAP), a protein phosphorylation inhibitor, cleaved and reached blastocyst stage at 84 and 50%, respectively, which increased significantly compared with those activated with 1, 4, 5-triphosphate alone (30 and 0%) or multiple electrical pulses (62 and 30%). On the contrary, several studies observed that the pre-treatment with electrical stimulation significantly increased cleavage rates when oocytes of different species were activated with 6-dimethylaminopyridine compared to those treated with cycloheximide (pigs: Grupen *et al*., 2002; cattle: Holm *et al*., 2003; sheep: Alexander *et al*., 2006 and camel: Khatir *et al*., 2009). It was noted that the combination of electrical stimulation with chemical activation was markedly increasing the percentage of oocytes developing to the blastocyst stage in comparison to individual treatment with electrical pulses (rabbit: Yin *et al*., 2000; ferret: Li *et al*., 2002; Liu *et al*., 2005; sheep: Alexander *et al*., 2006), cycloheximide (ferret: Li *et al*., 2002; sheep: Alexander *et al*., 2006) or 6-dimethylaminopyridine (ferret: Li *et al*., 2002; sheep: Alexander *et al*., 2006).

An alternative agent served to induce $(Ca^{2+})_i$ oscillations and oocyte activation in various species is strontium chloride (Sr^{2+}) (Kline and Kline, 1992; Okada *et al*., 2003; Meo *et al*., 2004; Tomashov-Matar *et al*., 2005). According to Zhang *et al*. (2005), strontium chloride action mimicked the effects of sperm on the oocyte, and seemed to be mediated through the inositol trisphosphate receptors. For the activation of murine oocytes, it was shown that the concentration of 10 mM and the exposure time of 2.5 to 3 hours were the best conditions (Ma *et al*., 2005; Loren and Lacham-Kaplan, 2006). However, it was reported that a higher concentration seemed to be necessary for the activation of bovine oocytes (Meo *et al*., 2005). Okada *et al*. (2003) reported that the microinjection of several micoliters of 0.1 M strontium chloride solution into metaphase II arrested oocytes induced an increase in $(Ca^{2+})_i$ and hence promoting oocyte activation. Koo *et al*. (2005) showed no significant changes in the levels of maturation promoting factor (MPF) and mitogen activated protein (MAP) kinase of chemically activated porcine oocytes. Longer exposure of porcine oocytes to chemical activation agents might be detrimental for their activation; however, the co-application of a protein synthesis inhibitor could improve oocyte activation by inhibiting the synthesis of maturation promoting factor.

It has been demonstrated that the age of the oocytes is crucial as aging improves the response to stimuli (Nagai, 1987; Ware *et al*., 1989). This is further proven by Collas and Robl (1990) as well as Stice and Robl (1988), who observed more efficient activation with aged oocytes by multiple electrical pulses than younger oocytes, but the potential of activated oocytes to develop into blastocysts decreases with age. By evaluating the pronuclear formation, a better response of bubaline oocytes to ethanol activation was observed at 30 vs. 24 hours post-maturation (Miranda *et al*., 2002). Also, post-maturation of bovine oocytes for 30 and 40 hours had significantly higher pronuclear formation after electrical activation than that of 22 hours (Suzuki *et al*.,

1999). Besides, Kubiak (1989) showed that murine metaphase II oocytes progressively develop the ability for full activation, as indicated by their increasing responsiveness to activation stimuli with time.

5.4 AN ATTEMPT TO CRYOPRESERVE CAPRINE AND BOVINE EMBRYOS USING VITRIFICATION TECHNIQUE (EXPERIMENT 4)

By using the combination of ethylene glycol and dimethyl sulfoxide in this preliminary and exploratory experiment, unfortunately there was no positive result obtained in both toxicity screening and vitrification of bovine embryos using cryoleaf. Using ethylene glycol and the combination of ethylene glycol and dimethyl sulfoxide, Campos-Chillon *et al*. (2009) obtained 42.70% and 14.70% of blastocyst per 8-cell in open pulled straw vitrification at bovine 8-cell embryo, respectively. However, using caprine embryos in this experiment, the survival rate of toxicity screening and vitrification of blastocyst were 75.00% and 33.33% of hatched blastocysts, respectively. The caprine embryo survival results were comparable with those of El-Gayar and Holtz (2001) and Yacoub *et al*. (2010) which were 64.00% and 70.00%, respectively, after open pulled straw vitrification. Therefore, it is suggested that the current protocol of vitrification in this experiment is more suitable for the caprine species.

Several studies have shown that the culture system has a dramatic effect on embryo survival following cryopreservation (Galli and Lazzari, 1996; Lonergan *et al*., 2001; Rizos *et al*., 2001, 2002). The ability to survive in culture after the cryopreservation-warming process appeared to be one useful indicator to measure the *in vitro* produced embryo cryoinjury. According to Rodriguez-Dorta *et al*. (2007), the pregnancy rate (92.00%) as well as embryo survival rate (62.00%) obtained after transfer of fresh *in vitro* produced embryos cultured in SOF were similar to those previously reported by Cognié *et al*. (2001) with *in vivo* fresh embryos (89.00% of

pregnant goats and 71.00% embryo survival), suggesting that the SOF medium could provide a high viability in non-cryopreserved *in vitro* produced caprine embryos.

Cognié *et al*. (2003) reported that *in vitro* produced embryos are more sensitive to cryopreservation than *in vivo* embryos. Rodriguez-Dorta *et al*. (2007) found that goat oviduct epithelial cells (GOEC) co-culture significantly improved the *in vitro* produced embryo survival rate after vitrification. Indeed, after direct transfer (without morphological selection nor removal of cryoprotectants) of *in vitro* produced vitrified embryos co-cultured with goat oviduct epithelial cells, the kidding rate (56.00%) and the embryo survival rate (33.00%) were similar to those obtained by Guignot *et al*. (2006) after direct transfer of *in vivo* vitrified embryos which were 56.00 and 32.00%, respectively. The culture system plays a crucial role in determining embryos quality cultured *in vitro* and, consequently, embryos ability to survive cryopreservation (Martino *et al*., 1993; Lonergan *et al*., 2001). It was clearly shown that culture conditions do not fit all embryo requirements within the female reproductive tract (Bavister, 2000). By using SOF culture and goat oviduct epithelial cells co-culture, Rodriguez-Dorta *et al*. (2007) obtained an embryo survival rate of 9.00% and 33.00% from vitrified embryos, respectively. This proved that embryos cultured with goat oviduct epithelial cells acquired a better quality in terms of cryotolerance and embryo survival rate than embryos developed in SOF culture without cell support. These effects may rely on the production of embryotrophic factors by oviduct cells, the depletion of potentially harmful substances, the modification of the medium constituents to levels more appropriate for embryo development or the modification of physico-chemical environment parameters such as pH or oxygen concentration (Hernandez-Ledezma *et al*., 1996; Thompson, 1997; Bavister, 2000; Rizos *et al*., 2001).

The differences obtained in this experiment with other research groups might be due to the different combination of cryoprotectants used. According to Campos-Chillon

et al. (2009), ethylene glycol was superior to the combination of ethylene glycol and dimethyl sulfoxide designed for the durations, temperatures and embryo developmental stages studied. Compared to glycerol, dimethyl sulfoxide, propylene glycol, or acetamide, ethylene glycol has minimal toxicity and is more permeable to embryos (Kasai, 1996). The original formulations of ethylene glycol (Selman and El-Danasouri, 2002) and the combination of ethylene glycol and dimethyl sulfoxide (Vajta *et al*., 1998) used sucrose in the vitrification and warming solutions. Due to differences between the 2 sugars in molecular weight and viscosity, Campos-Chillon *et al*. (2009) replaced sucrose with galactose to further facilitate dilution of cryoprotectants (McWilliams *et al*., 1995). Galactose, a non-embryotoxic 6-carbon monosaccharide, acts as an osmotic agent as well as maintains structure and functional integrity of membranes. Cell lysis is likely prevented by galactose during warming through reducing water movement across membranes (Kuleshova *et al*., 1999). Shaw *et al*. (1997) added Ficoll 70 to promote vitrification by increasing the surface tension of the solution and protecting cellular membranes and hence lowering the amount of ethylene glycol needed.

By using straw, survival rates (re-expansion) increased when embryos were loaded at 24 compared to 4ºC. The dynamics of equilibration between water moving out of the cell and cryoprotectant moving inside the cell likely were faster at room temperature with no apparent toxicity (Campos-Chillon *et al*., 2009). However, this observation varies among studies and concentrations of cryoprotectants used (Vajta *et al*., 1996; Van Wagtendonk-de Leeuw *et al*., 1997; Vajta *et al*., 1998; Pugh *et al*., 2000). Blastocysts favour longer exposure durations but not morulae. This may correspond with the larger amount of water content in the blastocoele cavity. Also, the favourable results achieved with cryopreserved blastocysts might be associated with the fact that its cells are greater in number, smaller and being arranged more or less in a single layer around the periphery, are exposed to cryoprotectants and low temperature in a more

uniform fashion than the cells of the morulae, which are few, bigger, and arranged as a solid ball. This was further proven by other studies, whereby the *in vitro* produced morulae have poor survival rates (Holm and Callesen, 1998; Hasler, 2003). Zona pellucida is a protective coat known to control osmotic pressure (Bronson and McLaren, 1970), transport and diffusion of nutrients and metabolites (Leoni *et al*., 2002) as well as cryoprotectants. The zona is not a static structure and changes its character as a result of several factors, for example, tubal passage (Kolbe and Holtz, 2005), sperm penetration (Yanagimachi, 1988; Suzuki *et al*., 1996) and cryopreservation (Moreira da Silva and Metelo, 2005). According to Yacoub *et al*. (2010), this might be partly responsible for the poor performance of cryopreserved morulae.

The developmental competence and visual selection of the *in vitro* produced morulae may be important. The morphological selection of normal morulae is extremely difficult as only about 65% of selected morulae develop into blastocysts in the study of Campos-Chillon *et al*. (2009). It has the possibility that morulae appear to be more sensitive to cryoprotectant exposure and thus needing shorter exposure durations. Cell death may possibly be attributed to exposure of morulae to toxic levels of cryoprotectant before cryopreservation. Vitrification requires high levels of cryoprotectants to achieve glass transition. A successful vitrification procedure requires optimisation of cryoprotectant concentration and exposure duration as well as exposure temperature. In this experiment, based on the positive results of toxicity screening in caprine blastocyst, the vitrification protocol seems to be more suitable for this stage of embryo development. Therefore, with limited embryos available, we chose the vitrification of blastocyst as the stage of embryos to be cryopreserved in this experiment.

5.5 GENERAL DISCUSSION

The results of the present study showed that the possibility of the production of embryos via *in vitro* fertilisation as well as parthenogenetic activation in both caprine and bovine species. However, more studies are needed to overcome various aspects contributing to the improvement of results of the present study. These include intrinsic and extrinsic factors such as animal breed and condition, hormonal treatment, oocyte quality, oocyte retrieval condition as well as *in vitro* culture system. For example, the main problem faced by the author in the study is difficulty in obtaining a large number of good quality oocyte for the *in vitro* fertilisation technique causing the obstacle to carry out proper experiment in *in vitro* fertilisation, parthenogenetic activation and vitrification. Therefore, special attention must be given to address the issue pertaining to oocyte quality specifically in the caprine species. In addition, the attempt to cryopreserve embryos via vitrification technique resulted in positive post-warming survival rate in caprine embryos at blastocyst stage. Nevertheless, more studies on factors affecting embryo survival after vitrification need to be carried out. These include type of cryoprotectant used, exposure duration with special reference to *in vitro* versus *in vivo* produced embryos, embryo developmental stage and *in vitro* versus *in vivo* (embryo transfer) development after devitrification.

5.5.1 Oestrus Synchronisation and Hyperstimulation

By using pregnant mare's serum gonadotrophin, the oestrus synchronisation and hyperstimulation techniques were generally successfully performed in the present study. However, the number of oocytes retrieved was still low. This may be due to the type of hormones that is pregnant mare's serum gonadotrophin, used for the oocyte donor goats, which tended to be variable and lower in the superovulatory response compared to follicle stimulating hormone (Amoah and Gelaye, 1990). The poor superovulatory

response would also suggest the possibility of antibody formation to chorionic gonadotrophin treatment following repeated oestrus synchronisation cycles and hyperstimulation in the same donor goats (Baril *et al*., 1996; Roy *et al*., 1999; Drion *et al*., 2001; Herve *et al*., 2004) or the small follicles are not responsive to gonadotrophin stimulation (Bruck *et al*., 2000).

The dosage and timing for the hormone injection on the donor goats need to be refined. However, goats under good nutrition condition are crucial before any other factors further being looked into. In this experiment, the management of goats was merely satisfactory as the management of the animals was at the farm level which was beyond the scope of responsibility of the author. For an example, in some laparoscopic oocyte pick-up, it has been observed that the oocyte donor goats were in a poor body conformation. Consequently, this may affect the findings of this study. Even though many protocols of hormonal synchronisation and hyperstimulation suggested in the literature, specific protocols are needed for specific environments particularly under tropical condition. Another major issue faced by the author of this study was the limitation in the number of goats available that resulted in sharing of goats among the many researchers in our laboratory. Due to these limitations, the scope of the study was limited with little modification. For an example, the author chose Controlled Intravaginal Drug Release device, Estrumate® (125 μg cloprostenol), pregnant mare's serum gonadotrophin (1500 IU) and Ovidrel® (250 IU chorionic gonadotrophin) as the only protocol for the synchronisation and hyperstimulation.

5.5.2 Temperature

The source of oocytes from abattoir was highly affected by the collecting saline temperature for the transportation of the ovaries. During the collection of ovaries from abattoir which located at Shah Alam and Senawang, warmed saline was brought along

in the thermos with the temperature of 35 to 37ºC. However, due to the long distance between university and abattoirs, the temperature of the saline in thermos sometimes decreased while reaching the abattoirs or university. This might be the factor causing the dysmorphic oocytes. Moreover, due to many researchers sharing the limited microscopes, the ovaries obtained from abattoir tended to be placed in the water bath for quite a long duration before ovary slicing was carried out. Although the temperature of the water bath could be maintained, the exposure of ovaries to the open environment might also be the factor of dysmorphic oocytes which led to low maturation, fertilisation and embryo developmental rates.

There were few reports of the effect of slaughter-to-culture intervals on the quality of oocytes. Storage of ovaries at 37 to 39ºC for 5 to 8 hours decreased both the maturation rate and the potential of follicular oocytes to develop into blastocysts after *in vitro* fertilisation (Yang *et al*., 1990; Nakao and Nakatsuji, 1992; Abe and Shioya, 1996). On the other hand, the storage of ovaries at 20ºC (Abe and Shioya, 1996) or 25ºC (Yang *et al*., 1990) for 8 hours did not reduce the maturation rate or the potential of *in vitro*-fertilised oocytes to develop into blastocysts. Although Solano *et al*. (1994) reported that the maturation competence of oocytes was not reduced or early cleavage was obtained after *in vitro* fertilisation by storing ovaries at 4ºC for 24 hours, the potential for these oocytes to develop into blastocysts is unknown.

In the present study, the oocytes retrieved from laparoscopic oocyte pick-up were transported to another laboratory (from NaTuRe to EMiL) for the subsequent experiments due to facilities problem at NaTuRe laboratory. The immature oocytes were placed in the *in vitro* maturation medium with the temperature of 35 to 38ºC during the transportation. When reached the EMiL laboratory, the oocytes were transferred from the tube to the *in vitro* maturation droplets for subsequent culture. During the process of transferring, the quality of the oocytes was affected as the oocytes

were often exposed to a wide range of temperatures that may ultimately affect subsequent development. According to several reports, the cooling of mammalian oocyte to sub-physiological temperatures is well known to affect its viability through the induction of various abnormalities at all stages of meiosis (Moor and Crosby, 1985; Heyman *et al*., 1986; Pickering *et al*., 1990; Aman and Parks, 1994). Didion *et al*. (1990) also reported that oocytes at the germinal vesicle stage are highly sensitive to the decreased temperatures.

5.5.3 *In Vitro* Maturation

In this study, oocytes were selected and characterised by the layers of cumulus cells. Oocytes with more than 5 cumulus cell layers were assumed to be more competent compared to those with lesser cumulus cell layers. This could be explained by the functions of cumulus cells in which the cumulus cells transmit molecules through gap junctions that are required to support oocyte growth and development as well as regulate meiosis and the global transcriptional activity in the oocyte genome (Tanghe *et al*., 2002). In turn, the oocyte secretes soluble paracrine factors called oocyte secreted factors (OSFs) that act on granulosa cells and direct them to perform multiple functions such as: stimulation of mural granulosa cells and deoxyribonucleic acid (DNA) synthesis and cellular proliferation of cumulus cells (Gilchrist *et al*., 2003; Hickey *et al*., 2005); differentiation of these 2 sub-populations in relation to steroidogenic production (Li *et al*., 2000); stimulation of cumulus expansion (Ralph *et al*., 1995; Procházka *et al*., 1998; Zhang *et al*., 2008); as well as prevention and protection of cumulus cell apoptosis (Hussein *et al*., 2005). Hussein *et al*. (2006) has reported that the addition of oocyte secreted factors to the *in vitro* maturation medium was able to improve embryo development (Hussein *et al*., 2006).

5.5.4 *In Vitro* Fertilisation

The major constraints in this study were the insufficiency of oocyte sources and poor quality oocytes for the *in vitro* fertilisation protocol to produce embryos using various treatments. For an example, in caprine *in vitro* fertilisation, related technical problems could not be studied and overcome due to the limited number of oocytes available per replicate. Consequently, in many cases, more definitive conclusions were not able to be made on the effects of the treatments studied on the embryo development. Even though more oocytes obtained for the bovine compared to caprine oocytes, the quality of the former oocytes was poorer, thus resulting in poor embryo development after *in vitro* fertilisation in the bovine in the present study.

In this study, frozen-thawed sperm were used. The freezing-thawing process has been found to cause some damage on the sperm membrane (Bailey *et al*., 2002; Rodriguez-Almeida *et al*., 2005). This could be due to poor calcium release during the process of fertilisation resulting in a low fertilisation rate and subsequent decreased embryo developmental competence *in vitro* (Bailey and Buhr, 1994). Furthermore, semen from different bulls have been shown to affect fertility (Sumantri *et al*., 1997). Therefore, it is possible that using cryopreserved sperm in the study may have negative effect on the *in vitro* fertilisation performance.

Although completion of oocyte maturation is not an absolute requirement for sperm penetration and for sperm nuclear decondensation (Chian *et al*., 1992; Niwa *et al*., 1991), development is impaired following premature exposure to sperm; hence, it is generally thought that the optimum time for *in vitro* fertilisation is at metaphase II stage. In bovine oocyte, the attainment of the metaphase II stage is known to occur 18 to 24 hours after the beginning of *in vitro* maturation. Different research groups (Monaghan *et al*., 1993; Ward *et al*., 2002) have reported that the optimal duration of maturation of bovine oocytes *in vitro* to optimise blastocyst yield is 24 hours. On the other hand,

Semple *et al*. (1993) have reported higher blastocyst production from oocytes matured for only 14 hours. However, Park *et al*. (2005) reported an improved blastocyst development and pregnancy rate with the *in vitro* maturation duration of 18 hours. These conflicting findings may be accounted for the presence of many factors affecting embryo development *in vitro* (Dominko and First, 1992; van der Westerlaken *et al*., 1994).

It has been suggested that prolonged gamete co-incubation under the conditions of *in vitro* fertilisation, in which high concentrations of sperm are incubated in small volumes of medium resulted in the production of high levels of hydrolytic enzymes (Rehman *et al*., 1994) and free radicals (Aitken, 1994) which damage the oocytes. Furthermore, increasing the insemination duration has been found to be correlated to a higher incidence of polyspermy which was in agreement with Sumantri *et al*. (1997). These observations suggest that it would be advisable to remove the presumptive zygotes from the *in vitro* fertilisation medium as early as possible, once the sperm penetration has been accomplished, to minimise the time the presumptive embryos are exposed to adverse conditions. Due to this reason, 2 different insemination durations were chosen in this study to compare the effect. Our result has shown that 8 to 14 hours of insemination duration is preferred to 18 to 24 hours to produce better *in vitro* fertilisation performance alongside with other factors such as good quality of oocytes and sperm as well as *in vitro* culture media.

5.5.5 Parthenogenetic Activation

Most of the currently used oocyte activation protocols rely on the methods to induce an intracellular Ca^{2+} increase, as naturally elicited by the sperm during fertilisation process. Different types of activation agents were studied based on this principle. According to Jellerette *et al*. (2006), caprine metaphase II (MII) oocytes are shown to generate $[Ca^{2+}]_i$

oscillations at activation, similar to that of other mammals. Nevertheless, different methods of activating caprine oocytes were shown to result in different $[Ca^{2+}]_i$ release patterns. Significant differences were observed in the amount of Ca^{2+} released by each method of activation and a decrease in $[Ca^{2+}]_i$ released in *in vivo* matured oocytes as compared to *in vitro* matured oocytes. Parthenogenetic activation by ionomycin initiated a single $[Ca^{2+}]_i$ rise, whereas sperm at fertilisation-induced multiple $[Ca^{2+}]_i$ oscillations. A single $[Ca^{2+}]_i$ rise brought detrimental effect on embryo development compared to a series of calcium oscillations during oocyte activation (Lorca *et al*., 1993; Susko-Parrish *et al*., 1994).

In this study, same combination of activation agents was used, which was combination of calcium ionophore and 6-dimethylaminopurine for both caprine and bovine species. Consequently, one of the reasons for the lower embryo development obtained for the species studied may be due to different activation agents used by other research groups. For examples, there were reports showing that the similar cleavage rate in all treatments (ethanol + cycloheximide, ethanol + 6-dimethylaminopurine and ethanol + cycloheximide + 6-dimethylaminopurine) suggest that ethanol (7%) plays a significant role in oocytes activation, as it suddenly increases intracellular Ca^{2+} (Presicce and Yang, 1994) and probably lead to the development of embryos. Identical cleavage rates of oocytes, Liu and Yang (1999) observed that similar signaling events were followed with ethanol + cycloheximide and ethanol + 6-dimethylaminopurine. The combination treatment of ionomycine or ethanol and 6-dimethylaminopurine that first initiates a calcium flux and then inhibits protein phosphorylation induces pronuclei formation without completion of meiosis in bovine oocytes (Liu *et al*., 1998b).

Therefore, it is suggested that the combination of activation treatment plays a critical role in the signaling pathways of embryo development for a particular species as the development of morula and blastocysts in combined treatment (ethanol +

cycloheximide + 6-dimethylaminopurine) was significantly higher (50.9 and 30.9%, respectively) than respective *in vitro* fertilisation (39.4 and 15.2%), ethanol + cycloheximide (35.7 and 4.8%, respectively) and ethanol + 6-dimethylaminopurine (26.4 and 9.4%, respectively) (Mishra *et al*., 2008). The activation methods used in their trial have been proven to be effective for activation in bovine (Presicce and Yang, 1994) and buffalo (Kitiyanant *et al*., 2003; Parnpai and Tasripoo, 2003) oocytes. By using similar activation agents, Gasparrini *et al*. (2004) observed development of bubaline embryos up to 32.6% morula and 22.9% blastocyst, whereas Singla *et al*. (1997) reported 30.4% blastocyst development following activation with cytochalasin B in *Bubalus bubalis*.

Kline *et al*. (1992) reported that among the activation agents, ionomycine induces an increase in intracellular Ca^{2+} in oocytes and the source is both external and from the internal stores. However, ethanol induces a Ca^{2+} rise whose main source is external, although a minor intracellular release is also involved (Shiina *et al*., 1993). The mature oocytes are arrested at metaphase of second meiotic division with an elevated maturation promoting factor activity, which is maintained by a cytostatic factor (CSF), essential components of which are the products of the c-mos proto-oncogene, methylacetoxyprogesterone kinase, and possibly Cdk2 kinase. Deoxyribonucleic acid replication is avoided as these proteins maintain the condensed status of chromatin (Verlhac *et al*., 1994). The elevated Ca^{2+} levels induced by ionomycine or ethanol treatment released the oocytes from the meiotic arrest by inactivation of maturation promoting factor and cytostatic factor (Collas *et al*., 1993; Lorca *et al*., 1993). However, these substances induce a single Ca^{2+} rise in oocytes while as, the initial Ca^{2+} rise is followed by Ca^{2+} oscillations during the normal fertilisation process. This single Ca^{2+} rise causes only a transient decline in maturation promoting factor and cytostatic factor activity, which gets restored with recondensation of chromosomes and re-entry of

activated oocytes into metaphase-II (Susko-Parrish *et al*., 1994; Collas *et al*., 1995). Thus, sequential approaches have been developed that involve the transient inactivation followed by persistent inhibition of maturation promoting factor by addition of protein synthesis, protein phosphorylation or specific maturation promoting factor inhibitors (Presicce and Yang, 1994; Susko-Parrish *et al*., 1994; Bos-Mikich *et al*., 1995; Liu *et al*., 1998b; Mitalipov *et al*., 2001).

Besides the different activation agents used that may contribute to the differences in results, different exposure timings are also probably one of the factors causing the variation. The exposure durations used for oocytes activation in different species ranging from 2 to 5 minutes (cattle: Liu *et al*., 1998; monkey: Mitalipov *et al*., 2001; goat: Ongeri *et al*., 2001; buffalo: Gasparrini *et al*., 2004; buffalo: Saikhun *et al*., 2004), which seems to vary with different species and different laboratory conditions.

Several reports suggesting that the activation rate is highly dependent on the age of oocytes, with ageing improving the response to stimuli (Nagai, 1987; Ware *et al*., 1989). Suzuki *et al*. (1999) has proven that with higher activation rate for the matured oocytes with 30 and 40 hours compared to those with 22 hours after electric activation, whereas Pimentel *et al*. (2002) has reported that parthenogenetic activation rates (pronucleus/i formation) of equine oocytes after treatment with ionomycine/6-dimethylaminopurine increased from 13 to 64% when the duration of *in vitro* maturation was increased from 24 to 42 hours. In bubaline oocytes activation, evaluated based on pronuclear formation, Saikhun *et al*. (2004) showed that at 30 hours post maturation was better when compared with 24 hours using ethanol in their study, whereas in another study using ethanol as activating agent, the highest efficiency of activating the oocytes was observed at 24 hours post-maturation (Gasparrini *et al*., 2004).

As sperm is the natural activating agent of the oocyte, the *in vitro* fertilisation group was expected to have a higher proportion of activated oocytes. However, a higher proportion of oocytes was activated in groups activated by parthenogenesis. The reasons behind this can be due to the low efficiency of *in vitro* production system for *in vitro* fertilisation, which is yet to be optimised. The inefficiencies of *in vitro* production may be attributed to many factors including the semen quality, sperm preparation as well as the *in vitro* fertilisation and *in vitro* culture media.

5.5.6 Follicle Diameter

The outcome of *in vitro* production of embryo involves numerous factors including oocyte quality, sperm quality and *in vitro* culture system. For an example, good oocyte quality comes from good follicles on the ovaries. The follicle diameter has been widely used as a non-invasive parameter to select the cumulus oocytes complexes (COCs) for *in vitro* embryo production (IVP). In cattle (Lequarre *et al*., 2005) and goat (Crozet *et al*., 1995), the oocytes from large follicles are more competent than oocytes from small follicles. Thus, there were some reports (Kauffold *et al*., 2005; Bagg *et al*., 2007) showing that no significant differences were found on embryo development between adult and prepubertal animals when oocytes were recovered from the largest follicle size. However, follicle population in the ovaries from prepubertal females has been shown to have smaller diameter compared to their adult counterparts (Armstrong, 2001). Martino *et al*. (1994) reported that most of the follicles in mature female goats have a diameter between 2.5 while 3 mm in prepubertal goat ovaries. Oocytes from prepubertal goats complete their growth into those follicles bigger than 3 mm (Martino *et al*., 1994). However, Romaguera *et al*. (2010a) observed that 50% of cumulus oocytes complexes from large follicles were at germinal vesicle breakdown (GVBD) nuclear stage just after

recovering, and they showed a higher percentage of late apoptosis than cumulus oocytes complexes from small follicles.

It is well documented that oocyte secreted factors are able to regulate numerous cumulus cells functions such as metabolism (glycolysis and cholesterol synthesis) (Sugiura and Eppig, 2005; Su *et al*., 2008), steroidogenesis (Vanderhyden *et al*., 1993), apoptosis (Hussein *et al*., 2005) and expansion (Buccione *et al*., 1990). These functions are critical during oocyte development (Li *et al*., 2008). Romaguera *et al*. (2010b) observed that the addition of denuded oocytes in the *in vitro* maturation medium significantly increased the blastocyst rate of oocyte secreted factors from small follicles although cleavage rates did not improve. Similar results were obtained by Hussein *et al*. (2006) in bovine, who observed that the oocyte secreted factors co-culture with denuded oocytes significantly increased the proportion of embryos that reached blastocyst stage. Therefore, it is important to consider the follicle diameter in relation to other factors in future studies involving *in vitro* fertilisation.

5.5.7 *In Vitro* Culture

To date, a lot of effort was made to increase the efficiency of *in vitro* culture system. The modification included the substitution of serum/BSA with chemically defined macromolecules such as polyvinyl alcohol (PVA) and sodium hyaluronate (SH) in *in vitro* culture systems (Biggers *et al*., 1997; Nowshari and Brem, 2000). However, according to some research groups, with these substitutes, inconsistent or inferior results were obtained compared to those obtained with serum/BSA (Biggers *et al*., 1997; Nowshari and Brem, 2000; Massip, 2001). In the present study, mSOF medium was used in caprine *in vitro* fertilisation while KSOM was used in bovine *in vitro* fertilisation as the culture medium. Although no blastocyst was obtained in both experiments, KSOM seemed to be better compared to mSOF medium as the percentage

of morulae obtained in bovine *in vitro* fertilisation was higher than in caprine, despite the different species and number of oocytes used. KSOM was not used in the caprine *in vitro* fertilisation as according to the understanding of author, none of the research has reported the usage of KSOM in the caprine *in vitro* fertilisation. However, the author would like to test on the efficacy of KSOM on caprine species; therefore, KSOM was used in the parthenogenetic activation. This was due to in parthenogenetic activation, several factors that contributed to the embryo development could be eliminated such as the preparation and quality of sperm as well as any other factors contributed to the fertilisation rate. According to Nedambale *et al*. (2004), sequential KSOM + bovine serum albumin culture followed by SOF + foetal calf serum resulted in improved morulae as well as early and day 7 blastocyst rates compared to KSOM + bovine serum albumin alone. On the other hand, the reverse sequential treatment by SOF followed by KSOM did not improve development in any criteria tested.

Amino acids (Basal Medium Eagle, BME and Minimum Essential Medium, MEM) were present in KSOM but not mSOF medium. According to Elhassan *et al*. (2001), the ratios of amino acids are critical for protein synthesis in living cells. This was supported by several reports that the embryo development was improved with the supplementation of non-essential amino acids alone (Liu and Foote, 1995; Pinyopummintr and Bavister, 1996) or in combination with lower concentrations or later addition of essential amino acids (Liu and Foote, 1995; Liu *et al*., 1996; Lane and Gardner, 1997; Steeves and Gardner, 1999) during the first 2 to 3 embryonic cleavages. Glutamine was found in KSOM but not mSOF medium. Rieger *et al*. (1992) has reported that glutamine has an important role in bovine embryo metabolism and blastocyst formation and may give an advantage to KSOM. Also, EDTA which was found in KSOM medium, was reported to have beneficial effects on murine embryos passing the 2-cell block (Abramczuck *et al*., 1977; Mehta and Kiessling, 1993).

The low number of blastocysts obtained in this study may be due to the lack of serum during the later stages of embryo development (Gardner, 1994; Lane and Gardner, 2003). Serum was shown to be able to stimulate early expansion of blastocysts by accelerating the blastulation process (Menino and Williams, 1987; Plante and King, 1994; Carolan *et al*., 1995; Van Langendonkt *et al*., 1996; Thompson *et al*., 1998; Tricoire *et al*., 1999) and improve blastocyst yield (Thompson *et al*., 1998). Furthermore, there was report showing that serum in culture media enabled the hatching to be promoted by providing a pool of plasminogen; bovine embryos convert it to plasmin that proteolytically degrades the zona pellucida and facilitates hatching (Kaaekuahiwi and Menino, 1990).

Nedambale *et al*. (2004) reported that cell numbers reduced in the culture medium of SOF + foetal calf serum alone and SOF + foetal calf serum changed to KSOM + bovine serum albumin during the second half of the culture period compared to those cultured with a sequential use of bovine serum albumin and serum (KSOM + bovine serum albumin then SOF + foetal calf serum). There were some other research groups trying to use serum in SOF media during the entire embryo culture; however, they found that serum induced early blastocyst formation without increasing cell proliferation (Yoshioka *et al*., 1997; Tricoire *et al.*, 1999; Kuran *et al*., 2001). From the literature, it appears that serum is beneficial only when used during later stages of embryo development, and inclusion of serum during the early stages will reduce embryo quality, which may explain the results obtained in the caprine *in vitro* fertilisation of this study.

5.5.8 Vitrification

There were many different protocols and tools used for vitrification of embryos. In this study, cryoleaf was used as a different tool of vitrification compared to other research

groups. With limited number of embryos obtained in this study, it was infeasible to test the different aspects affecting viability of cryopreserved embryo which include the exposure durations, combination of cryoprotectants, concentration of cryoprotectants, embryo developmental stages as well as different species. In addition, it was known that the viability of vitrified-devitrified embryos much depended on the culture system used. In the present study, the culture system consumed longer time to be modified in order to achieve a better culture system as a lot of trial was needed; however, the low number of good quality oocytes contributed to limited number of embryos which made the attempt of embryo vitrification harder. In addition, due to the limited number of late stage bovine embryos were obtained, thus toxicity screening and vitrification were only conducted on early stage bovine embryos. On the other hand, for caprine embryo vitrification study, since the toxicity screening conducted showed positive results on the late stage embryo, therefore, the vitrification of caprine embryos were focused only on late stage embryos. The vitrification results obtained in this preliminary study, even though limited, they were encouraging as shown in the survival of vitrification-devitrification of caprine blastocyst.

Among various cryoprotectants, due to the limitation of embryos and time constraint, only combination of ethylene glycol, dimethyl sulphoxide and sucrose was chosen as cryoprotectant in this study. The vitrification solution composed of 25% ethylene glycol + 25% dimethyl sulphoxide was shown to be effective for the cryopreservation of *in vivo* derived murine (Ishimori *et al*., 1992), rabbit (Vicente and Garcia-Ximenez, 1994) and bovine embryos (Ishimori *et al*., 1993) as well as *in vitro* produced bovine embryos (Vajta *et al*., 1995, 1996). Another vitrification solution which was non-toxic to murine embryos at room temperature and resulted in pregnancy rates in mice which were similar to those achieved after the transfer of non-frozen blastocysts was comprised of 3 cryoprotectants: ethylene glycol + dimethyl sulphoxide

+ 1, 3 butanediol (BD) (Valdez *et al*., 1992). Recently, Huang *et al*. (2006) compared the different concentration of cryoprotectants which consist of the combination of ethylene glycol and dimethyl sulfoxide to test on the vitrification efficiency in goats. In the vitrification study, the combination of ethylene glycol and dimethyl sulfoxide was usually used with different concentration: El-Gayar and Holtz (2001) with 20% ethylene glycol + 20% dimethyl sulfoxide; Mucci *et al*. (2006) with 3.56 M ethylene glycol + 2.6 M dimethyl sulfoxide + 0.5 M sucrose and Hochi *et al*. (2010) with 15% ethylene glycol, 15% dimethyl sulfoxide + 0.5 M sucrose. It was reported that butanediols have excellent glass-forming characteristics (Mehl and Boutron, 1988) and vitrify at low molar concentrations compared to ethylene glycol (Ali and Shelton, 1993a). The combination of 2 or 3 cryoprotectants allows vitrification of the solution to occur at lower concentrations of the individual cryoprotectants. Moreover, the permeability of each of the cryoprotectants in the vitrification solution was altered by the presence of another (Vicente and Garcia-Ximenez, 1994).

Saha *et al*. (1996) reported that further supplementation of 0.3 M trehalose and 12% polyvinylpyrrolidone (PVP) to ethylene glycol (40%) enhanced the *in vitro* survival of bovine blastocysts following vitrification. In Kasai *et al*. (1996), EFS (30% ethylene glycol, 18% ficoll and 0.3 M sucrose) was used whereby the combination of cryoprotectants contains a single permeable cryoprotectant (CPA), a macromolecule and a sugar. The addition of a macromolecule, such as polyvinylpyrrolidone or ficoll, was believed to promote vitrification (Fahy *et al*., 1984) as well as protect cellular membranes and the zona pellucida from damage imposed during the cooling and warming procedures (Leibo and Oda, 1993; Titterington *et al*., 1995). Kasai (1996) proposed that supplementation of the vitrification medium with small saccharides such as sucrose reduces its toxicity to embryos by reducing the intracellular concentration of the cryoprotectant.

In summary, there are numerous factors including internal and external factors influencing the embryo developmental competence after cryopreservation using vitrification technique in order to optimise the vitrification protocol for embryo cryopreservation to be used in research laboratories as well as application for the industry. For example, more studies in the future on the toxicity screening using different combinations of cryoprotectants with different molarities specifically for caprine species are timely. In addition, the culture system, for example the choice of culture media, where *in vitro* (*in vitro* fertilisation), as well as *in vivo* culture (embryo flushing) of the cryopreserved embryos probably specifically for the vitrified embryos should be optimised for different species. The resultant developmental competence of vitrified embryos derived from different sources of *in vitro* or *in vivo* will be only beneficial when they can be transferred at various stages of development that undergo positive pregnancy and ultimately giving birth to healthy offspring. In a nutshell, this study was conducted to pursue this goal, and our preliminary findings show positive and encouraging results of survivability after vitrification of the caprine embryos. However, more detailed and refined studies are needed in the future involving various factors intrinsically and extrinsically before it can be used routinely.

Chapter 6

6.0 CONCLUSIONS

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This study presents the production of bovine and caprine embryos via *in vitro* fertilisation and parthenogenesis as well as an attempt to vitrify *in vitro*- and *in vivo*-derived embryos. From the findings of the present study it could be concluded that:

a) Bovine and caprine embryos were successfully produced *in vitro* using *in vitro* fertilisation and parthenogenesis techniques.

b) Generally, the maturation, fertilisation and cleavage rates decreased as grading of oocyte decreased.

c) The oocyte grading system based on cellular vestments and cytoplasm uniformity was effectively being used to select oocytes in terms of further developmental competence after *in vitro* maturation, fertilisation and culture as well as parthenogenesis.

d) The cleavage rates up to morula were successfully obtained via *in vitro* fertilisation in bovine and caprine.

e) The cleavage rates up to blastocyst were obtained via parthenogenesis in bovine and caprine.

f) Shorter insemination duration (8 to 14 hours) was preferable in obtaining more cleavage rate in in *vitro* fertilisation.

g) Preliminary cryopreservation of embryos via vitrification method was successfully attempted by producing survival embryos at blastocyst stage after vitrification-devitrification.

h) In a nutshell, even though *in vitro* fertilisation is very successful in many domestic animals such as cattle, sheep and pigs, there are many intrinsic and extrinsic factors yet to be researched for goat *in vitro* fertilisation in order to

optimise the technique to be used routinely in laboratory and for the industry. Our limited findings obtained from this study contribute towards meeting this goal.