CHAPTER 7

GENERAL DISCUSSION

Due to the low cost, collecting oocytes from ovaries of slaughtered goats is preferred to flushing superovulated ones. However, in Malaysia, experience have shown that the number of ovaries obtained were few because of the low slaughtering activity. The low number of goats available for slaughter and the importation of lamb and mutton from other countries are two important contributing factors. The problem was further aggravated by the fact that in Malaysia the slaughtered goats are of low and varying reproductive status. Deb and Goswami (1990) have also found that in India, the ovaries used in their experiments were from uncontrolled animals of varying age and probably of low reproductive status, since the female animals were usually slaughtered when their reproductive performance is poor. However, inspite of the difficulty, the present study was successfully conducted involving cytoplasmic and nuclear changes in vitro (IVM), using oocytes from ovaries of slaughtered goat, and subsequently fertilized (IVF) with frozen-thawed sperm, and the embryos thus produced were further cultured (IVC) for development.

The mammalian primary oocytes are capable to resume spontaneously the process of meiosis when removed from the ovarian follicles and cultured in vitro (Chang, 1955). In this process, the oocyte is removed from the influence of the follicular microenvironment. Also, the physical contact with mural granulosa cells is ruptured, terminating intercellular communication via the gap junctions. This chemico-physical stimulation of the oocyte causes condensation of the chromatin and the breakdown of the nuclear membrane (germinal vesicle), leading to MII (Edwards, 1965). The knowledge of spontaneous resumption of meiosis in vitro has been beneficial for the invention of other subsequent embryo techniques including IVF-ET.

The criteria used to describe the development of oocytes either in vivo or in vitro were cytoplasmic and nuclear changes and cumulus cell expansion. Although studies in cattle (Fleming and Saacke, 1972; Kruip *et al.*, 1983; King *et al.*, 1986), sheep (Cran *et al.*, 1980) and pig (Norberg, 1973) have been performed, there was not much information on these aspects in goat oocytes. Development of goat oocytes in vitro has been studied (Kim *et al.*, 1984; Hanada, 1985; Song and Iritani, 1985; Younis *et al.*, 1991; De Smedt *et al.*, 1992; Pawshe *et al.*, 1994), but ultrastructural basis for the evaluation of such oocytes has not been

demonstrated. Similarly, data on the potentiality of goat oocytes to resume meiosis in vitro have been lacking. Deb and Goswami (1993) studied the chromosomal configuration of oocytes cultured in vitro using ovaries of slaughtered goats. However, in their studies, only one type of oocyte, COC, was used, and no information regarding the meiotic status of CFO undergoing development in vitro was reported. Furthermore, the maturation rate of 10 to 18.2% reported by these workers were far too low compared to that of the present study of 55.1%. It should be noted at this stage that the oocytes are said to attain maturation when their chromosomes reached MII stage (King *et al.*, 1979; 1986; Deb and Goswami, 1993; Rajikin *et al.*, 1995). The present study is the first to document the parallel changes involving the cytoplasmic organelles as well as the chromosomes in goat oocytes cultured in vitro (Rajikin *et al.*, 1994; 1995). Based on the findings, it was shown that the oocytes cultured for 20 hr in the TCM 199 medium has attained cytoplasmic and nuclear maturation. This timing was in agreement with the maturation of bovine oocytes of 20 to 24 hr (Shamsuddin *et al.*, 1993).

Light microscopic study of oocytes (Chapter 4) has revealed that 20 hr incubation is sufficient for the maturation of oocytes. It is interesting to note that the expansion and the loosening of cumulus and corona cells did

not change further even after 40 hr. This was supported by the finding of electron microscopic study (Chapter 4) that at 20 hr the mitochondria were fully developed, distributed evenly and usually in close proximity with dilated endoplasmic reticula, and this did not seem to change at 40 hr. The peripheral distribution of cortical granules strongly support the maturing process of oocytes since these granules will prevent polyspermy should subsequent fertilization take place.

Ultrastructural studies reported in Chapter 4 have shown that there were no apparent difference in the characteristic of cytoplasmic organelles between 20 and 40 hr incubation. Similarly, on the basis of chromosomal changes, the percentage of oocytes that attained MII stage at 40 to 48 hr was as high as that at 20 to 30 hr. However, studies by Shamsuddin *et al.* (1993) suggested that an incubation period of 20 to 24 hr is sufficient for IVM in bovine oocytes, and that incubation beyond 26 hr resulted in a reduced maturation rate, probably due to an increase in overmatured oocytes. Hyttel *et al.* (1986) who studied the ultrastrcture of in vitro maturation of oocytes in cattle has also reported that such oocytes begin to degenerate 30 hr after incubation. In view of these findings, it is felt that the subsequent fertilization rate for the oocytes

incubated more than 40 hr will be lower than those of 20 hr. However, a more intense study on goat oocytes between 20 and 40 hr incubation would be beneficial for further understanding of maturation process.

The importance of cumulus cells for the oocytes to attain their full development has been substantiated by both the cytoplasmic ultrastructural and nuclear finding on COCs. The inferiority of CFOs compared to COCs was not only due to the absence of cumulus cells, but also the fine structure of these oocytes does not seem to be able to support their development. For example, CFOs have very few lipid bodies. The mitochondria were sparsely distributed, lacking cristae and not well developed; there was absence of hooded mitochondria. Endoplasmic reticula which are thought to be involved in a low level of protein and nucleic acid synthesis (Baker, 1982) were not dilated and with no associated ribosome. In CFOs also, the cortical granules were few and had no definite pattern of distribution. In view of these findings, although 20% of the CFOs attained MII stage at 20 to 30 hr, it is doubtful as to whether such oocytes were fertilizable in the subsequent IVF procedure. This doubt is even greater for the CFOs incubated at 40 to 48 hr because at this period, time factor could probably set the oocytes to overmature (Shamsuddin *et al.*, 1993) or degenerate

(Hyttel *et al.*, 1986). Even if fertilization does take place on these oocytes, the subsequent embryos produced from such oocytes may not be viable (Critser *et al.*, 1986). Thus, the possible explanation for the presence of 20% CFO which attained MII stage at 20 to 30 hr was that they were of Graafian follicles that still remained just before ovulation after the LH surge. These oocytes were mixed with those from atretic follicles, although no distinction was made regarding the condition of the follicles at the time of slicing of ovaries.

Maturation study reported in Chapter 6 showed that out of a total of 544 COCs used, 198 oocytes (39%) undergone cumulus cell expansion. Since cumulus expansion was the only indication of oocytes viability after IVM procedure, the oocytes with this characteristic were chosen and used for IVF. However, from the chromosomal study reported in Chapter 5, 55% of COCs attained MII stage, i.e. more than the value which was based on cumulus expansion alone. Therefore, it is possible that 20 hr incubation of COCs in the IVM medium could result in the development of oocytes regardless of whether cumulus cells expand or not.

The fact that the oocytes undergone incubation in IVM medium for 20 hr had attained maturity was confirmed when 12.5 and 5.3% of these oocytes co-incubated with heparin-

treated sperm and oviductal epithelial cells were cleaved into 2- and 4-cell, respectively. This cleavage was definitely as a result of penetration of sperm into the matured oocytes and not due to parthenogenesis. This conclusion was taken based on the failure of maturing oocytes co-incubated with epithelial cells but in the absence of sperm to cleave (Group 2, Table 6.1). The present study also indicates that the failure of the inseminated oocytes to fertilize in an epithelial-free system suggests that the possible growth factor(s) secreted by these cells was necessary for fertilization and/or development. Therefore, the finding of 2- and 4-cell cleavage suggested that the IVM-IVF-IVC systems employed in the present studies were workable within the local set up.

The present study found that in the 2-cell embryo, one blastomere was always larger than the other. It was not clear from the present finding whether the larger blastomere divided first into two daughter blastomeres followed by the smaller one. Frequent examination on the developing goat embryos is needed to establish this possibility. This anomaly probably is a physiological one, because, in human, Sadler (1990) noted that at 2-cell stage, one blastomere is usually larger and probably divide first, resulting in a 3-cell stage. Subsequently, the small blasto-

mere divides and the embryo consists of two large and two small blastomeres.

The success of the IVF procedure described in this thesis not only depend on the viability and fertilizability of the oocytes but also on the sperm. It is beyond the scope of this thesis to deal further on the preservation of sperm. However, the fertilizability of the frozen-thawed sperms used by the present study, particularly their ultrastructure are worth discussing. According to Memon and Ott (1981), an ideal extender needed to meet certain requirements, namely: provide nutrients as a source of energy, contain constituents that protect against harmful effects of cooling and freezing, provide a buffer to prevent harmful shift in pH as lactic acid forms, maintain the proper osmotic pressure and electrolyte balance, contain antibiotic that inhibit bacterial growth, increase the volume of semen so that multiple insemination can be performed and provide an environment in which metabolic activities of the sperm can continue. The choice between skim milk or yolk extended in buffer mixed with a sugar and an antibiotic, depends on storage temperature (Colas, 1984) as the protective powers of both media against deleterious effect of chilling and freezing are different (Kampschmidt *et al.*, 1953); the protective powers of milk being less against changes in temperature.

Frozen sperm obtained through the methods employed by the present study did not show morphological difference, except occasional disappearance of undulating plasma membrane and acrosomal material (Appendix 2; Fig. A.4), compared with that of fresh ones (Fig. A.3). Since morphological characteristics may influence the fertilizability of sperms, it can be assumed that the lack of significant morphological differences suggests that the fertilizability of frozen-thawed sperm was not impaired. However, a further comparative study involving the use of frozen and fresh semen on the rate of fertilization should give more information regarding their fertilizability.

Data on the use of frozen-thawed semen for IVF procedure in goat is lacking. However, in some farm animals, this procedure has been frequently used particularly in bovine (Fayrer-Hosken *et al.*, 1989; Utsumi *et al.*, 1991; Fuku *et al.*, 1992; Goto *et al.*, 1994). The present finding, therefore, offers a basis for the future use of frozen-thawed semen for the routine IVF procedure in goats to produce a high number of embryos either for experiments or commercial purposes.

Fertilization is the common parameter by which capacitation of sperm may be judged. Since fertilization did take

place, it can be assumed that the sperm, by the action of heparin (0.05 mg/ml), was capacitated. Parrish *et al.* (1988) showed that glycoseaminoglycans (GAG), including heparin, can induce acrosome reaction in bovine spermatozoa and improve both the frequency and quality of fertilization in vitro. From the present study, however, it remains a question as regards to the different heparin concentrations which can produce optimum capacitation. Parrish *et al.* (1988) found that the fertilization frequency achieved with semen from different bulls and with different heparin concentrations have been variable. Beside this, it is worthy of studying also the use of other methods of capacitation including employing high ionic strength (Brackett *et al.*, 1982; Bondioli and Wright, 1983), bovine follicular fluid (Fukui *et al.*, 1983; Sanbuissho and Threlfal, 1989), caffein (Aoyagi *et al.*, 1988) and calcium ionophore (Byrd, 1981; Ben-Av *et al.*, 1988).

The present study has demonstrated the importance of oviductal epithelial cell for both IVF and IVC. However, what is more important is not the epithelial cells *per se*, rather, the possible secreted growth promoting factor (s) and/or the removal of embryo-toxic factor(s) from the medium (Heyman *et al.*, 1987; Loutradis *et al.*, 1987). Therefore, research involving, for example, studies leading to the identification of such factor(s) is necessary, particularly

when obtaining the epithelial cells at the right stage of oestrous cycle and bacterial contamination are the two important problems commonly encountered by the present procedure.

The successful production of embryos from maturing oocytes in vitro (IVM) followed by IVF using frozen-thawed sperms and co-cultured with oviductal epithelial cells warrants further studies such as further development of embryos beyond 4-cell stage. The absence of further development beyond 4-cell stage implies that there is a developmental block between 4- to 8-cell stage. Prolonged 8- to 16-cell stage in sheep embryos in vivo has been reported (Crosby *et al.*, 1988). In goat also such phenomenon has been reported (Sakkas *et al.*, 1989). However, in those studies, the embryos were obtained through in vivo fertilization instead of in vitro as was obtained by the present study. The differences in the procedure of whether IVF or in vivo should be considered because in hamsters, the 2-cell block was reported only in vitro and not in vivo (Nakayama *et al.*, 1993) . The prolonged cleavage stage has also been demonstrated in mouse at the 2-cell stage (Goddard and Pratt, 1983) and in pigs at 4-cell stage (Bavister, 1988).

The mechanisms of the prolongation of cleavage stage is still not clear, however, this period appears to coincide with the timing of the switch from maternal to embryonic

genome control (Crosby *et al.*, 1988; Telford *et al.*, 1990). Using light microscope autoradiography, [^{3}H]uridine incorporation was first detected in the nucleoplasm and nucleoli at the end of the 8-cell stage, suggesting that both pre-mRNA and rRNA synthesis are initiated at this stage (Camous *et al.*, 1986). It is also suggested that there is a loss or decay of mRNA molecules of maternal origin, developmental arrest in the presence of transcriptional inhibitors, and qualitative changes in protein synthetic patterns from one stage to the next (Telford *et al.*,1990). Recently, Nakayama *et al.* (1993) suggested that the embryonic developmental block might be related to oxygen toxicity, which results from exposure to high oxygen concentrations and light exposure during the manipulation of oocytes and embryos.

The transfer of embryos (ET) to the recipient females should also be considered. Hitherto, the birth of offspring through the above procedures has never been reported. The birth of kids reported by Hanada (1985) and Jufen *et al.* (1991) was a result of IVF procedure using oocytes matured in vivo. Attempts to transfer the in vitro fertilized embryos obtained from in vitro matured goat oocytes has been carried out by Younis *et al.* (1991; 1992) but in both studies the pregnancies did not continue to term.

The importance of IVM medium other than TCM 199 for the maturation of oocytes, and IVF and IVC media

other than m-Kreb for the fertilization of oocytes and culture of embryos, respectively, should also be considered. This is to determine which medium can produce the highest maturation, fertilization and cleavage rate. In their studies on IVF in goat, Younis *et al.* (1991) compared three different media, namely, modified defined medium (Brackett and Oliphant, 1975), modified TALP (Parrish *et al.*, 1986) and modified hepes-buffered M 199, and found that the first medium produced the best result whether oocytes were matured in vivo or in vitro.

The present study used body temperature of goat (39.0°C) rather than 37.0°C as most IVF in mammals was performed (Lavy *et al.*,1988). However, Eng *et al.*(1986) sug gested that the optimal fertilization occurs at body temperature rather than an arbitrary 37.0°C. Nevertheless, it is not possible from the present study to ascertain which temperature would produce better results. Therefore, further studies involving the comparison between these two sets of temperature in IVMFC procedures are warranted.

Finally, despite constraints and limitations right from slaughter house, government, species interest, existing knowledge, financial, laboratory facilities and countries research status, the present study has revealed and contributed significantly on general pool of knowledge on IVM-IVF-IVC in goats. The objective of the study to

evelop procedures for the maturation and fertilization of ne oocytes has been achieved: morphological finding togeth- r with chromosomal evident on mature oocytes has shown a ignificant result; the fertilizability of matured oocytes as confirmed by the cleavage of embryos following further mbryonic incubation. The ultimate proof of success is the birth of kids after transfer of in vitro fertilized embryos o recipient mother, and this should be a next logical step. or this end, we still embarking on this project, and in he forseeable future it is not impossible that the knowl- edge on this species would be of the same status with that of cattle and sheep.