Chapter 1

1.0 INTRODUCTION

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1.1 BACKGROUND

In vitro embryo production (IVEP) technology has been widely used to increase the population of superior genetic merit of livestock animals including goats. To achieve optimised success, sufficient number of oocytes is the pre-requisite for various reproductive techniques related to *in vitro* production procedures. In order to obtain abundant source of oocytes for large-scale production of embryos by *in vitro* production procedure, getting ovaries from abattoir is one of the ways (Agrawal *et al*., 1995). However, in Malaysia, the number of female goats slaughtered is few and consequently it is quite difficult to carry out proper experiments in goat biotechnologies. Another source of oocytes could be alternatively obtained through laparoscopic oocyte pick-up (LOPU). It has been shown that the ovaries could be hyperstimulated several times and followed by aspiration of oocytes for various embryo techniques (Baldassarre *et al.*, 2007).

There are 3 main steps involved in the method of *in vitro* production of embryos, namely maturation of primary oocytes from large antral follicles, fertilisation of matured secondary oocytes with frozen-thawed semen and *in vitro* culture (IVC) of the putative embryos for up to 7 days until the formation of blastocysts. Conventionally, *in vitro* production in goats involves the superstimulation procedure in matured female animals either for flushing of embryos and oocytes or laparoscopic oocyte pick-up. Various gonadotrophins such as follicle stimulating hormone (FSH), pregnant mare's serum gonadotrophin (PMSG), equine chorionic gonadotrophin (eCG) and human chorionic gonadotrophin (hCG) have been introduced to the hormone regime for superovulation and superstimulation procedures. There are several methods to collect

the *in vivo* embryos. Collection of late stage embryos by laparotomy involves the flushing of uterine horns with flushing medium. Due to the post-operative adhesions that frequently occur, this procedure allows only 2 to 3 collections per goat. However, according to Baril *et al*. (1996), laparoscopic embryo collection is less invasive and thus allows up to 7 laparoscopic sessions. Another way of collecting embryos non-surgically is via cervix using a rigid catheter which is described by Sohnrey and Holtz (2000).

Caprine embryos *in vitro* could be obtained from various reproductive techniques such as *in vitro* fertilisation (IVF) (Amir, 2006; Phua, 2006; Chan, 2008; De *et al.*, 2011), intracytoplasmic sperm injection (ICSI) (Jimenez-Macedo *et al*., 2007; Rahman, 2008; Kong, 2010) and somatic cell nuclear transfer (Liu *et al*., 2010; Abdullah *et al*., 2011; Wang *et al*., 2011). For the caprine *in vitro* fertilisation technique carried out in our laboratory, the *in vitro* embryo developmental competence was satisfactory. For examples, Phua (2006) obtained 42.6% embryo cleavage rate, while Amir (2006) and Chan (2008) obtained 9.9% and 39.0% of morula, respectively. In other laboratory, De *et al.* (2011) recently reported to obtain 68.0% hatching blastocyst in the caprine. Therefore, it is apparent that for their studies are needed to improve the performance of *in vitro* fertilisation in the caprine.

In order to achieve successful *in vitro* production systems, oocyte quality is one of the important factors. The assessment of oocyte quality is, therefore, crucial to distinguish between developmentally competent and incompetent oocytes for the use of subsequent assisted reproductive technology (ART) techniques. A simple and convenient method of oocyte morphological assessment is based on cumulus cell criterion to determine the competence of an oocyte.

A reduction of the period of sperm-oocyte exposure has been suggested to increase the incidence of monospermy (Coy *et al*., 1993; Ocampo *et al*., 1994a). In fact, most of the current *in vitro* fertilisation systems use 5 to 6 hours sperm-oocyte

insemination duration (Abeydeera and Day, 1997a; Funahashi *et al*., 1999; Wang *et al*., 1999; Abeydeera *et al*., 2000; Gil *et al*., 2003), compared to 12 to 18 hours insemination duration used in the original porcine *in vitro* fertilisation systems (Iritani *et al*., 1978; Nagai *et al*., 1983; Toyoda *et al*., 1984). Such information is not readily available in the literature and this issue is still controversial.

Parthenogenetic activation (PA) of mammalian oocytes has been induced by electrical and/or chemical methods to elevate Ca^{2+} levels, followed by the application of an inhibitor of protein phosphorylation (6-dimethylaminopurine, 6-DMAP) or an inhibitor of protein synthesis (cycloheximide) to accelerate and enhance the formation of pronuclei in non-aged metaphase II oocytes. The electrical method involves electrical pulses while chemical method involves either ethanol or ionomycin (Presicce and Tang, 1994; Cibelli *et al*., 1998; Leal and Liu, 1998; De Sousa *et al*., 1999, Wells *et al*., 1999; Zakhartchenko *et al*., 1999). In our laboratory, we are now able to produce cleaved murine and caprine parthenogenetic embryos (I. Siti Khadijah, research postgraduate scientist of ABEL laboratory, personal communication, 2011).

During the 1970s, the cryopreservation of murine and bovine embryos was developed (Whittingham *et al*., 1972; Wilmut and Rowson, 1973). The technique has since been modified to cryopreserve embryos from other species at different developmental stages, *in vitro* produced embryos as well as cloned or biopsied embryos (Massip, 2001; Dobrinsky, 2002; Kasai, 2002; Squires *et al*., 2003; Kasai and Mukaida, 2004; Guignot, 2005). Vitrification method implies the use of high concentration cryoprotectants and an extremely rapid cooling rate. By this, a glass-like solidification of liquids without ice crystals is formed and thus preventing cell damage (Rall, 1987; El-Gayar and Holtz, 2001). Bilton and Moore (1976) were the first to successfully cryopreserve caprine embryos. The first successful transfer of vitrified caprine embryos was reported by Yuswiati and Holtz (1990) by using a standard vitrification protocol

with ethylene glycol (EG) and propanediol in straw (0.25 ml). El-Gayar and Holtz (2001) obtained the first successful transfer of caprine embryos using open pulled-straw (OPS) technique. There were various methods for embryo vitrification utilised in the past, for example, straws (Baril *et al*., 2001), electron microscopy grids (Martino *et al*., 1996), open pulled straw (OPS) (Vajta *et al*., 1997), cryo-tops (Kuwayama and Kato, 2000), cryo-loops (Lane *et al*., 1999) or the tips of micropipettes (Cremades *et al*., 2004).

The successful rates of vitrification using straws obtained in earlier vitrified-devitrified study by Baril *et al.* (2001) were morula to early blastocyst (33.0%) and blastocyst to expanded blastocyst (55.0%) in sheep. Martino *et al.* (1996) used electron microscopy grids for bovine oocytes and obtained satisfactory cleavage rate (30.0%) after vitrified-devitrified of *in vitro* fertilised embryos and half of these developed into blastocysts (15.0%). Al Yacoub *et al*. (2010) obtained 70.0% embryo survival rate after vitrification using open pulled straw in caprine blastocyst. The hatching rate of bovine blastocyst after vitrification-devitrification was 41.0% using cryotop (Abdalla *et al*., 2010). Lane *et al.* (1999) successfully obtained blastocyst to hatching blastocyst (95.5%) after vitrification using cryoloops. Gibbons *et al.* (2011) obtained 64.0% caprine embryo survival rate after vitrification of blastocyst by using tips of micropipettes. In our laboratory, some efforts to cryopreserve embryos using mouse models were made such as conventional freezing (Hashim, 1996), quick freezing (Ramli, 1996), direct plunging (Ramli, 1995) and vitrification (Sarbandi, 1994).

1.2 STATEMENT OF PROBLEMS

1) Which internal and external factors affect the performance of *in vitro* production of embryos in caprine and bovine?

2) Are there any differences in oocytes quantity and quality between those obtained from abattoir and recovered from laparoscopic oocyte pick-up?
3) What are the differences in the developmental mechanisms of oocytes and embryos between *in vitro* fertilisation produced and parthenogenetic activation produced embryos?
4) How to minimise the toxicity to the embryos due to high cryoprotectant concentration exposure during cryopreservation protocol?
5) Which cryoprotectant combinations give the optimal survival rate and the least toxicity?
6) What is the optimal exposure duration to cryoprotectant for vitrification of caprine and bovine embryos?
7) How can *in vitro* fertilisation performance still be improved in bovine and caprine species?

1.3 JUSTIFICATION

Goat production and commercialisation is given high priority worldwide including Malaysia. This upsurge in interest is due to its economic and social importance for the production of diversified animal products such as meat, milk and fibre. Moreover, many types of forages can be used for goats as they are able to tolerate unfavourable climates and adverse environments. In addition, goats have short gestation period (5 months), small size, easy to breed and relatively high milk production and good meat quality for human consumption. In order to increase the population of genetically superior goats at a rapid rate, application of reproductive techniques such as *in vitro* fertilisation and embryo cryopreservation is essential in animal production in a country such as Malaysia that has a low population of goat (less than 500,000 heads).

The continuous improvements of *in vitro* production and vitrification of caprine embryo techniques are crucial to achieve high success rate of embryo transfer to produce offspring for the industry. *In vitro* fertilisation programmes vary from one laboratory to another, for example, media and protocols should be suited to specific laboratories due to intrinsic and extrinsic factors typical for the laboratories, and thus causing different outcomes. As a result, caprine *in vitro* embryo production systems have been increasingly refined over the years, developed for specific laboratories.

The *in vitro* fertilisation system of goat has not achieved optimal, and not yet well developed although sperm acts as a natural activator of oocytes, and hence should be the most efficient. In bovine *in vitro* fertilisation where the system is better developed, percentage of blastocyst development obtained from oocytes that were activated with a calcium ionophore (CI) followed by 6-dimethylaminopurine was similar to that of *in vitro* fertilisation controls (Liu *et al*., 1998a). Oocytes that undergo parthenogenetic activation could potentially develop to blastocyst, whereas the competent oocytes that undergo *in vitro* fertilisation have to overcome the various deficiencies that come along, for example sperm quality and capacitation.

Vitrification protocol is simpler and faster to complete compared to other embryo cryopreservation protocols such as conventional freezing, quick freezing and direct plunging. In other words, the vitrification process is easy to master and needs little skill as vitrification involves simply by transferring the embryos between 2 vitrification solutions, loading them into the vitrification device and finally plunging them into liquid nitrogen for storage. Moreover, the formation of ice crystal which causes the cell injury can be minimised or prevented. In addition, vitrification is cost effective as there is no expensive equipment needed for the implementation of vitrification procedure.

1.4 APPLICATION

In vitro production technology increases the embryos from high genetic value female as oocytes can be recovered from prepubertal, pregnant, dead or slaughtered animals. Besides, low cost embryos can be provided for basic research, embryo biotechnology studies and all kinds of embryo research, which need high number of embryos for manipulation.

Cryopreservation allows the usage of multiple ovulation and embryo transfer (MOET) schemes more widely to increase the genetic gain, resulting from selection. Moreover, the populations threatened by extinction can be conserved. In addition, embryo cryopreservation eases the transportation of superior breed of animals as well as aids in solving the infertility problems.

The parthenogenetic activation of oocytes serves a tool to investigate the comparative roles of paternal and maternal genomes in controlling early embryo development. Moreover, parthenogenetic activation is related to cloning research as the artificial activation of oocytes is an important component of nuclear transfer protocols (Kim *et al*., 1996). Therefore, an optimised activation protocol may enhance better or complete reprogramming of the reconstructed embryo and thus increases the success rate in cloning.

1.5 OBJECTIVES

The main objectives of the present study were to obtain sufficient amount of immature oocytes via laparoscopic oocyte pick-up performed on superovulated donor goats, ovariectomy and abattoir source for caprine and bovine for *in vitro* maturation, fertilisation and culture (IVMFC) as well as parthenogenetic activation experiments. An attempt was also made to cryopreserve embryos using vitrification technique. This study was conducted according to the following specific objectives:

a) To produce bovine and caprine embryos *in vitro* using *in vitro* fertilisation technique.

b) To produce bovine and caprine embryos *in vitro* using parthenogenetic procedure.

c) To cryopreserve caprine embryos with bovine embryos as samples for learning curve using cryoleaf as carrier tool in vitrification technique.

d) To determine the effect of optimal duration of cryoprotectant exposure duration on embryo vitrification performance in caprine (bovine as a model animal).

e) To determine the effect of different insemination duration on *in vitro* fertilisation performance in bovine and caprine.

f) To determine the effect of oocyte grading on bovine and caprine *in vitro* fertilisation and parthenogenetic activation derived embryos.

Chapter 2

2.0 REVIEW OF LITERATURE

Chapter 2

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2.1 BACKGROUND

Table 2.1 shows the significant milestones in *in vitro* maturation, fertilisation and culture (IVMFC), parthenogenetic activation and vitrification in caprine and bovine.

Table 2.1: Timeline of selected significant findings of oestrus synchronisation, oocyte recovery, *in vitro* maturation, fertilisation and culture, parthenogenetic activation and vitrification in caprine and bovine

Year	Author	Species	Significant finding
1998	Iwata *et al.*	Bovine	Free oxygen radical generation was induced by high glucose concentration and that low glucose concentration was beneficial for embryo development.
1998	Romano	Caprine	62.5 mg cloprostenol by the intramuscular route was luteolytic in Nubian goats, and that a scheme of 2 doses 12 days apart should be an effective method for oestrus synchronisation.
1999	Regueiro *et al.*	Caprine	Using eCG to increase little size in goats may be influenced by body weight and breed.
2001	El-Gayar and Holtz	Caprine	Tested the suitability of the open pulled-straw (OPS) method applied from bovine for vitrifying caprine embryos and found the OPS vitrification was a suitable method for cryopreserving goat day 7 blastocysts.

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Year	Author	Species	Significant finding
2001	Kusina *et al.*	Caprine	Ovarian activity was reduced in goat does fed below maintenance requirements and feeding above maintenance could lead to poor reproductive performance.
2001	Ongeri *et al.*	Caprine	Oocytes activated with either ethanol or ionomycin followed by incubation in 6-DMAP resulted in higher blastocyst development than IVF controls.
2001	Rho *et al.*	Caprine	Percoll density-gradient centrifugation was superior method for separating caprine sperm from frozen-thawed semen in preparation for IVF.
2004	Nedambale *et al.*	Bovine	Replacing KSOM with SOF after 4 days of culture produced better quality blastocysts.
2005	Simone *et al.*	Bovine	Sr may be efficiently applied for bovine oocyte activation at 20 mM in Ca^{2+}- and Mg^{2+}-free TALP medium for 6 hr.
2006	Huang *et al.*	Caprine	Vitrified *in vivo* derived caprine embryos (morula and blastocyst) by the microdrop method obtained similar survival rate with the OPS method.

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Year	Author	Species	Significant finding
2006	Nedambale *et al.*	Bovine	IVF-M199 was successfully used for IVF, compared favourably to BO medium, and offered the advantage of an extended IVF period for up to 18 hr that requires only one-half a dose of semen, and resulted in better quality blastocysts that endured vitrification with a hatching rate comparable to that of control groups.
2007	Bhojwani *et al.*	Bovine	G6PDH activity could prove to be a useful marker for determining the oocyte quality.
2007	Bhuiyan *et al.*	Bovine	Fructose up to 5.6 mM concentration could be used as an alternative for energy substrate in culture media without any detrimental effect on pre-implantation development in bovine IVF embryos.
2007	Katska-Ksiazkiewicz *et al.*	Caprine	The morphological quality of COCs appeared to be the most important factor that influenced the efficiency of *in vitro* embryo production in goats.
2007	Rodríguez-Dorta *et al.*	Caprine	The co-culture of IVP caprine embryos with GOEC significantly improved the pregnancy and embryo survival rates leading to the birth of healthy offspring.

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Year	Author	Species	Significant finding
2008	Hosseini *et al.*	Bovine	Exposure of IVM bovine oocytes to electrical pulse, followed by exposure to CI-6-DMAP or ET-SR-6-DMAP could be regarded as the optimal artificial activation protocol for *in vitro* development of parthenogenic bovine oocytes.
2008	Wang *et al.*	Bovine	ET+CHX+CB treatment was more favourable protocol for parthenogenesis of bovine oocytes.
2009	Dhali *et al.*	Bovine	Follicle size for oocyte aspiration was effective for determining IVC success and that IVF may not discriminate among bulls of different field fertility.
2009	Karaca *et al.*	Caprine	Divided multiple injections of a total of 950 IU eCG are effective without progestagen pre-treatment in the induction of oestrus and obtaining successful pregnancy and live kids in the Coloured Mohair goats during the anoestrus season.
2009	Kharche *et al.*	Caprine	The use of fatty acid free albumin resulted in a significantly higher cleavage rate, compared to unmodified albumin, and the supplementation of OGS (20%) in the fertilisation medium, significantly increased the cleavage rate of IVM caprine oocytes, compared to defatted albumin.

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Year	Author	Species	Significant finding
2009	Lehloenya and Greyling	Caprine	The route of superovulatory hormone administration had no effect on the initial oestrus response.
2011	De *et al.*	Caprine	The cleavage rate and blastocyst yield can be successfully increased by cysteamine supplementation in IVM medium or IVC medium, or both IVM and IVC media, without compromising the quality of the zygote.
2011	Leivas *et al.*	Bovine	Adding FCS to the culture medium increased the efficiency of IVP of bovine embryos.

2.2 OESTRUS SYNCHRONISATION AND SUPEROVULATION

Oestrus synchronisation is a useful management tool that has been successfully employed in enhancing reproductive efficiency, especially in goats (Vilariño *et al*., 2011; Zarazaga *et al*., 2011). The mean advantage of oestrus synchronisation is to facilitate a large number of does to be bred over a short period of time and, therefore, the management of animals could be organised systematically to meet the demand of goat industry. Oestrus synchronisation would also allow the schedule of kidding to take advantage of feed supplies, labour, upward price trends and implementation of breeding technologies such as artificial insemination (AI). Oestrus synchronisation not only benefits the large animal breeding establishments, but also brings benefit to the small holder producers.

2.2.1 Methodology of Oestrus Synchronisation and Superovulation in Goats

During the anoestrus season in goats, some methods have been introduced to induce the oestrus. They are both hormonal and non-hormonal treatments. Hormonal treatment involves the injection of hormones to the does. On the other hand, the sudden introduction of sexually active bucks (Veliz *et al*., 2002) and the use of an artificially altered photoperiod (BonDurant, 1986) are non-hormonal methods.

In non-seasonal breeding goats, intravaginal progesterone sponges, norgestomet ear implants, cloprostenol or a combination of progesterone sponges and cloprostenol are effective in synchronising oestrus (Kusina *et al*., 2000). Due to the treatment of anoestrus goats with progestagen alone is known to be inadequate in inducing either a behavioural oestrus or a luteinising hormone surge with a consequent ovulation, progestagen treatment is proposed to be followed by gonadotrophin administration 48 hours prior to sponge removal in order to stimulate oestradiol secretion by the ovary (Ritar *et al*., 1984; Gordon, 1997; Zarkawi *et al*., 1999). According to Armstrong *et al.* (1983a), when goats are treated with eCG (1000 IU) subcutaneously on day 12 of the oestrous cycle without progestagen pre-treatment and synchronised by cloprostenol (50 mg) applied 2 days after the start of gonadotrophin treatment, the incidence of large follicles that fail to ovulate is significantly elevated. Also, many of ovulated ones of these goats exhibit luteal phase failure.

Administration of cloprostenol (125 to 250 pg) 10 days apart induced oestrus within 18 to 48 hours in nondescript indigenous goat breeds (Perera *et al*., 1978; Taylor, 1978). The percentages of Red Sokoto does being synchronised with the administration of 2 doses of PGFlα 10 days apart and by using intravaginal medroxyprogesterone acetate pessaries were 84% and 91 to 95%, respectively (Molowuku *et al*., 1980; Karatzas *et al*., 1997). Potent analogues of progesterone, mainly Cronolone (flurogesterone acetate, FGA) and methylacetoxyprogesterone (MAP) have been

incorporated into commercial intravaginal sponges and devices as they are effective in small amounts. The use of progestagens has been found to have no effects in goats (Molowuku *et al*., 1980; Akusu and Egbunike, 1984; Robin *et al*., 1994).

The donor goats for laparoscopic oocyte pick-up (LOPU) session were conventionally synchronised by means of intravaginal sponges containing medroxyprogesterone acetate (60 mg) (MGA, Veramix®, Upjohn, Canada; progesterone) inserted 10 days prior to laparoscopic oocyte pick-up and an injection of Estrumate® (125 ug) (Malinkrodt, Canada; prostaglandin) on the morning of the day 8. Sponges were removed at the time of laparoscopic oocyte pick-up. There are 2 methods of hormone administration, which are multi-injection and "one-shot" injection. In the multi-injection regime, the goats received a total equivalent to NIH-FSH-PI of Folltropin®-V (133 mg) (Vetrepharm, Canada; FSH) given twice daily over the 48 hours prior to laparoscopic oocyte pick-up. In the "one-shot" regime, goats were injected with NIH-FSH-PI of Folltropin®-V (80 mg) and equine chorionic gonadotrophin (300 IU) (Equinex®, Ayerst, Canada; eCG; FSH) in a single application at either 36 or 48 hours prior to laparoscopic oocyte pick-up. By using this protocol, a total of 21, 219 oocytes was recovered from 1580 laparoscopic oocyte pick-up procedures (13.4 oocytes per goat) which represent an average recovery rate of around 80% (Baldassarre and Karatzas, 2004).

According to Koeman *et al.* (2003), oestrus was synchronised in goats with intravaginal sponges (Veramix1, Upjohn Co., Orangeville, Ont., Canada), containing medroxyprogesterone acetate (60 mg), inserted for 10 days prior to oocyte collection. Goats were given cloprostenol (125 mg) intramuscularly (Estrumate®, Schering Canada Inc., Pointe-Claire, Que., Canada), equine chorionic gonadotrophin (eCG) (300 IU) intramuscularly (Folligon®, Intervet Canada Ltd., Whitby, Ont., Canada) and NIH-FSH-

PI (80 mg) intramuscularly (Folltropin® -V, Vetrepharm Canada Inc., London, Ont., Canada) at 36 hours before oocyte collection.

In order to carry out the laparoscopic oocyte pick-up, there was another procedure of using different hormones and synchronisation method. According to Abdullah *et al.* (2008), oestrus was synchronised with an intravaginal controlled internal drug release device (CIDR, progesterone, 0.3 g; Pharmacia and Upjohn Ltd., Auckland, New Zealand) for 10 days, with a cloprostenol (Estrumate, 125 mg; Schering-Plough, NSW, Australia) 36 hours prior to controlled internal drug release device removal. At device removal, does were given intramuscular treatments of Ovagen (follicle stimulating hormone, 70 mg; Immuno Chemicals Products Ltd., Auckland, New Zealand) and Ovidrel (human chorionic gonadotrophin, 500 IU; Serono, Switzerland), and were allocated to undergo laparoscopic oocyte pick-up. It was found out that the fewest oocytes per goat were retrieved at 36 hours, and the proportion of Grade A oocytes was greater as the interval from follicle stimulating hormone (FSH)/human chorionic gonadotrophin (hCG) to laparoscopic oocyte pick-up increased. Similarly, rates of maturation, cleavage, and morula formation increased significantly as the interval from hormonal treatment to laparoscopic oocyte pick-up was prolonged to 60 to 72 hours.

Superovulation in goats is frequently restricted by the cost of gonadotrophin or the handling requirements. The major commercial products applied are equine chorionic gonadotrophin or pregnant mare's serum gonadotrophin (PMSG) and follicle stimulating hormone used in higher doses to elicit a superovulatory response. Pregnant mare's serum gonadotrophin is used to stimulate ovarian activity during seasonal anoestrus (Gordon, 1997), and is usually used concurrently following oestrus synchronisation. In most countries, pregnant mare's serum gonadotrophin is available and tended to be a practical hormone to administer for superovulation (Amoah and Gelaye, 1990). Pregnant mare's serum gonadotrophin is preferred for its lower cost and

easy availability; it can be more easily administered than follicle stimulating hormone, usually as a single injection (1500 to 2000 IU), but the superovulatory response to pregnant mare's serum gonadotrophin can be quite variable, and is usually lower than in a follicle stimulating hormone-induced superovulation (Amoah and Gelaye, 1990). Problems associated with pregnant mare's serum gonadotrophin-induced superovulation include a high number of non-ovulated follicles, early regression of corpus luteum, short or irregular oestrous cycles and embryo expulsion (Amoah and Gelaye, 1990).

In order to superovulate does, a combination of equine chorionic gonadotrophin and human chorionic gonadotrophin has also been widely used (Medan *et al*., 2003). Follicle stimulating hormone is a better choice than pregnant mare's serum gonadotrophin of in terms of superovulating does, as it provides more stimulated oocytes. Follicle stimulating hormone is usually administered in decreasing doses of 1 to 5 mg, injected at 12 hours intervals over a period of 3 to 5 days around the time of termination of the progestagen treatment. There were a number of experiments have been performed to compare the superovulatory response between follicle stimulating hormone and pregnant mare's serum gonadotrophin, in which the evidence favoured the use of follicle stimulating hormone than pregnant mare's serum gonadotrophin (Tsunada and Sugie, 1989; Pendleton *et al*., 1992). Tsunada and Sugie (1989) reported that average number of oocyte revovery (OR) per doe was significantly higher in follicle stimulating hormone-treated does (9.4) than that of pregnant mare's serum gonadotrophin-treated (5.7).

A route of administering a superovulation treatment contributes to variation in superovulation response, as it determines the rate of gonadotrophin absorption (Dobbs *et al*., 1994). The superovulatory treatment (follicle stimulating hormone) is generally administered intramuscularly (i.m.) in most trials conducted in goats. There is very few information on goats, regarding the route of the superovulatory gonadotrophin treatment

administration (Mahmood *et al*., 1991; Pendleton *et al*., 1992; Selvaraju *et al*., 2003; Goel and Agrawal, 2005). According to Dobbs *et al*. (1994), the route of gonadotrophin administration could be vital in the superovulatory treatment timing, positioning of the time of ovulation and determining the superovulation efficiency.

Synthetic analogue of luteolytic agents such as cloprostenol (62.5 µg) was shown to be effective in synchronising goats via intravulvo-submucossal route (Mgongo, 1987, 1988). These reports were from different region to region, that normally use completely different breeds. For example, Mason (1981) reported that different ovulation rates obtained for different breeds of goats: 1.2 in Angora goat (Shelton, 1969); 1.4 to 1.7 in Boer goat (Greyling *et al*., 1985; Greyling and van Nierkerk, 1990) and 1.7 in African goat (Dhindsa *et al*., 1971).

Superovulation of donors involves the usage of multiple injections of follicle stimulating hormone (Nuti *et al*., 1987; Krisher *et al*., 1994) or with large doses which is usually more than equine chorionic gonadotrophin (1000 IU) (Battye *et al*., 1988; Cameron *et al*., 1988; Kumar *et al*., 1991). There were some research showing follicle stimulating hormone was more superior compared to equine chorionic gonadotrophin in both superovulation and embryo recovery in goats (Armstrong *et al*., 1983a; Pendleton *et al*., 1992; Rosnina *et al*., 1992). However, low doses of equine chorionic gonadotrophin are commonly used under field condition with large herds because of the ease of administration (only one injection) and low cost.

Several reports claimed that by using doses of equine chorionic gonadotrophin that ranged from 200 to 600 IU gave good results (Greyling and Van Nierkerk, 1990; Ritar *et al*., 1994; Menegatos *et al*., 1995; Freitas *et al*., 1996; Selvaraju and Kathiresan, 1997). The usage of large dose of equine chorionic gonadotrophin causes early luteal regression and subsequently shorten the oestrous cycles (Armstrong *et al*., 1983b;

Pendleton *et al*., 1992; Rosnina *et al*., 1992) which could possibly be due to the long half-life of equine chorionic gonadotrophin.

2.2.2 Age of Donor

Embryo competence from oocytes of prepubertal females is lower than that of adult females (Armstrong, 2001). Izquierdo *et al.* (2002) obtained 10% of blastocysts using oocytes from 2 months old females from abattoir source and 8% of blastocysts was obtained by Koeman *et al.* (2003) with laparoscopic oocyte pick-up sourced oocytes from 2- to 5- months old hormonally stimulated goats. There was only a small proportion of the oocytes recovered from 2 to 3 mm diameter follicles can support embryonic development as the capacity to complete cytoplasmic maturation develops beyond the acquisition of meiotic competence (Crozet *et al*., 1995). This was proven by their results of obtaining 6% goat blastocyst production with oocytes from follicles of 2 to 3 mm in adult goats.

Crozet *et al.* (1995), Keskintepe *et al.* (1998) and Cognie *et al.* (2003) obtained 26%, 32% and 36% blastocysts from adult goat oocytes, respectively. This proved that the percentage of blastocysts obtained from oocytes of adult goats is higher than those oocytes of prepubertal goat. The factors leading to these results are the lower male pronucleus formation (Mogas *et al*., 1997b), a high rate of haploid (Villamediana *et al*., 2001), polyspermic zygotes (Mogas *et al*., 1997b), abnormal distribution of cortical granules (Velilla *et al*., 2004) and mitochondrial morphology (Velilla *et al*., 2006) that have been found in oocytes from prepubertal females.

2.3 RETRIEVAL OF OOCYTES AND EMBRYOS

2.3.1 Laparoscopic Oocyte Pick-up (LOPU)

Laparoscopic oocyte pick-up is the starting point for the *in vitro* embryo production. It allows multiple recoveries of oocytes from the same donor animal as laparoscopic oocyte pick-up is less invasive compared to the laparotomy surgery used for the recovery of *in vivo* zygotes and *in vivo* matured oocytes. The laparoscopic oocyte pick-up technique is very reliable and allows for the recovery of a predictable number of oocytes during each session. *In vitro* production of embryos using immature oocytes recovered by laparoscopy has the potential to overcome some of the problems associated with standard multiple ovulation and embryo transfer (MOET) techniques (Tervit, 1996; Baldassarre *et al*., 2002; Cognié *et al*., 2003), as the oocytes derived from laparoscopic oocyte pick-up are recovered from animals of known health status. While individual variation in the response to gonadotrophin treatment remains, laparoscopic oocyte pick-up, almost always results in more than 5 oocytes aspirated per donor (Baldassarre and Karatzas, 2004). Laparoscopic oocyte pick-up provides a good yield of oocytes as well.

For the laparoscopic oocyte pick-up procedure described by Baldassarre *et al*. (1994), donor goats are restrained on a standard laparoscopy table under general anaesthesia follow by the aspiration of follicles under laparoscopic observation using a 20 gauge needle mounted in a plastic pipette connected to a collection tube and vacuum line. In the hands of an experienced operator, 10 and 20 minutes were needed for the whole procedure per goat, depending on the number of follicles to be aspirated, which allows the recovery of over 100 oocytes in a 2 to 3 hours session.

2.3.2 Ovary Slicing and Follicle Aspiration

Caprine and bovine ovaries could be obtained from abattoir and ovariectomy. Conventionally, in adult goat ovaries, oocytes are recovered by follicle aspiration selecting follicles bigger than 3 mm diameter. From prepubertal goat ovaries, ovary slicing allows the collection of more oocytes per ovary than by follicle aspiration (n=6.05 and 1.27, respectively); however, the oocyte morphological quality is lower in the former (Martino *et al*., 1994b). According to Agrawal (1992), ovaries containing a corpus luteum have yielded lower numbers of oocytes than ovaries without a corpus luteum. Although ovary slicing has been found to be a more efficient method for recovering a high number of caprine cumulus oocyte complexes, there could be extra oocytes obtained from small follicles, in which these oocytes have been reported to be less developmentally competent after *in vitro* fertilisation (IVF) (Keskintepe *et al*., 1994a; Pawshe *et al*., 1994). When ovaries were sliced, cumulus oocyte complexes may be recovered not only from antral follicles on the surface (Alm *et al*., 1998) but also from smaller antral follicles from the inside of the ovary, which may be in earlier stages of follicular development after antrum formation (Bhojwani *et al*., 2007).

2.3.3 Classification and Selection of Oocyte

Immatured oocytes obtained from ovaries are routinely selected for *in vitro* maturation (IVM) by visual assessment of morphological features. Although morphological criteria provide reasonable means of identifying oocyte quality and suitability for *in vitro* maturation, there is evidence that morphological criteria are insufficient for the identification of oocytes that are competent for *in vitro* development to the blastocyst stage. Such a conclusion can be based on the results that showed frequent failures of up to 60% of *in vitro* matured-fertilised oocytes to reach the blastocyst stage *in vitro*, both in goats and in other mammalian species (Parrish *et al*., 1986; Crozet *et al*., 1995;

Katska-Ksiazkiewicz *et al*., 1996, 2004, 2005, 2006; Cognié, 1999; Galli *et al*., 2003; Pujol *et al*., 2004; Alm *et al*., 2005; Han *et al*., 2006; Nedambale *et al*., 2006). Brilliant cresyl blue (BCB) test was applied in a selection of prepubertal caprine oocytes competent for *in vitro* maturation (Rodriguez-González *et al*., 2002, 2003; Urdaneta *et al*., 2003). The test allows the determination of the activity of glucose-6-phosphate dehydrogenase (G6PDH) which is the enzyme synthesised in growing oocytes but inactive in oocytes that have finished their growth phase. According to Tian *et al.* (1998), Brilliant cresyl blue is a dye that can be broken down by glucose-6-phosphate dehydrogenase. Therefore, oocytes undergoing growth and those that have completed their growth phase will have different levels of glucose-6-phosphate dehydrogenase. The oocytes that have completed the growth phase are blue (BCB+) as the glucose-6-phosphate dehydrogenase activity is too low for stain reduction, while the growing oocytes become colourless (BCB-) due to enzyme activity in living oocytes. Rodriguez-González *et al.* (2002) have shown that Brilliant cresyl blue positive oocytes of prepubertal goat were larger in diameter (136.6±6.3 µm) and performed higher maturity and fertilisability rates (81.4 and 23.5%, respectively) compared to those that remained unstained (125.5±10.2 µm; 52.5 and 8.2%, respectively).

Several studies demonstrated that mitochondria number and quality are closely related to the fertilisability of the oocyte and to the developmental capacity of the embryo (Larsson *et al*., 1998; Huo and Scarpulla, 2001; Stojkovic *et al*., 2001; Hsieh *et al*., 2004; Van Blerkom, 2004; May-Panloup *et al*., 2005; El Shourbagy *et al*., 2006; Spikings *et al*., 2007). Spikings *et al.* (2007) strongly indicated that in incompetent BCB- oocytes, mitochondrial deoxyribonucleic acid (mtDNA) replication were delayed, resulting in either failed fertilisation or developmental arrest. Significant differences in nuclear-encoded regulatory protein expression were observed between BCB+ and BCB- oocytes. This indicates that mitochondria biogenesis and mitochondrial deoxyribonucleic

acid copy number can be used as a marker of oocyte competence. From the results obtained by Opiela *et al*. (2008), there was no significant difference in the blastocyst rate among embryos obtained from BCB+ and control oocytes.

Grading oocytes based on the cumulus investment and cytoplasmic distribution also performed by Kharche *et al*. (2009) in Table 2.2 as well as Li *et al*. (2009) in Table 2.3 and Figure 2.1, whereas the modified classification of oocytes from Blondin and Sirard (1995) was based on the expansion of cumulus and texture of ooplasm (Table 2.4). According to Habsah (2006), Groups A, B and B' were oocytes with compact and dense cumulus cell layers, oocytes with compact but not dense cumulus cell layers, and oocytes with thin or small remnants of cumulus cell layers or partially naked oocytes.

Table 2.2: Grading of oocytes based on the cumulus investment and cytoplasmic distribution (adapted from Kharche *et al*., 2009)

Oocyte grade	Cellular vestments and cytoplasm uniformity
Excellent (A)	Oocytes with more than 4 layers of compact cumulus cell masses with an evenly granulated cytoplasm.
Good (B)	Oocytes with at least 2 to 4 layers of compact cumulus cell masses with an evenly granulated cytoplasm.
Fair (C)	Oocytes with at least 1 complete compact layer of cumulus cell mass with an evenly granulated cytoplasm.
Poor (D)	Oocytes with no cumulus cell or an incomplete layer of cumulus cells or expanded cumulus mass with or without a dark or unevenly a granulated cytoplasm.

Table 2.3: Grading of oocytes based on the cumulus investment and cytoplasmic distribution (adapted from Li *et al*., 2009)

Oocyte grade	Cellular vestments and cytoplasm uniformity
GI	COCs with granular cytoplasm and more than 5 layers of cumulus cells
GII	COCs with homogeneous cytoplasm and more than 5 layers of cumulus cells
GIII	COCs with granular cytoplasm and between 3 and 5 layers of cumulus cells
GIV	COCs with homogeneous cytoplasm and 3 to 5 layers of cumulus cells

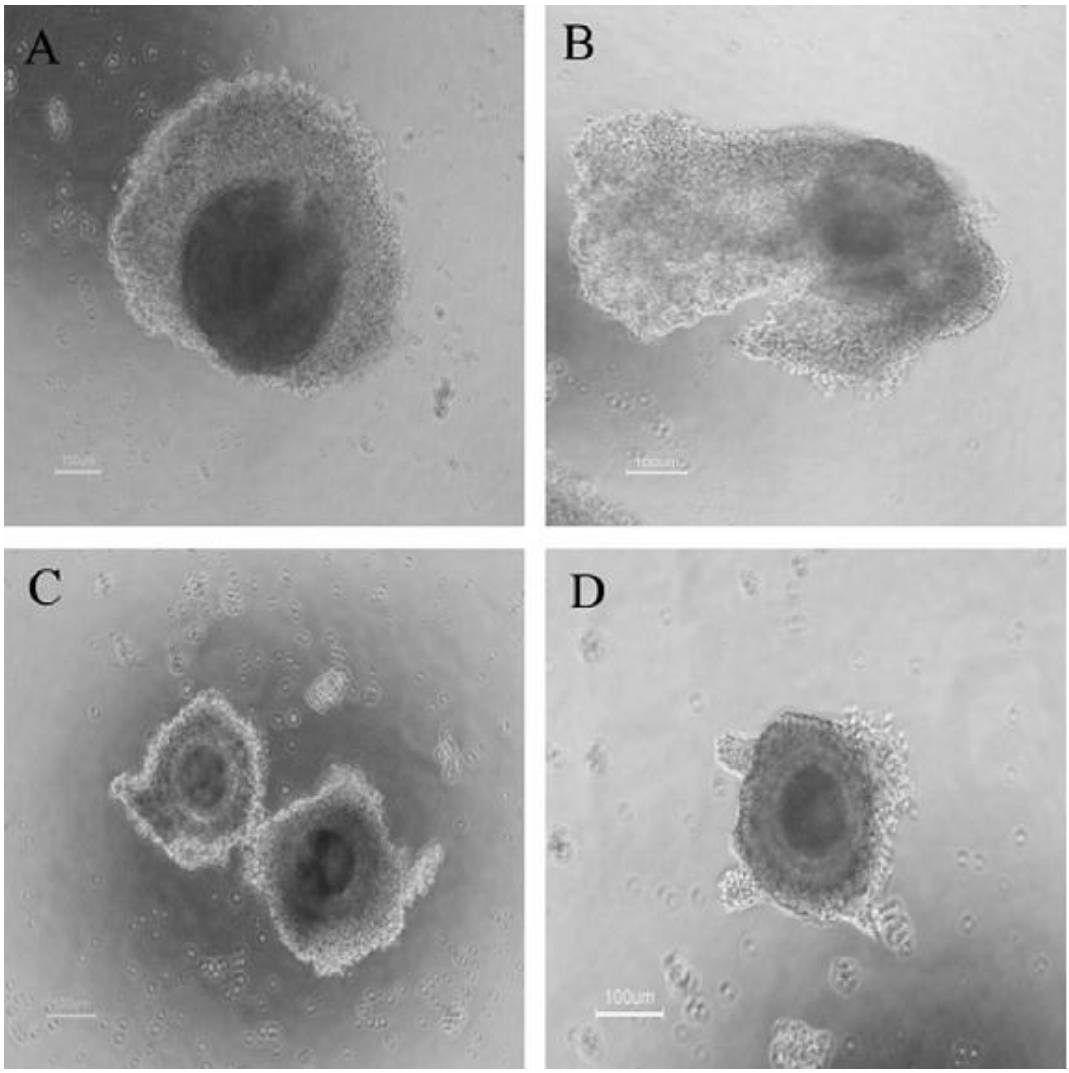

Figure 2.1: Gradings of oocytes (Table 2.3; adapted from Li *et al*., 2009).

Table 2.4: Classification of bovine COC[a]

Class	No. of cumulus layers	Expansion of cumulus	Texture of ooplasm
I	≥5	Compact	Homogeneous
II	≥5	Slight expansion AND/	OR Slight granulation
III	<5	No or slight expansion	Homogeneous
IV	<5	No or slight expansion	Granulation
V	0	No cumulus	Variable
VI	≥1	Full expansion with dark clumps	Heavy granulation

[a]Modified from Blondin and Sirard (1995); Adapted from Bilodeau-Goeseels and Panich (2002)

2.3.4 Embryo Flushing

A poor synchrony of ovulation after sponge withdrawal in follicle stimulating hormone-treated females is a possible cause of fertilisation failure. In goats, the potential for a gonadotrophin releasing hormone (GnRH) injection to synchronise ovulation and to increase the production of embryos is controversial. In a small-scale experiment, ovulation rate and the number of embryos obtained increased in goats treated with gonadotrophin releasing hormone 24 and 48 hours after the removal of progestagen (Akinlosotu and Wilder, 1993). In another trial, Krisher *et al.* (1994) reported an increase in ovulation rate in gonadotrophin releasing hormone-treated goats, but with a low fertilisation rate. There was another alternative which was the use of a gonadotrophin releasing hormone antagonist 12 hours after the removal of sponge, followed by a treatment with porcine luteinising hormone (pLH) 24 hours later to mimic the preovulatory surge (Baril *et al*., 1996).

According to Holtz (2005), the variability in number of ovulations and yield of viable embryos remains the main drawback. The variability was caused by both intrinsic and extrinsic factors. The intrinsic factors include genetic (Nuti *et al*., 1987), age (Mahmood *et al*., 1991) and stage of the cycle at which the treatment applied (Wani *et al*., 1990), whereas the extrinsic factors comprise of season, nutrition, health state and type of gonadotrophin administered. Therefore, vigorous research efforts are directed for the establishment of suitable superovulation regimes to augment the development of associated technologies based on them.

2.4 *IN VITRO* MATURATION (IVM)

2.4.1 Medium

The relatively low efficiency rate achieved after *in vitro* maturation of oocytes compared to *in vivo* produced is almost certainly related to the oocytes quality.

Acquisition of oocyte developmental competence occurs continuously throughout folliculogenesis. The follicular size and follicular atresia influence the developmental competence of the oocytes (Mermillod *et al.*, 1999). However, the heterogeneous nature of immatured oocytes maturation could also be influenced by the components of culture media and culture conditions used for *in vitro* maturation.

Most *in vitro* maturation system for oocytes commonly used hormones such as follicle stimulating hormone, luteinising hormone (LH) and oestradiol (E_2) in the medium and supplemented with foetal calf serum (FCS) (Moor and Trounson, 1977). Follicular fluid (FF) recovered from large follicles (>4 mm) could be used as a supplement in caprine oocytes *in vitro* maturation medium containing TCM 199 and follicle stimulating hormone (100 ng/ml). it has been shown that there was an enhancement effect when follicular fluid was recovered from non-atretic (Cognié *et al*., 1995b) or gonadotrophin-stimulated follicles (Cognié and Poulin, 2000).

The kinetics of oocyte nuclear maturation was modified and the blastocyst yield on day 8 post-insemination increased with the maturation medium supplemented with follicular fluid (10%), follicle stimulating hormone (100 ng/ml) and cysteamine (100 µM) (Cognie *et al*., 2003). Compared to the control medium without cysteamine supplementation, caprine cumulus oocyte complexes that were matured in defined medium (not containing serum or bovine serum albumin) containing TCM 199 and epidermal growth factor (EGF) (10 ng/ml) supplemented with cysteamine (50 to 100 µM) had significantly increased ($P<0.001$) in the intracellular glutathione content of oocytes after *in vitro* maturation as well as blastocyst yield (Cognie *et al*., 2003).

Bovine *in vitro* maturation under undefined or semi-defined conditions that other factors contained in follicular fluid, such as growth hormone (Izadyar *et al*., 1997), activin or inhibin (Stock *et al*., 1997), could stimulate oocyte maturation and that the inhibin α-subunit could decrease the developmental potential of oocytes (Silva *et al*.,

1999). Mizushima and Fukui (2001) have achieved significant higher rates of normal fertilisation and lower rates of polyspermy by adding hypotaurine and β-mercaptoethanol to the maturation medium of single oocytes, compared to the supplementation of hypotaurine alone. A high glucose concentration seemed to be beneficial for *in vitro* maturation under low oxygen tension conditions, whereas the fertilisability, developmental competence, and cryoresistance were improved with the addition of epidermal growth factor and/or cysteamine to the maturation medium. Besides, the blastocyst rate was not affected when epidermal growth factor was added to a group-*in vitro* production system, whereas epidermal growth factor addition clearly increased the number of blastocysts in the individual-*in vitro* production system (Oyamada *et al.*, 2004).

The *in vitro* culture (IVC) was maintained at a higher oxygen level (20%) than that of the *in vivo* environment (3 to 9%), hence, the development of mammalian oocytes *in vitro* was negatively affected by the increased oxidative stress occurring during the *in vitro* culture environment (Rangasamy *et al*., 2009). According to Takahashi *et al*. (1993), the increase in embryonic hydrogen peroxide production levels, attributable to *in vitro* culture, could be due to a species-specific block to embryonic development.

Glutathione (GSH), a major sulphydryl compound found in mammalian cells, protects the cell from these oxidative damages (Gasparrini, 2002). Cysteamine, a low molecular weight compound, has been shown to enhance cysteine mediated glutathione synthesis in bovine embryos (Takahashi *et al.*, 1993). The glutathione synthesis is subsequently enhanced by the addition of cysteamine in the maturation medium (De Matos and Furnus, 2000) and this improved oocyte maturation by protecting the oocytes from oxidative stress (Meister, 1983; Gasparrini *et al*., 2003). The supplementation of

cysteamine in the culture medium of oocytes has also been found to improve the *in vitro* maturation rate (Roushandeh *et al*., 2007).

The presence of insulin-like growth factor (IGF-I) and epidermal growth factor in the follicular fluid has been reported in several species at concentrations that are usually used for supplementation of the maturation media (Guler *et al*., 2000). The receptors that present in the granulosa cells or oocytes suggest the involvement of these factors in the maturation process of the oocytes (Watson *et al*., 1992). Epidermal growth factor is a polypeptide with potent mitogenic activity in several types of cells, both *in vivo* and *in vitro*. It was originally isolated from the sub-maxillary gland of mice, human urine and other sources.

During *in vitro* maturation in caprine cumulus cells, epidermal growth factor triggered the signaling mechanism via the mitogen activated protein kinase (MAPK) pathway (Gall *et al*., 2005). The epidermal growth factor receptors were expressed by caprine cumulus cells (Gall *et al.*, 2004). Therefore, epidermal growth factor was expected to act on the cumulus cells surrounding the oocyte undergoing maturation, and could possibly help in the oocyte maturation process. Using epidermal growth factor in caprine maturation media improved the maturation rate of oocytes (Nagar and Purohit, 2005).

2.4.2 Duration

The duration of *in vitro* maturation may play a critical role for subsequent development of the oocytes. This could be due to an inappropriate timing of maturation led to abnormal chromatin (Dominko and First, 1997b), oocyte aging (Hunter, 1989; Hunter and Greve, 1997) and reduced development (Marston and Chang, 1964). Even though sperm could penetrate oocytes prior to completion of oocyte maturation (Niwa *et al*., 1991; Chian *et al*., 1992), subsequent development rate was generally lower, hence, it

appeared that the optimum time for *in vitro* fertilisation was at completion of meiosis. It was known that the time necessary for accomplishment of maturation *in vitro* differed among species, varying from 22 to 24 hours in bovine (Picco *et al*., 2010; Rios *et al*., 2010) to 27 hours in caprine (Romaguera *et al*., 2010a; De *et al*., 2011). The time required for maturation of oocytes *in vitro* was slightly longer (27 hours) in caprine than in ovine and bovine (De Smedt *et al*., 1992, 1994; Keskintepe *et al*., 1994a; Martino *et al*., 1994a; Crozet *et al*., 1995; Cognie, 1999).

2.4.3 Follicle and Oocyte Size

A positive relationship has been found between follicle diameter, oocyte diameter and the developmental competence of oocytes. Embryo development improved with the increase in follicle and oocyte diameters (Gandolfi *et al*., 2005). Lonergan *et al.* (1994) showed that follicles larger than 6 mm provided the most competent oocytes in bovine. This is further proved by Fair *et al.* (1995) and Harada *et al.* (1997) with the results of obtaining the most competent oocytes when their diameter was 135 mm. Bovine oocyte diameter seems to be smaller than that of caprine oocytes. Hence, bovine full meiotic competence was achieved with a follicle size of about 3 mm, which corresponds to an oocyte diameter of about 110 mm (Fair *et al*., 1995). Hyttel *et al.* (1997) has evaluated the relationship between bovine cleavage rate and oocyte diameter: for bovine oocytes smaller than 100, 100 to 109, 100 to 119 mm and >120 mm, the cleavage rates obtained were 7, 41, 55 and 71%, respectively. The blastocyst rates of 20 and 30% were obtained from oocytes smaller than 100 mm and from 100 to 109 mm, respectively, while oocytes from 100 to 129 mm triggered 60% of blastocyst rate. On the other hand, oocytes with diameter more than 120 mm triggered 49% of blastocyst rate.

According to De Smedt *et al.* (1994), adult goat oocytes sequentially acquired meiotic competence in follicles ranging from 0.5 to 2-3 mm in diameter, and Crozet *et*

al. (2000) observed that the mean oocyte diameter increased from 96±0.3 to 136±0.6 mm as follicle size increased from 0.5 to 2-3 mm. In prepubertal goat oocytes, Martino *et al.* (1994a) also observed that meiotic competence acquisition was achieved with a follicle diameter of 2 to 3 mm with an oocyte diameter of 134±5.37 mm. Both Martino *et al.* (1994a) and De Smedt *et al.* (1994) observed that oocytes smaller than 110 mm corresponded to incompetent meiotic oocytes, from 110 to 125 mm they corresponded to partially competent oocytes and oocytes larger than 125 mm had full meiotic competence.

According to Crozet *et al.* (1995), significant differences have been found in the percentage of blastocysts obtained from oocytes recovered from follicles of 2 to 3 mm (6%), follicles of 3.1 to 5 mm (12%), follicles >5 mm (26%) and from ovulated oocytes (41%) in adult goats. In prepubertal goats, most of the oocytes recovered from 2 to 3 mm diameter follicles. A higher blastocyst rate was obtained in oocytes diameter larger than 135 µm (12.50%) compared to oocytes of 125 to 135 µm diameter (1.95%) (Anguita *et al*., 2007).

Since the largest follicles in prepubertal females never will ovulate, they could appear to be the most atresic since they never will ovulate. Higher blastocyst rates were yielded by cumulus oocyte complexes with no signs of atresia (Yuan *et al.*, 2005). On the other hand, Li *et al*. (2009) concluded that early signs of atresia improved blastocyst yield. Apoptosis (the programmed cell death process) mediates follicle and oocyte atresia. It was characterised by chromatin aggregation, nuclear and cytoplasmatic condensation and partition of cytoplasm and nucleus into membrane-bound vesicles (Liu *et al*., 2000).

2.5 *IN VITRO* FERTILISATION (IVF)

2.5.1 Sperm Preparation Methods

Several techniques were used to separate motile sperm from semen samples. This includes swim-up, glass-wool filtration and the use of bovine serum albumin/Percoll density-gradient centrifugation procedures in each with its advocates for improving sperm quality and oocyte penetration rates (Keefer *et al*., 1985; Ectors *et al*., 1989; Stubbings and Wosik, 1991; Palomo *et al*., 1999). Parrish *et al.* (1986) reported beneficial effects of the swim-up technique in which swim-up consistently yielded samples showing highly motile sperm and the fertilisation frequency was increased from 46 to 59%. Catt. (1990) also observed that the swim-up technique resulted in higher fertilisation rates from the sperm fractions obtained. However, according to Mac Lean *et al.* (1998), although this technique yielded highly motile sperm, the overall yield of sperm was very poor.

From the comparison of glass-wool filtration and swim-up procedures for sperm preparation, Stubbings and Wosik (1991) obtained similar results with both. However, glass-wool filtration has the advantage for its rapidity. This procedure depends on the adherence of nonviable sperm with abnormal membranes to the glass-wool.

Percoll, a colloidal suspension of silica (SiO_2) particles coated with polyvinylpyrrolidone (PVP), until recently was widely used for sperm preparation in human *in vitro* fertilisation. Percoll density-gradient centrifugation for sperm preparation has also been used in bovine *in vitro* fertilisation systems (Ectors *et al*., 1989; Utsumi *et al*., 1991). Percoll resulted in higher sperm recovery rates compared to the swim-up and glass-wool filtration methods (Parrish *et al*., 1995; Mac Lean *et al*., 1998). Gorus and Pipeleers (1981) suggested that the centrifugation step in this technique aligned all sperm parallel to the centrifugal force so that in such an orientation,

progressively motile sperm could move toward the bottom of the centrifuge tube more readily than could the non-motile or poorly motile sperm.

Sperm washing by centrifugal sedimentation and re-suspension in fresh medium was generally held to be the quickest and most effective method to remove seminal plasma; however, it caused cell damage in the sperm (Harrison and White, 1972). From the observation of the decreased sperm motility over time, Alvarez *et al.* (1993) concluded that centrifugation severely damages sperm. Centrifugation in Ficoll medium was found to be able to eliminate the seminal plasma with a minimum of mechanical damage (Harrison, 1976).

Swim-up and centrifugation in Percoll gradient were the 2 most widely used methods to select motile sperm cells. Guerin *et al.* (1989) concluded that although the cleavage and transfer rates were similar for the 2 protocols, centrifugation of sperm on a Percoll gradient yielded nearly double higher for the clinical pregnancy rate and the birth rate. Akerlof *et al.* (1987) also showed that sperm selected by Percoll gradient was found to be more superior compared to those separated by the swim-up procedure. On the other hand, Ng *et al.* (1992) reported that Percoll was not superior to swim-up with respect to identifying sperm parameters. Using frozen-thawed bovine semen, Parrish *et al*. (1986) found that the penetration rate of oocytes by sperm that separated by swim-up was higher than those selected by Percoll but Percoll was faster procedure and the recovery of motile sperm increased 6-fold. There was no significant difference found in the percentage of cleaved oocytes developing to blastocysts between swim-up and Percoll methods. Swim-up technique was used to prepare fresh caprine semen for *in vitro* fertilisation based on the selection of the most motile sperm. (Thompson and Cummins, 1985; DeSmedt *et al*., 1992; Crozet *et al*., 1993, 1995; Keskintepe *et al*., 1994a, 1996).

2.5.2 Sperm Preparation Medium

The fluid surrounding the gametes during fertilisation *in vivo* or *in vitro* is not only chemically but also physically different. Different chemicals were used in various *in vitro* fertilisation media, attempting to mimic the composition of the oviduct fluid (Abeydeera and Day, 1997b; Long *et al*., 1999; Coy and Romar, 2002; Yoshioka *et al*., 2008). However, information regarding the physical properties of both physiologic and artificial fluids is still relatively scarce.

Viscosity is an important physical characteristic of the organic fluids. The viscous glycoprotein secretions in the oviduct accumulating in the caudal portion are thought to regulate sperm ascent, contributing critically to formation of a sperm reservoir and thereby to a preovulatory sperm gradient along the isthmus. Under the influence of increasing titers of plasma progesterone, such secretions undergo a progressive reduction in extent and viscosity during the postovulatory interval, dissipation being largely completed before passage of embryos into the uterus 46 to 48 hours after ovulation (Hunter, 1999, 2002, 2008).

Under *in vitro* conditions, by contrast, the viscosity of the medium is usually close to the viscosity of water and much lower than that of the oviduct fluid. Foetal calf serum, bovine serum albumin (BSA) or polyvinyl alcohol (PVA) are frequent components of such media (Bavister, 1981; Eckert and Niemann, 1995; Kim *et al*., 2004; Suzuki and Yoshioka, 2006) to provide a protein source and/or increase the viscosity, but never achieving the presumable levels in oviduct fluid as precise viscosity measurements are not available.

The viscosity of the medium brings either a beneficial or detrimental factor on sperm motility, depending on the site or status of the sperm. In the oviduct environment, bovine sperm in the caudal isthmus develop hyperactive motility with frequent changes in direction helping them to detach from the epithelium, progress to the ampulla,

encounter the cumulus mass and more easily penetrate the cumulus matrix and the zona pellucida (Suarez *et al*., 1992; Suarez, 1996; Suarez and Ho, 2003). Such events for bovine sperm differ from the hyper-viscoelasticity in human seminal fluid which is associated with a lower percentage of motile sperm and infertility (Elzanaty *et al*., 2004). Thus, a certain degree of viscosity is desirable once sperm have escaped from seminal plasma and are ready to interact with the oocyte, either under *in vivo* (oviduct) or *in vitro* (culture medium) conditions.

2.5.3 Sperm Capacitation

The most crucial components affecting the performance of *in vitro* fertilisation are the source of sperm and methods of their capacitation. Freshly ejaculated semen is usually used in carpine *in vitro* fertilisation (De Smedt *et al*., 1992; Cox *et al*., 1994; Keskintepe *et al*., 1994a; Crozet *et al*., 1995; Mogas *et al*., 1997a; Villamediana *et al*., 1998; Malik *et al*., 1999; Palomo *et al*., 1999; Wang *et al*., 2002; Romaguera *et al*, 2010b; De *et al*., 2011). Frozen-thawed sperm was used in several study for caprine *in vitro* fertilisation (Cognié *et al*., 1995a; Cognié and Poulin, 2000; Ongeri *et al*., 2001; Rho *et al*., 2001; Magalhães *et al*., 2011). The possibility of applying the same method of capacitation for both fresh and frozen-thawed caprine sperm has not yet been determined.

There are a number of chemical agents for stimulating and maintaining sperm motility. These include caffeine (Younis *et al*., 1992; Keskintepe *et al*., 1994a,b) and a mixture of penicillamine, hypotaurine and epinephrine (PHE) (Gordon and Lu, 1990; Susko-Parrish *et al*., 1990; Vergos, 1990; Miller *et al*., 1992,1994) that were used to stimulate sperm motility. Other than the heparin and other chemicals as agents of sperm capacitation, capacitation is also induced, at least partially, with the modifications of medium osmolarity, pH of medium and a period of time of sperm pre-incubation (Luciano *et al*., 2004).

Heparin was found to be able in improving the efficiency of sperm capacitation in bovine (Parrish *et al.*, 1988) and caprine (Cognié *et al*., 1995a; Mogas *et al*., 1997a; Palomo *et al*., 1999; Rho *et al*., 2001). This is due to the addition of heparin in the *in vitro* fertilisation medium stimulates the fertilisation rate, cleavage rate and embryo developmental kinetics (Cox *et al*., 1994; Cognié *et al*., 1995a; Mendes *et al*., 2003). However, according to Cognié *et al.* (1995a), the higher fertilisation rate could be associated with a lower viability of embryos after transfer. Heparin has been reported to be the most potent glycosaminoglycan in improving the efficiency of fertilisation by facilitating the utilisation of calcium for sperm capacitation in goats (Cox *et al*., 1994), and it has been used with success in goats (Zhang and Li, 1998). This ability is related to the sulphate content of the molecule (Ax and Lenz, 1987). According to Miller and Ax (1989), the desulphation of heparin negates its ability to bind and capacitate sperm, whereas the resulphation allows the molecule to regain its ability to bind sperm. Sulphate residue may also mediate the anticoagulant effect of heparin (Ofosu *et al*., 1989), and hence the bioassay used to grade the molecule may be affected. According to Ax and Lenz (1987), heparin may have 1.6 to 3.0 sulphates per disaccharide, so the source of heparin and the extraction and purification procedures used by commercial companies may become a source of variation in the efficiency of the molecule to stimulate sperm ability to fertilise matured oocytes. In goat, Grade I heparin has been demonstrated by Cox *et al.* (1995) to be superior to obtain improvement in the *in vitro* fertilisation medium due to a better utilisation of calcium induced by the molecule. Development of an efficient method for capacitation *in vitro* of both fresh and frozen caprine sperm is vital for further biotechnological applications in caprine *in vitro* production.

In vitro capacitated sperm are endowed with the ability of penetrating the cumulus layers and the binding to the zona pellucida that elicits the occurrence of the acrosome

reaction. In order to reach this status in ejaculated sperm, bound proteins from the cauda epididymidis and the seminal plasma are to be removed from the sperm surface, particularly over the acrosomal region, a matter that is usually done *in vitro* by washing and centrifugation of the sperm suspension or by cleansing through discontinuous density gradients of silica beads. Cleansed sperm are usually incubated in media that are enriched with bicarbonate, which stimulates a series of events in the sperm (Gadella and van Gestel, 2004), including the stimulation of a special form of a soluble adenylyl cyclase (sAC) (Litvin *et al*., 2003), which within minutes of exposure, by increasing levels of cyclic adenosine monophosphate (cAMP) intra-sperm, activates a protein kinase A (PKA)-dependent protein phosphorylation cascade (Harrison, 2004).

Bicarbonate also induces a rapid increase in merocyanine fluorescence, indicating that the phospholipid packing of the sperm plasma membrane becomes disordered by the ion and hence, such disordering of the phospholipids increases membrane fluidity and enables intercalation of merocyanine into the hydrophobic core of the sperm plasma membrane, within minutes of exposure (Harrison and Miller, 2000). However, only in a proportion (20 to 40%) of the tested sperm (Harrison and Miller, 2000) does phospholipid scrambling occur (Gadella and van Gestel, 2004). In parallel with these events, but significantly later than the membrane fluidity changes (>90 minutes versus <10 minutes) and owing to the usual presence of albumin (bovine serum albumin) in the incubation medium, the removal or redistribution of cholesterol in the plasma membrane of the sperm head domain takes place (Flesch *et al*., 2001; Gadella and van Gestel, 2004).

Bicarbonate also involved in this albumin-driven efflux of cholesterol, as well as in the escape of the sperm-specific glycolipid ‘‘seminolipid’’ present in the outer lipid leaflet of the sperm plasma membrane (Vos *et al*., 1994) from the apical membrane area to the equatorial domain (Gadella *et al*., 1994, 1995). By stabilising the plasma

membrane, the seminolipid is considered to prevent the acrosome reaction in the apical domain of unprimed sperm during low bicarbonate levels. Bicarbonate exposure causes the relocation to the equatorial segment which diminishes the protective role of the seminolipid and local membrane destabilisation renders fusogenic effect for the acrosome reaction after contact with the zona pellucida. According to Gadella *et al*. (1995), the remaining plasma membrane over the post-acrosomal domain, where seminolipid concentrates after *in vitro* capacitation, keeps stability; and it is not reactive to bicarbonate.

Another late effect (>60 minutes) of bicarbonate *in vitro*-induced sperm capacitation is the activation of tyrosine kinases, following by the tyrosine phosphorylation (Ficarro *et al*., 2003). This slow response is caused by its dependence on previous events which are lipid scrambling and cholesterol removal. Lastly, bicarbonate seems necessary to prime the sperm for zona pellucida-binding and to render the apical sperm head membrane fusogenic to enable the capacitated sperm acrosome reaction (Gadella and van Gestel, 2004).

When ejaculated sperm are inseminated *in vivo*, the sperm surface domain, covered by seminal plasma spermadhesins (Jonakova and Ticha, 2004), is exposed and becomes accessible to lipid-binding components of the female intra-luminal fluids either in the uterus or the oviduct. These in turn enables the removal of cholesterol from the sperm plasma membrane, thus enhancing membrane fluidity, which in turn causing lipid scrambling, and initiate further capacitation changes such as the uptake of extracellular Ca^{2+}, tyrosine phosphorylation and the reorganisation of the sperm membrane (Töpfer-Petersen *et al*., 2002; Tardif *et al*., 2003).

2.5.4 Acrosome Reaction

The acrosome reaction of mammalian sperm is a prerequisite for sperm penetration of the zona pellucida and fusion with the oocyte plasma membrane. It involves fusion and progressive vesiculation between the outer acrosomal membrane and the overlying plasma membrane, with the subsequent release of acrosomal enzymes.

The presence of the cumulus oophorus or components of the extracellular matrix is capable of inducing acrosome reaction *in vitro* (Bavister, 1982; Lenz *et al*., 1982; Tesarik, 1985; De Jonge *et al*., 1988; Siiteri *et al*., 1988). According to Suarez *et al.* (1986) and Siiteri *et al.* (1988), acrosome reaction is also induced by human follicular fluid components. Since sperm acrosome reaction is induced near the site of fertilisation, oocyte layers, which include the matrix of the cumulus oophorus and the zona pellucida, may promote acrosome reaction (Cross *et al*., 1988; Sullivan *et al.*, 1990; Kligman *et al*., 1991). There were several studies implicated the cumulus mass (Siiteri *et al*., 1988; Sullivan *et al*., 1990) and the zona pellucida (Hoshi *et al*., 1993) in human sperm acrosome reaction. The acrosome reaction in goats may be induced by *in vitro* incubation in the presence of uterine fluid (Kusunoki *et al.*, 1984, 1988). A 16% rate of acrosome-reacted cells was found in samples incubated for 5 hours in the presence of rat uterine fluid (Kusunoki *et al*., 1988).

There were several techniques have been carried out for evaluation of the acrosome reaction of mammalian sperm. Contrarily, most of them do not differentiate between true acrosome reaction (live sperm) and degenerative acrosome reaction (dead sperm). Some specific reagents were used for binding to the inner acrosomal membrane: fluorescein-isothiocyanate concanavalin A (Holden and Trounson, 1991) or monoclonal antibodies reacting with internal antigens (Braun *et al*., 1991), without distinction of true or degenerative acrosome reaction. The combination of sperm with the viability stain Hoechst 33258 can distinguish the true acrosome reaction and degenerative

acrosomal loss. De Leeuw *et al.* (1991) described the acrosomal state from viable sperm using fluorescent dye in combination with phase contrast and fluorescence microscopy. The simultaneous determination of acrosomal status and viability of sperm can determine the true acrosome reaction. Several intermediate steps in the mouse zona pellucida induced acrosome reaction were defined by using the fluorescent probe chlortetracycline (CTC) (Kligman *et al*., 1991). The triple-stain tecnique of Talbot and Chacon (1981) is an inexpensive and easy method allowing the evaluation of the true acrosome reaction of viable cells.

2.5.5 Oocyte Quality

Good quality oocytes is a prerequisite for success for *in vitro* production in domestic animals (Hasler, 1998; Galli *et al*., 2001; Merton *et al*., 2003). There were different sources that oocytes can be derived from, including post-mortem oocyte recovery from abattoir ovaries as well as (repeated) transvaginal ultrasound-guided follicle aspiration (laparoscopic oocyte pick-up) in living donors (Bols, 2005). The final intrinsic developmental capacity and quality of the retrieved oocytes can be determined by several factors which are mostly related to the donor, for example, stage of the oestrous cycle at the time of oocyte retrieval, follicular status in relation to oocyte growth and final maturation, as well as the physiological condition and breed of the donor (Merton *et al*., 2003). Moreover, during the technical procedures to obtain the immature cumulus oocyte complexes, the important technical factors, such as temperature and time of storage for abattoir ovaries, have an impact on oocyte quality (Blondin *et al*., 1997). Factors affecting laparoscopic oocyte pick-up, including follicle visualisation, needles and aspiration vacuum used and the processing of cumulus oocyte complexes before *in vitro* production, all of which, can jeopardise the quality of the oocyte cumulus cell investment (Bols, 2005). There were several studies showing that the importance of an

intact cumulus cell investment for oocyte maturation and *in vitro* embryo development cannot be overestimated (Zhang *et al*., 1995; Konishi *et al*., 1996; Boni *et al*., 2002). In addition, prediction of the developmental potential of the harvested cumulus oocyte complexes is crucial for an effective oocyte selection. Oocyte quality is most commonly assessed by the ability to further develop to the blastocyst stage *in vitro* (Sirard *et al*., 2006), which is known to differ in several ways from *in vivo* development. Generally, *in vitro* production is performed in large groups of cumulus oocyte complexes as group culture is still a prerequisite to achieve acceptable blastocyst rates. Unfortunately, that is impossible to make studies on *in vitro* oocyte quality linked to the biological data of the donor, or the originating follicle, with group *in vitro* production. In order to solve this problem, individual *in vitro* production might be the solution. However, the need for *in vitro*, non-invasive oocyte quality assessment techniques remains, even when the immediate link between the individual oocyte and its specific follicular environment is lost or of less interest as only with such a technique in place, good quality oocytes will be able to be selected prior to *in vitro* production (IVP) procedures in order to economise the *in vitro* production procedure as a whole, and to increase its efficiency that remains limited to about 30 to 40% blastocysts.

2.5.6 Co-incubation Duration

The duration of gamete co-incubation during *in vitro* fertilisation contributes to the embryo development performance. Based on studies on the kinetics of the fertilisation events, the suggested optimum duration for sperm-oocyte co-incubation for maximising blastocyst yield is 18 to 24 hours (Long *et al*., 1993; Rehman *et al*., 1994). However, prolonged gamete co-incubation during *in vitro* fertilisation exposes the oocytes and embryos to high levels of reactive oxygen species (ROS) produced by dying sperm (Baker and Aitken, 2004), which results in zona hardening and may bring detrimental

effects on embryo development (Gianaroli *et al*., 1996b). Furthermore, Sumantri *et al*. (1997) reported that the incidence of polyspermy increases with longer gamete co-incubation times during *in vitro* fertilisation. In order to improve the low blastocyst formation rates in bovine (Fukuda *et al*., 1990; Nedambale *et al*., 2006) with prolonged sperm-oocyte incubation (18 hours) in the traditional fertilisation medium (Brackett-Oliphant, BO medium), it has been suggested that using Brackett-Oliphant medium for fertilisation requires the removal of oocytes after 6 hours of *in vitro* fertilisation (Fukuda *et al*., 1990; Brackett and Zuelke, 1993; Jaakma *et al*., 1997), due to the presence of dead and dying sperm (Nedambale *et al*., 2006). The appropriate insemination intervals for caprine *in vitro* maturation-fertilisation-culture have been reported as 18 to 24 hours (De Smedt *et al*., 1992; Keskintepe *et al*., 1993). According to De Smedt *et al.* (1992), the best time to evaluate the state of penetration of the oocytes is 17 hours. Nonetheless, the exact minimum time needed for capacitated caprine sperm to penetrate the cumulus investments and the zona pellucida, finally fusing with the oolemma has been reported to be less than 4 hours (Mogas *et al*., 1997a).

2.6 PARTHENOGENETIC ACTIVATION

2.6.1 Activation Chemicals

Parthenogenesis can be induced by ionomycin (Lan *et al*., 2005), ethanol (ET) (Yang *et al*., 1994b; Yi and Park, 2005), calcium ionophore A23187 (CI) (Liu *et al*., 2002a; Liu *et al*., 2002b) and strontium (Sr) (Méo *et al*., 2004, 2005). Wang *et al.* (2008) demonstrated that the blastocyst rates were low and not significantly different between ethanol, strontium, ionomycin and A23187 treatment alone. This is due to the incomplete activation of oocytes by single stimulation (Ju *et al*., 1997; Liu *et al*., 1998a,b).

The combined treatments, especially ionomycin + 6-dimethylaminopurine and ethanol + cycloheximide (CHX) + cytochalasin B (CB), have a significant increase in pronuclear formation, cleavage, blastocyst rate and cell number of blastocyst than treatment alone (Wang *et al*., 2008). Presicce and Yang (1994) reported that activation rates achieved with the combined ethanol and cycloheximide treatment were high for both young and aging oocytes. It is known that, during fertilisation of the oocyte, an increase in intracellular calcium concentration is a universal response elicited by the sperm (Whitacker and Patel, 1990).

The capability of calcium rises to release oocytes from the meiotic arrest is related to the ability to trigger persistent inactivation of maturation promoting factor (MPF) and cytostatic factor (CSF), which is the result of c-mos and mitogen activated protein kinase activity. A single calcium rise causes only a transient decline of maturation promoting factor and cytostatic factor activity which is insufficient for full oocyte activation (Collas *et al*., 1993; Lorca *et al*., 1993). The most effective stimuli are shown to be those promoting multiple intracellular calcium peaks (Vitullo and Ozil, 1992). Based on these, alternative activation methods have been developed that combine a transient inactivation of maturation promoting factor obtainable with a single $[Ca^{2+}]_i$ rise, with a persistent inhibition of maturation promoting factor, induced by addition of either protein synthesis inhibitors, for example, cycloheximide (Bos-Mikich *et al*., 1995) or non-specific kinase inhibitors, for example, 6-dimethylaminopurine (Liu *et al*., 1998a). The synthesis or re-accumulation of cyclin B is usually restricted by the protein synthesis inhibitors, thereby, the re-synthesis of maturation promoting factor activity is blocked (Presicce and Yang, 1994). Whereas, by inactivating mitogen activated protein kinase, kinase activity of maturation promoting factor is inhibited by non-specific protein kinase inhibitors (Motlik *et al*., 1998; Gordo *et al*., 2000).

2.6.2 Mechanisms Inhibiting Parthenote Development

The absence of paternal genome causes the mammal parthenotes fail to develop to term: this peculiarity leads to an abnormal regulation of differentiation and proliferation mainly in extra-embryonic lineages, resulting in a poor support of embryonic growth (Surani *et al*., 1984). There was evidence showing that parthenogenetic cells lines do not contribute efficiently to mesodermal lineages and to extraembryonic tissues, such as trophectoderm and primitive endoderm lineages, with the studies with mouse chimaeras and teratomas (Sturm *et al.*, 1994).

Complementary epigenetic modifications which lead to differential roles and gene expression in the embryo causing maternal and paternal genomes quantitatively identical but not functionally equivalent (Barton *et al*., 1984; Surani *et al*., 1984), therefore the unbalanced development typical of parthenotes is due to the lack of expression of the paternal copies of some genes which are vital for extra-embryonic tissues development (Surani, 2001). The separate contribution of the maternal and paternal inherited alleles of some genes to the correct development of the embryo is mediated by an epigenetic phenomenon that selectively silences or promotes the expression of genetic imprinting. Gene imprinting does not limit its effect to early embryonic development but imprinted genes are associated with human diseases, including disorders affecting development and behaviour, for example, Beckwith-Wiedemann, Prader-Willi, Angelman and Russel-Silver Syndromes (Hall *et al*., 1990; John and Surani, 1996).

2.7 *IN VITRO* CULTURE (IVC)

2.7.1 Medium Supplementation

Serum contains proteins, amino acids, carbohydrates, trace elements, hormones, growth factors, cell attachment and spreading factors, and some, as yet, undefined factors (Takagi *et al.*, 1991). It has been commonly used as a component of co-culture media.

Serum supplementation during *in vitro* production of embryos can enhance blastocyst production (Thompson *et al*., 1998; Holm *et al*., 1999; Gordon, 2003). Foetal calf serum has a discrete action in early stages of embryonic development which is from the morulae to the blastocyst stages. In addition, it increased the number of cells and stimulated blastocyst hatching. However, reducing serum concentrations during some steps of *in vitro* production were needed to sustain embryo quality (Holm *et al*., 1999).

Bovine serum albumin contains variations in energy substrates and in growth factor concentrations among batches (Rorie *et al*., 1994). This leads to a variety of batch-dependent embryotrophic properties. Bovine serum albumin in *in vitro* culture increases the embryonic development rate (Dobrinsky *et al*., 1996; Holm *et al*., 1999; Lazzari *et al*., 2000; Rizos *et al*., 2003). Some authors included bovine serum albumin or serum at defined *in vitro* culture periods (Pinyopummintr and Bavister, 1991; Rorie *et al*., 1994; Liu and Foote, 1995; Nedamdable *et al*., 2002; Westberg *et al*., 2002; Lim *et al*., 2003; Mucci *et al*., 2006). However, abattoir-derived oocytes were used in the majority studies, with few data concerning oocyte pick-up of *in vivo* oocytes. In order to obtain high quality embryos, serum concentration needs to be reduced during various phases of *in vitro* production.

There were no significant differences in cleavage rates but the blastocyst development was improved, by adding either β-mercaptoethanol or both compared to culture without additions (6.4% and 6.8% versus 1.6%) (Mizushima and Fukui, 2001). Single bovine embryo development could be enhanced by adding amino acids to a

chemically defined culture medium, at concentrations found in the female reproductive tract (Li *et al.*, 2006). Lim and Hansel (1996) demonstrated the positive effects of the addition of platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and transforming growth factor (TGF β1β2) on the developmental capacity of singly cultured 8-cell embryos. Arachidonic acid, β-mercaptoethanol and glutathione stimulated the subsequent development of 8-cell embryos in the presence of platelet derived growth factor and transforming growth factor β1β2.

2.7.2 Co-culture

As an alternative to medium supplementation, culture environment can be enriched by culturing somatic cells together with the zygote. There were various types of cells used for co-culture. These comprise of oviduct cells (Eyestone and First, 1989), feeder layers of different cell types such as Buffalo rat liver (BRL) (Hasler *et al.*, 1995) or Vero cells (Carnegie *et al*., 1997). Co-culture has many well-defined embryotrophic effects which include: faster cleavage (Bongso *et al*., 1989), higher blastocyst rate (Rexroad, 1989), reduced fragmentation (Wiemer *et al*., 1989b), increased blastocyst cell numbers (Smith *et al*., 1992), improved morphological appearance/grade and expanded blastomeres (Wiemer *et al.*, 1989b), reduced apoptosis (Xu *et al*., 2000), facilitated hatching (Ellington *et al.*, 1990), maintenance of viability prior to transfer (Bongso and Fong, 1993), increased human chorionic gonadotrophin secretion (Turner and Lenton, 1996), improved pregnancy rates (Wiemer *et al.*, 1989a) and live births (Marcus and Brinsden, 1996).

Poor quality embryos, those grown in less supportive media (Carney *et al*., 1990) as well as those subjected to centrifugation and micromanipulation procedures (Thomas and Seidel, 1993; Lai *et al*., 1996), can be 'rescued' by co-culture (Dirnfeld *et al*., 1997), and the impact of cryopreservation techniques upon subsequent viability can also be

minimised (Tucker *et al*., 1995). These effects are most pronounced with the increase of co-culture duration (Wiemer *et al*., 1989a; Lai *et al*., 1996), especially during the early cleavage stages (Yeung *et al*., 1992), which may be mediated by the accumulation/delayed expression of growth factors (Xia *et al.*, 1996). The selection of the best embryos for transfer is allowed by such extended culture (Rubio *et al*., 2000).

Somatic cells are used to improve *in vitro* culture of early stage embryos by overcoming the *in vit*ro developmental block of cultured mammalian embryos. This is because somatic cells could provide embryonic growth factors and remove embryotoxic substances from the culture medium (Rexroad, 1989; Kane *et al*., 1992). In caprine *in vitro* matured-*in vitro* fertilised oocytes, cumulus cells (Younis *et al*., 1991c; Younis *et al*., 1992; Keskintepe *et al*., 1994b; Keskintepe *et al*., 1996; Mogas *et al*., 1997a) and oviductal epithelial cells (Sakkas *et al*., 1989; Prichard *et al*., 1990; Prichard *et al*., 1991; Buggin-Daubie *et al*., 1992; Crozet *et al*., 1995) have been used to support initial cleavage rates as well as progression to compact morulae and to blastocysts. There were studies carried out on animal co-culture systems to suggest that co-cultures are neither cell nor species specific (Goto *et al*., 1992; Pavasuthipaisi, 1994).

The reported embryotrophic effects of co-culture are influenced by the interaction of various parameters, including: somatic cell origin (Galli *et al*., 2000, 2003) and treatment (for example, cryopreservation, hormonal exposure, perifusion), base medium composition (Ellington *et al*., 1990; Lim *et al*., 1997; Izquierdo *et al*., 1999), culture method (Xu *et al*., 1992), timing of co-culture (Desai and Goldfarb, 1998), presence of serum (Fukui *et al*., 1991; Rief *et al*., 2002), microdrop/open culture (Sherbahn *et al*., 1996), gas/oxygen tension (Yang *et al*., 1994a), temperature (Ocampo *et al.*, 1994b), somatic cell substrate (Piekos *et al*., 1995) and the inclusion of supplements, viz.: glutathione (Luvoni *et al*., 1996), glycine and alanine (Moore and Bondioli, 1993), haemoglobin (Lim *et al*., 1999), retinol (Lima *et al*., 2004), b-mercaptoethanol (Geshi *et*

al., 1999) and heavy metal ion chelators (Fukui *et al*., 1991). Maternal and paternal influences, including animal strain, also contribute to the outcome of co-culture (Boediono *et al*., 2003).

Physical contact between embryo and oviduct epithelial cells might be essential (Rexroad *et al*., 1988; Bongso *et al*., 1990; Ellington *et al*., 1990; Boccart *et al*., 1991; Naqvi *et al.*, 1992; Rieger *et al*., 1992; Trounson *et al*., 1994; Hernandez-Ledezma *et al*., 1995; Rieger *et al*., 1995). However, co-culture with somatic cells presents several disadvantages as well. These include time factors, contamination risks and dubious quality control (Eyestone *et al*., 1991; Bavister *et al*., 1992; Mermillod *et al*., 1993).

Izquierdo *et al.* (1999) obtained the best results by co-culturing embryos with oviduct epithelial cells compared to cumulus cells of prepubertal goats. The proportion of zygotes that complete their development till the blastocyst stage, however, was low (10%). Mogas *et al.* (1997a) have obtained 8.3% of blastocysts by co-culturing embryos with granulose cells for oocytes from pre-pubertal goats; by culturing the embryos with oviduct cells, Crozet *et al.* (1995) and Pawshe *et al.* (1996) have obtained up to 26 and 40% of blastocysts with caprine oocytes, respectively. On the other hand, Katska-Ksiazkiewicz *et al.* (2007) obtained blastocyst by co-culturing with goat oviduct epithelial cells (GOEC) (34.3%) and Vero (33.3%), whereas Goovaerts *et al*. (2009) tested the effect of cumulus cell co-culture on single embryo culture and showed that it brought significantly ($P<0.001$) beneficial to blastocyst rates in single culture conditions (21.8% with cumulus cell versus 2.9% without cumulus cell), but not in group culture conditions (24.6% with cumulus cell and 30.7% without cumulus cell).

Virtually all existing co-culture systems rely on working with a single culture medium to address the different, and changing, requirements of somatic cells and preimplantation embryos throughout the culture period (Gardner, 1998). Moreover, ill-timed medium changes can cause embryos and somatic cells competing for nutrient

resources, in addition to the obvious complications of medium acidification. While many co-culture systems attempt to resolve this dilemma by using embryo culture media, this neglect of the specific requirements of somatic cells - oviduct epithelial cells in primary culture in particular - results in the loss of their *in vivo* morphological and functional properties, this includes absorption, transcytosis and secretion (Rodriguez-Boulan and Nelson, 1989), in which their embryotrophic properties may be impacted (Rief *et al*., 2002). Indeed, the simple polarisation of epithelial cells depends on culture conditions, for example confluence, cell support and perifusion (Minuth *et al*., 1992; Reischl *et al*., 1999).

Co-culture has the potential risk of undetected somatic cell-embryo-surrogate mother/dam viral disease transmission, for example, bovine viral diarrhoea virus, bovine herpes virus and caprine arthritis-encephalitis virus (Givens *et al*., 2000; Lamara *et al*., 2002). Since viral infection can adversely alter conditioned medium composition, screening cell sources is still crucial (Vanroose *et al*., 2001), although trypsin treatment of embryos and/or the use of safe antiviral agents are able to minimise the risk of recipient infection (Edens *et al*., 2003; Givens *et al*., 2006). However, disease transmission is often overlooked by co-culture critics in non-human mammalian embryo *in vitro* production, where oocytes or embryos are often matured, fertilised and cultured in heterologous groups regardless to in cell-free conditions or in ligated oviducts *in vivo*. In respect to this, disease transmission could potentially be overcome by using screened immortalised cell lines (Desai *et al*., 1994) or autologous cells that have been either previously cryopreserved or harvested at the time of oocyte retrieval (Simon *et al*., 1999; Broussard *et al*., 1994).

While live births have been obtained following *in vitro* maturation, fertilisation and/or culture in association with co-culture in various species (Camous *et al*., 1984; Abeydeera *et al*., 1998), Sinclair *et al*. (1999) reported that foetuses derived from co-

cultured ruminant embryos have greater foetal and organ weights, organ immaturity and a greater incidence of polyhydramnios, although this is not consistent across all parameters investigated and/or studies (Jacobsen *et al*., 2000).

2.7.3 Gas Atmosphere

The biological microenvironment can be changed by gas atmosphere during *in vitro* development, and thus embryo metabolism is affected (Krisher, 2004), due to the potential link with the production of reactive oxygen species. Hagemann *et al.* (1998) showed a non significant difference in developmental rates by using 7 or 20% oxygen during maturation or fertilisation of single bovine oocytes, while Oyamada *et al.* (2004) showed significant higher cleavage and blastocyst rates following maturation at 20% oxygen compared to 5%. Compared to 20% oxygen, blastocyst rates and embryo cell numbers were significantly increased in the presence of a cumulus cell co-culture in synthetic oviductal fluid (SOF) medium with 5% oxygen, in both group and single culture (Goovaerts *et al*., 2009), although in general, 20% oxygen is preferred in somatic cell co-culture used case (Yuan *et al*., 2003).

2.8 VITRIFICATION

As early as 1985, ice-free cryopreservation of mouse embryos at -196ºC by vitrification was reported as an alternative approach to cryostorage (Rall and Fahy, 1985). Since then, vitrification techniques have become increasingly main stream in animal reproduction as an alternative cryopreservation method to traditional slow-cooling or rapid-thaw protocols. In addition, the last few years have seen a significant resurgence of interest in the potential benefits of vitrification protocols (Juergen *et al*., 2002).

Vitrification is a process that induces a glass-like solidification of living cells during cryopreservation. It involves 5 critical steps: exposure of the cell to

cryoprotectants; cooling of the cell to temperatures below 0ºC; storage at the 'glass' transition temperature of water below -130ºC; warming and thawing; dilution and removal of cryoprotectants prior to incubation (Luyet and Rapatz, 1970; Leibo, 1986; Mazur, 1988; Leibo and Songsasen, 2002; Leibo, 2004). Vitrification involved the simplest method of cooling whereby the embryos were transferred from room temperature directly into liquid nitrogen (LN_2). The unique advantage of the vitrification process is elimination of lethal ice crystal formation which causes the cryoinjury. This ice formation can be prevented by short exposure to a cryoprotectant medium containing permeable (for example, glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, methanol and ethanol) and impermeable (sucrose, galactose, mannose, lactose and raffinose) cryoprotectants.

However, high concentrations of cryoprotectants could lead to toxic and osmotic injuries, the extent of which also depends on the permeability of the solutions used and the embryos developmental stage (Smorag and Gajda, 1994). Several efforts have been invested to decrease the toxicity of cryoprotectants by applying less toxic and more permeable chemicals, lowering total concentration of chemicals and using 2 or more cryoprotectants to decrease the specific toxic effect of each. By using vitrification solution containing 20% ethylene glycol and 20% dimethyl sulfoxide, El-Gayar and Holtz (2001) obtained good results in caprine embryos vitrification. Huang *et al.* (2006) was the first to test the vitrification efficiency in goats using 16.5% ethylene glycol (EG) and 16.5% dimethyl sulfoxide (DMSO) compared with 20% ethylene glycol and 20% dimethyl sulfoxide.

2.8.1 Cryoprotectant

Vitrification is a highly unstable process in which solidification is achieved by rapid elevation of viscosity, until the level is high enough that the material can be considered

solid for all practical purposes. Highly viscous solution, known as ‘cryoprotectants’ are introduced into the biological specimen to suppress crystallisation and promote vitrification.

Permeating cryoprotectant is an organic solute responsible for protecting the intracellular organelles of the embryos during cooling and warming prior to and after storage in liquid nitrogen (Bautista and Kanagawa, 1998) by interacting intracellularly to influence microfilament and microtubule dynamics; however, cryoprotectant is not consistent in their interaction with cellular organelles and the cytoskeleton of embryos across species (Parks and Ruffing, 1992; Vincent and Johnson, 1992). Non-permeating cryoprotectant is a macromolecule or sugar which is added to reduce ice formation during freezing (Bautista and Kanagawa, 1998), facilitate dehydration of cells prior to cooling and protect the cellular membrane (Massip *et al*., 1995). Although cryoprotectant protects cells from damage during cryopreservation and storage, it could be detrimental to the cells due to chemical toxicity (Fahy, 1986) or osmotic stress (Mazur and Schneider, 1984). Depolymerisation caused by cryoprotectant could be reversible upon dilution, rehydration and subsequent culture (Massip *et al*., 1995).

Numerous mechanisms including osmotic expression of water from the cells into the solute-rich medium, stabilisation of the cell membrane by the cryoprotectant (Wolkers *et al*., 2002; Sum *et al*., 2003) and suppression of ice crystallisation in the medium and/or within the cells (Conrad *et al*., 2000) have been proposed for the cryoprotection imparted to the cells. The morphology of the interface (Hubel *et al*., 1992) and the surface energy between the cell and the ice interface can also be affected with the addition of cryoprotectants (Van Oss *et al*., 1991).

The addition of cryoprotectant results in an initial cell shrinkage and subsequential volume recovery due to the chemical potential or osmotic pressure differences across cell membranes and the difference in membrane permeability to water and

cryoprotectant. On the other hand, an initial swelling of the cell is caused by the removal of cryoprotectant. According to Gao *et al.* (1995), the excursion of cell volume should not go beyond a damaging threshold in order to maintain cell viability.

Although the cell membrane integrity has been used as an empirical indicator to evaluate cell viability, the detailed mechanisms of cell damage due to cryoprotectant addition or removal remain unclear. Significant changes of intracellular condition, accompanied by the cell volume change which is regulated by changing in the intracellular electrolyte and non-electrolyte concentrations through a cell signaling system (Cala, 1983; Hallows and Knauf, 1994; Lang *et al*., 1995), during the cryoprotectant addition and removal may reveal the mechanisms of osmotic/toxic damages from the cryoprotectant to cells.

While complete vitrification can potentially prevent the devasting effect of ice crystallisation (Song *et al*., 2000), the high concentration of the cryoprotectants involved in it potentially very toxic (Luyet, 1937; Karow, 1981; Taylor, 1984; Fahy, 1987; Taylor, 1987). Cryopreservation subject to a low concentration cryoprotectants and sub-critical cooling rates to promote vitrification, is widely known as classical cryopreservation. In classical cryopreservation, ice crystals first nucleate at about the heterogenous nucleation point for the specific cryoprotectant composition, while the concentration of the remaining solution elevates. The progress of crystal formation and elevation of the concentration of the remaining solution continues with cooling until the remaining solution becomes so viscous as to form glass at the particular cooling rate (Pegg *et al*., 1997).

Upon cooling to -196ºC, injury associated with formation of ice is eliminated during complete vitrification as the concentration of cryoprotectants is high enough to guarantee no ice forms in vitrified suspensions during the cooling, storage, or warming steps (Rall, 1987). Several rapid cooling procedures, suspending embryos in mixtures of

sucrose and penetrating cryoprotectants such as glycerol, dimethyl sulfoxide or ethylene glycol have yielded good survival rates despite the formation of ice in the suspending medium itself (Takeda *et al*., 1984; Szell and Shelton, 1986, 1987; Shaw and Trounson, 1989; Leibo and Oda, 1993). These data suggested that conditions were probably adequate to prevent the formation of large amounts of intracellular ice.

2.8.2 Cooling and Warming Rate

Vitrification cryopreservation has an advantage of fast cooling and warming rates that prevents ice formation within embryos. Increasing the cooling/warming rate (Vajta and Kuwayama, 2006) or decreasing the sample volume will increase the probability of vitrification (Arav, 1992). An increase in cooling rate would decrease chilling injury (Rall, 1987; Dobrinsky and Johnson, 1994) and permit a cryoprotectant concentration reduction (Vajta, 2000). A successful vitrification requires rapid cooling rate (Le Gal and Massip, 1999) as it renders the ice-crystal free in the specimen which prevents cryoinjury due to the extracellular and intracellular crystallisation (Arav *et al*., 1993; Hurtt *et al*., 2000).

Minimising the volume of the sample decreases the likelihood of ice crystal formation and thereby promotes vitrification (Arav, 1992; Arav *et al*., 2002; Yavin and Arav, 2007). The low volume of cryoprotectant medium surrounding embryos and the direct contact with liquid nitrogen provides very high cooling rates (for example, 24000ºC/minutes with open pulled-straw; OPS technology). Faster cooling is achieved by holding the embryos in a small volume of cryoprotectant and by reducing the thickness of the straw wall, allowing closer contact with the liquid nitrogen (Vajta *et al*., 1998). Dobrinsky (2002) has proposed that rapid cooling of embryos during vitrification could bypass or ‘out-race’ detrimental chilling-induced cellular changes that take place

during slow cooling by conventional freezing and eliminate the possibility of intracellular and extracellular ice formation.

The concentration and combination of cryoprotectants as well as the addition and removal protocols have been almost fully exploited (Vajta and Kuwayama, 2006). A variety of carriers are utilised to reduce the volume of the vitrification solution containing the embryos in order to increase cooling and warming rates to gain real benefit over conventional methods. Embryos in a small amount of vitrification solution reduces the exposure to unsuitable temperatures and concentrated cryoprotectants as embryos could be directly warmed and immediately diluted into the dilution solution (Chen *et al*., 2001).

Considerable effort has been invested to develop alternative methods to increase cooling and warming rates by minimising the vapour formation around the sample at cooling. Cooling the liquid nitrogen slightly below the boiling point (-196ºC), from -200 to -205ºC, may minimise the boiling around the submerged sample and increase cooling rates considerably. Although the approach was sporadically used earlier, it was widely available only after the construction of the VitMaster (IMT, Israel), a device producing vacuum in the reinforced liquid nitrogen container (Arav *et al*., 2002). A considerable part of liquid nitrogen evaporates, but the remaining solution cools down and even starts to solidify to form liquid nitrogen slush. Straws, preferably thin straws, even in sealed form, can be cooled safely with an increased cooling rate in this mixture of nitrogen slush and cooled liquid nitrogen. Extremely small sample volumes are loaded to the inner wall of a standard insemination straw (0.25 ml) and the straw is immersed directly into the liquid nitrogen after sealing following by in-straw dilution as well as direct transfer (Hamawaki *et al*., 1999). Another way to avoid vapour formation around the sample is the solid surface vitrification (SSV) method (Dinnyes *et al*., 2000). This approach makes a shortcut to exclude entirely the vapour formation by cooling metal

surfaces in liquid nitrogen and placing the solution containing the biological sample on the surface of metal. It is proved that solid surface vitrification may provide almost as high survival and developmental rates as achieved with the open pulled straw or cryoloop devices.

The cryoloop and solid surface vitrification techniques have been reported to allow direct exposure of cryoprotectant containing embryos to the cooling environment, thus resulting in rapid cooling. However, the solid surface vitrification technique is technically more challenging as some specimen would stick to the pipette or be lost due to random drop dispersion caused by the flipping movement (Begin *et al*., 2003). The cryoloop vitrification and solid surface vitrification techniques share the advantage of using a containerless system which favours rapid heat exchange during cooling, thus chilling injury can be prevented. The use of very small amounts of solutions (1 to 2 µl) in both techniques facilitate a rapid cooling rate while reducing risks of cracking which may occur during cooling or warming procedures. Cracking, which leads to rupture of the zona pellucida or plasma membrane lysis, is caused by extreme temperature gradients between the outer layer and the core of the vitrified embryo (Dinnyes *et al*., 2000).

The cryotop has been reported to be the current minimum volume vitrification approach (Kuwayama and Kato, 2000; Kuwayama *et al*., 2005a,b) and thus it is at present the most efficient technique for cryopreservation of sensitive samples (Vajta and Kuwayama, 2006), including human oocytes (Kuwayama *et al*., 2005a) and pieces of mouse ovarian tissue (Vajta and Kuwayama, 2006). The minimal volume approach of the cryotop technique increases the cooling and especially the warming rates (up to 40000ºC/minute) which may contribute to the improved and consistent survival as well as both *in vitro* and *in vivo* developmental rates (Kuwayama, 2007).

2.8.3 Embryo Cell Stage

Embryo developmental stage (Cocero *et al*., 1996; Leibo *et al*., 1996) is considered to be a critical factor for the viability of the embryo after vitrification. Several studies have shown that the post-thawed survival of caprine morulae after conventional freezing was poor (Li *et al*., 1990; Puls-Kleingeld *et al*., 1992). Le Gal *et al.* (1993) came to the same conclusion with *in vitro* studies but, upon transfer, did not record much of a difference between morulae and blastocysts. In the very first published attempt to vitrify caprine embryos (Yuswiati and Holtz, 1990), 1 of 2 kids born originated from a morula. In 2 recent investigations on open pulled straw vitrification of caprine embryos that used ‘‘morulae and early blastocysts’’, pregnancy rates of 14% (Guignot *et al*., 2006) and 33% to 51% (Hong *et al*., 2007) were obtained, respectively, but no mention was made as to what stage the surviving embryos belonged to.

It was known that vitrification and warming procedures bringing lower deleterious effects on the viability rate of embryos in advanced stages of development than those in earlier stages (Ali and Shelton, 1993b; Szell and Windsor, 1994). The reason for this difference may be due to the cellular membranes of embryos become more resistant to osmotic and toxic stress after the formation of the blastocoelic cavity. The diversification of cell types and in particular the increase of Na/K ATPase activity which occurs during blastocoelic formation in trophoblastic cells (Watson and Kidder, 1988) may determine more active transport mechanisms of cryoprotectants. Differences in blastomere size may explain the hatching rate. The cells of pre-compacted and compacted morulae are slightly larger than the cells of blastocysts, and this may render them become more sensitive to the osmotic stress which was induced by the permeated cryoprotectant removal (Tachikawa *et al*., 1993). However, embryo exposure to a highly concentrated solution of cryoprotectants during vitrification can produce

irreversible damage to the cytoskeletal organisation of embryos, in particular at the earlier stages.

According to Schneider and Mazur (1984), the increase in cryosurvival rates with more advanced stage embryos was related to the higher number and smaller size of cells which improve chilling tolerance when compared to earlier stage embryos with fewer and larger cells (Szell and Shelton, 1986), thus with scarce junctional contacts between blastomeres (Ziomeck and Johnson, 1980). Permeation of cryoprotectant may, therefore, be lower in early stage embryos (Mazur *et al*., 1976) and may damage intracellular components thus causing cryoinjury (Mohr and Trounson, 1981).