CHAPTER 2

REVIEW OF LITERATURE

2.1. INTRODUCTION

Process of in vivo fertilization involves multiple physiological changes, particularly the ovary, oocytes, oviducts and uterus. During folliculogenesis, the preovulatory oocyte undergoes meiotic maturation, a process which is not only halves the number of chromosomes, but also results in the cytoplasmic and chromosomal changes. This is followed by ovulation, the release of oocyte from the ovary, after which the oocyte is transported to the oviduct for fertilization. During their transport to the site of fertilization, spermatozoa undergo two specific changes, capacitation and acrosome reaction which enable them to penetrate the oocyte. The successful fertilization, which restores the diploid sets of chromosomes, produces embryo, which, while transported along the fallopian tube, it undergoes further embryonic development beginning from 2-cells to become 4-, 8-, 16-cells, morula and blastocyst. Depending on the species, the developing embryo will finally implant within the uterus between 1 day to several weeks after (Whittingham, 1979).

Understanding on the mechanisms of fertilization in mammals through the in vivo process is however, difficult.

Therefore, simulating such natural processes within a defined environmental condition will enable researchers to study not only the mechanisms of fertilization, but also to the extent of micromanipulating the embryos obtained.

Techniques for producing animal preimplantation embryos involving in vitro maturation (IVM) and in vitro fertilization (IVF) are being used in many laboratories worldwide. It is remarkable that within a decade of tremendous interest and rigorous work in the area of the reproductive technology, mechanisms regulating oocyte maturation and embryo development have been increasingly understood. One of the subsequent outcomes of this advancement is the production of viable embryos from ovarian oocytes collected hours after the death of animals. Indeed, hitherto, the basic studies of IVM-IVF have already facilitated the development of other studies such as micromanipulation, cryopreservation and transfer of embryo.

The purpose of the present review is therefore, to discuss the conditions involved in the in vivo fertilization, and how such conditions can be simulated within the in vitro situation. Following this, it will be meaningful to review the extent of knowledge that has been acquired so far through such research.

2.2. FOLLICULOGENESIS

Most part of this section was adapted from Nalbandov (1976) and Austin and Short (1982). In the ovary, after being formed, the oocytes are surrounded by a single layer of flattened epithelial cells which arise either from the rete ovarii or from the coelomic covering of the ovary. Follicular growth involves a change in the shape of the epithelial cells, which become cuboidal in form, and an increase in their number by mitosis. The follicle thus becomes two and three layered, and after a finite number of divisions, fluid accumulates in spaces between the epithelial cells and gathers to form a single antrum; and the follicle is now described as being vesicular.

The ovary is a dynamic structure in which vesicular follicles are constantly developing from those of primordial type. The primary follicles already appear in the ovaries during foetal life (Brambell, 1956). When the hormonal balance is established, that is at the time of puberty, the process of follicular growth is permitted to culminate in ovulation. The majority of vesicular follicles that are produced after puberty and all of those before puberty, undergo degeneration. The number of follicles that attained ovulation is almost fixed for every species. In human, for example, out of 20 recruited, only one follicle usually was

selected each month; the rest atretic or degenerated (Austin and Short, 1982).

Factors initiating follicular growth in primordial follicles remain unknown. It is difficult to explain how certain follicles grow, while seemingly identical neighbouring follicles remain unaffected. It is possible that the oocyte may produce a 'messenger' (possibly RNA) at a specific time in its growth phase. Alternatively, the oocytes may be programmed in some way (Baker, 1982). Perhaps the time of onset of meiotic prophase in germ cells during embryonic or foetal life affects the timing of their subsequent growth phase. The first cells to enter meiosis would also be the earliest to embark on follicular growth. This 'production-line' hypothesis may in part account for germinal selection in ovaries of cattle, monkeys and women in which meiosis is not synchronized and occupies a period of months in the foetal life (Baker, 1982).

The population of germ cells has a fixed upper limit which, with the disappearance of oogonia, is rapidly depleted with increasing age by the process of atresia. Due to the lack of information in farm animals, the situation in human female is referred. The number of germ cells increases from about 1700 during migration to 600,000 during the second month of gestation, and subsequently to almost

7,000,000 by mid-term. The population of germ cells then declines rapidly to about two million of which half are already degenerate at the time of birth. The decline is due mainly to the elimination of large numbers of cells by the process of atresia. Another contributing factor is the eventual cessation of mitosis in oogonia and their transformation into oocytes at the leptotene stage of meiosis (Baker, 1982). The number of oocytes continue to decline with increasing age until the time of menopause in women (or the cessation of reproductive function in animals). Of the 7,000,000 oocytes that were at one time present in the human ovary, only about 400 to 500 will have been ovulated; the remainder of the germ cells have atretic.

The mechanisms responsible for the atresia are obscure, although genetic defects and errors in metabolism are probably involved. The importance of genetic defects in the induction of atresia was shown from studies of mutant mice. Mutation at the *W* locus appears to induce degeneration of germ cells shortly after they have colonized the genital ridges (Austin and Short, 1982). Similarly, human females devoid of one X-chromosome per cell (XO or Turner's syndrome) are characterized by the absence of oocytes after birth (Austin and Short, 1982). Other errors in the germ cells, attributable to defects in metabolism or shortage of nutrients (due to inadequate follicular cells or paucity of blood supply) are probably important contributing factors.

The rate of atresia is controlled by the gonadotrophic hormones (Austin and Short, 1982). After unilateral ovariectomy the level of circulating FSH in mice is unaffected and the remaining ovary undergoes compensatory hypertrophy so that many growing and Graafian follicles occur as in two ovaries of controls. The rate of ovulation for the ovary doubles and the number of offspring per litter is unaffected. But, the reproductive life span of these mice are reduced since no new oocytes can be formed. Hitherto, however, the precise mechanism of gonadotrophic control on atresia still not clear.

2.3. PREOVULATORY MATURATION

2.3.1. Nuclear Maturation

For ovulation to occur, the Graafian follicle and the oocyte within it, undergo further changes which can only occur under precisely controlled hormonal condition. The level of follicle stimulating hormone (FSH) is elevated for only a short time at the beginning of the growth phase, after which the circulating gonadotrophin (FSH and luteinizing hormone, LH) remaining fairly constant until a short time before impending ovulation (Speroff and Vande Wiele, 1971). In cattle, this corresponds to approximately 6 hr after the initial rise of the LH surge or the onset of the

oestrus (Bernard *et al.*, 1983) or 22 hr after the LH peak (Callesen *et al.*, 1986). The interval between the LH peak and ovulation varies from 12 to 15 hr in the mouse, rat and rabbit, through 36 hr in the human female to about 42 hr in the pig (Speroff and Vande Wiele, 1971). The onset of preovulatory maturation is marked by LH surge. Shortly before LH peak, there is a sharp increase in oestrogen level and this is considered to be the stimulus for its production.

The LH surge and also the increase in oestrogen induce the increase in the number of granulosa cells to the optimum for the ovulatory Graafian follicle (Baker, 1982). The quality of follicular fluid in the antrum increase, and the follicle thus enlarge, its final size varies between species, e.g. 0.9, 8.0, 25 mm and 30-50 mm in rat, pig, women and horse, respectively (Baker, 1982). Information on other farm animals such as goat is lacking.

The changes in the structure of Graafian follicle are accompanied by a resumption of meiosis within the oocyte. Meiosis has been described into various stages namely prophase (subdivided into five stages, leptotene, zygotene, pachytene, diplotene and diakinesis), metaphase, anaphase, telophase and second meiotic division. The following detailed explanation of these stages were modified from Swanson *et al.* (1973) and Baker (1982).

2.3.1.1. Leptotene

During pre-leptotene, the nucleus acquires a peckled appearance and short segments of chromosomal threads may be seen. During leptotene (thin thread) the cells enlarges and shows an increased affinity for nuclear dyes until eventually the diploid number of chromosome can be identified in cytological preparation. The chromosomes are longer and more slender, and it is impossible to follow a single chromosome throughout its entire length.

2.3.1.2. Zygotene

At zygotene (yolk thread), the homologous maternal and paternal chromosomes are attached together to form pairs. The pairing process (or synapsis) may begin either at the end of the chromosomes or at some point in between. Pairing is accomplished in a short period and hence zygotene stage is transitory.

2.3.1.3. Pachytene

Pachytene (thick thread) is of relatively long duration. The chromosomes are paired along their entire length and they shorten and thicken by spiralization of the threads. As the cells grow and with a more complex cyto-

plasmic organelles (late pachytene stage), the lateral arms of the synaptonemal complex splits longitudinally to form four threads arranged in pairs (the complex now being term tetrads or bivalents). The nucleoli are evident at this stage.

2.3.1.4. Diplotene

Diplotene (double thread) now shows mutual repulsion from each other so that the bivalents separate except at chiasmata where 'crossing over' has occurred during the phase of chromosomal breakage and reunion. The exchange of genetic material between maternal and paternal threads results in reassortment of genes, which ensures that the chromosomes of the oocyte are different from those of either parents. The homologous chromatids constituting each bivalent still remaining attracted to each other, and hence the pattern characteristic of the diplotene stage is maintained.

2.3.1.5. Diakinesis

At the onset of preovulatory maturation, the oocyte is still at the primary stage whose development is arrested immediately following diplotene. At the time of LH surge, the oocyte contains lampbrush chromosomes, but the synthesis of RNA that is characteristic of early stage growth is now terminated. The chromosomes subsequently shorten and thicken, and their lampbrush loops are withdrawn. Chiasmata

move along the chromosomes to become terminal and the threads resemble 'cross' and 'chain' within the nucleus. This event occurs at the germinal vesicle stage of meiosis (diakinesis) and complete the prophase of the first meiotic division. Diakinesis is characterized by a more contracted state of chromosomes, by the disappearance or detachment of the nucleolus from its associated chromosomes, and by the even distribution of the bivalents throughout the nucleus.

2.3.1.6. Metaphase

Metaphase rapidly ensues, in which the bivalents arrange themselves on microtubule of the equator of the meiotic spindle. There is a complete disappearance of the nuclear membrane and the completed formation of the spindle.

2.3.1.7. Anaphase

During anaphase the bivalents move to opposite ends of the spindle, which rotate through 90^{o}, so that the axis becomes radially oriented. The two centromeres of each bivalent in meiosis are each functionally undivided as they move poleward, with the result that whole chromosomes instead of chromatids aggregate. Each anaphase group, therefore, is made up of a haploid number of chromosomes.

2.3.1.8. Telophase

This is then rotated by early telophase when the surface of the oocyte near to the chromosomes may form slight elevation. The repulsion of the two sets of chromosomes is now complete and the area of the spindle between them is elongated. Division of the cytoplasm of the oocyte rapidly occurs as a furrow around the more peripheral set of chromosomes. This division produces two daughter cells, with one (secondary oocyte) receiving most of the cytoplasm while the other (first polar body, Pb1) the minimum.

2.3.1.9. Second Meiotic Division

Immediately after the extrusion of the Pb1 the oocytes continue on the second meiotic division. Prophase is very short, and the chromosomes condensed to form a half-moon shaped mass at the periphery of the oocyte. Spindle microtubules subsequently appear adjacent to the chromatin and the chromosomes arrange themselves on the metaphase plate. Metaphase II is a stage of arrested development like the dictyate stage, and it is in that stage ovulation generally occurs. The subsequent meiotic maturation of the oocyte depends on penetration by spermatozoon during fertilization.

Germinal vesicle breakdown (GVBD) and resumption of meiotic changes within oocytes in Graafian follicles is induced by LH surge. But if oocytes are removed from their follicles prior to the release of LH in vivo and placed in a specific medium devoid of all hormones they can resume meiosis spontaneously (Brambell, 1956; Zuckermann, 1960). This suggests that Graafian follicle inhibits the maturation of the oocyte until it is triggered by LH (Baker, 1982).

2.3.2. Cytoplasmic Maturation

The cytoplasm of the oocytes, or ooplasm, is bounded by the plasma membrane and contains the nucleus, and a variety of organelles (Austin and Bishop, 1957; Austin, 1982). The main organelles in the oocytes are the mitochondria, endoplasmic reticulum and the Golgi system (Austin, 1982). In somatic cells, mitochondria commonly take the form of elongated double-walled sacs with incomplete partition or cristae, formed by the folding of inner membrane (Austin, 1982). In oocytes or 2-cell embryos, the mitochondria are more spherical in form, and the cristae are few in number. As the embryo goes through successive cleavage after fertilization, the mitochondria develop more cristae and become similar to somatic cell mitochondria. Concurrently, the metabolic capability of the system increases (Austin, 1982).

Endoplasmic reticulum in oocytes take the form of relatively sparse, smooth-walled, flattened vesicles, and thought to be involved in a low level of protein and nucleic acid synthesis (Austin , 1982). During cleavage the system becomes more complex, and rough endoplasmic reticulum, i.e. armed with ribosomes, comes to be a feature of secretory tissues as these differentiate during embryonic development. In the ovary, the oocyte Golgi system is involved in vitelloginesis or yolk synthesis and seems also responsible for the formation of the cortical granules. Cortical granules play an important role in the oocyte response to sperm penetration, essentially as part of the mechanism preventing polyspermy (see 2.10 for detail).

2.4 FERTILIZATION

According to Bedford (1982), fertilization which occurs in the oviduct, comprises a series of steps with penetration of the zona pellucida by spermatozoon followed by incorporation of it into the cytoplasm of the oocyte, and then the activation of the oocyte. Next there occurs a transformation of the sperm nucleus and the remaining haploid set of oocyte chromosomes so as to form, respectively, the male and female pronuclei. The approach of the pronuclei and the eventual pairing of the chromosomes that later differentiate within each pronucleus constitute syngamy - the final step which

occurs about 12 hr after the spermatozoon first enters the oocyte (Bedford, 1982).

The detailed stages of fertilization as described by Austin and Bishop (1957) was as follows: there is initial contact of spermatozoon with the cumulus oophorus soon after ovulation. This was followed by the binding of spermatozoon to the surface of the zona pellucida after it has passed through the cumulus cells. Then, there is a moment of sperm attachment and fusion with the oolemma after penetration of zona pellucida. This is accompanied by some contraction of the vitellus and so enlargement of the perivitelline space. This is followed by the swelling of sperm head and extrusion of the Pb2 as well as the decondensation of sperm chromatin. The following stage is the formation of pronuclei. After the fusion of pronuclei, the chromosomes of male and female are aligned on a mitotic spindle whose progress from metaphase through to telophase resulted in the first cleavage of new embryo.

Other component in fertilization such as sperm capacitation, acrosomal reaction, sperm concentration and sperm characteristics as an indicator of fertilization will be dealt in detail in the appropriate section (2.9).

2.5. EMBRYONIC DEVELOPMENT

2.5.1. Stages of Development

Once the embryo attained the 2-cell stage, it undergoes a series of mitotic divisions, resulting in a rapid increase in the number of cells. These cells which become smaller with each cleavage division, are known as blastomeres. Goat embryos surgically collected by Gardner *et al.* (1984) showed that the 4-, 8- to 16-cell stages were reached at 48 and 96 hr after ovulation, respectively. The morula and early blastocyst was reached at 120 hr, the expanded blastocyst at 144 hr and hatching blastocyst at 168 hr after ovulation (Gardner *et al.*, 1994). In their IVF studies in goat, Pawshe *et al.* (1994) showed that development of the cleaved embryos up to morula and blastocyst stages was observed on days 4 and 5, respectively.

2.5.2. Requirement of Nutrient

In conjunction with the rapid increase in cell number after compaction, embryos begin to utilize glucose as an energy source and increase amino acid uptake (Epstein, 1975; Flood and Wiebold, 1988). The high level of lactate dehydrogenase (LDH) is characteristic of mammalian preimplantation embryos, reflecting the embryos initial dependence upon pyruvate and lactate as energy source (Gardner *et al.*, 1994).

When the embryo is at 12- to 16-cells stage (morula), it travels from the ampullary part of the oviduct to the uterus. The travel of embryo in the oviduct is facilitated by ciliated cells (Odor and Blandau, 1973), whereas the nutrients which were mainly protein and glycoprotein were provided by the non-ciliated secretory cells (Oliphant, 1986). In the uterus, the embryo is dependent upon uterine secretion for its energy and growth. The uterine gland secretes 'uterine milk' which is composed of protein, fat, and glycogen. It is from uterine milk and cellular debris from the epithelial lining of the uterus that the young embryo derives its sustenance until it implants itself and forms permanent placental connection with the maternal circulatory system (Nalbandov, 1976).

2.5.3. Developmental Block

Cleavage of mammalian embryos in vivo is characterized by a prolonged 8- to 16-cell stage followed by a period of rapid cleavage after compaction. This has been reported in the sheep (Crosby *et al.*, 1988) and goat (Sakkas *et al.*, 1989). Such prolongation of 8- to 16-cell stage is associated with activation of the embryonic genome (Crosby *et al.*, 1988; Telford *et al.*, 1990). The lengthened cleavage stage in vivo and its link to embryonic genome activation is also

demonstrated in the mouse at the 2-cell stage (Goddard and Pratt, 1983) and in the pig at the 4-cell stage (Bavister, 1988). Beside activation of embryonic genome, it was found also that the loss or decay of mRNA molecules of maternal origin, developmental arrest in the presence of transcriptional inhibitors, and marked qualitative changes in protein synthetic patterns from one stage to the next become major features that characterize the timing of the switch from maternal to embryonic genome control (Telford *et al.*, 1990). Recently, in their attempt to overcome the 2-cell block phenomenon in hamster, Nakayama *et al.* (1993) suggested that the developmental block may be related to oxygen toxicity, which results from exposure to high oxygen concentrations and light exposure during the manipulation of oocytes and embryos.

2.5.4. Embryonal Mortality

It has been well known that the implantation rate and the number of young carried to term in most mammals are less than the number of oocytes fertilized. The discrepancy between the number of oocytes fertilized and the number of embryos found in the uterus varies greatly between the individuals of one species but is nearly constant for all polytocus species. Several studies of rats, rabbits, pigs and other litter bearers have shown that 30 to 50% of the fertilized oocytes are lost sometime during gestation

(Nalbandov, 1976). Most of the foetal mortality occurs during the first third of pregnancy, and probably most of the losses occur between fertilization and implantation. The physiological factors responsible for embryonal mortality are not known but several suggestions have been put forward (Nalbandov, 1976).

Ageing of germ cells is found to be an important factor for embryonal mortality. Based on work in pigs, Phillips and Zeller (1941) found that while there was no loss from failure to conceive, the loss during gestation, parturition and loss from birth to weaning were 31.3, 3.2 and 22.9%, respectively; and the live pigs at weaning was 42.6%.

The embryonal mortality among pigs is higher in animals with high ovulation rates (Nalbandov, 1976). This does not mean that high ovulation rates are undesirable; despite the higher mortality, the group with the highest ovulation rate produced one pig more than the group with the lowest ovulation rate and the lowest embryonal mortality.

Error of fertilization can lead to abnormal chromosome number in oocytes (Bedford, 1982). Polyspermy, although seen naturally in a low percentage of cases, it can be enhanced by ageing of oocyte before fertilization. Polygyny, the failure to extrude the second polar body resulting in an

extra set of female chromosomes, also a contributing factor for embryonic death.

In contrast to the ageing of oocyte in female, consideration has also to be made on sperm. It has been found that the chance for mutation in the sperm nucleus increase as a function of time, and that ageing brings an increase in genetic defects in spermatozoa (Bedford, 1982). The implication of this is that, in nature, the female in most mammals mates some hours before ovulation, or else, as in rabbits, ferrets and shrews, ovulation is reflexly induced by the act of copulation. This means that capacitated spermatozoa are waiting (therefore ageing) for oocytes which is yet to be ovulated.

Certain intrauterine factors may be conducive to greater embryonal mortality, but what these factors are remain unknown. In his studies in mice, Fekete (1947) compared the normal reproductive performance of two strains of mice, dba and C57 (black). They found that despite a lower ovulation rate, C57 has bigger litter sizes than dba. They concluded that the oocytes of dba are less viable and that dba uteri are less hospitable.

2.6. COLLECTION OF OVARIES AND FOLLICULAR OOCYTES

One of the important sources of ovaries are from the animals that are slaughtered in abattoirs. Dealing with ovaries from a commercial slaughter house, however, necessitates the worker to return to the laboratory in a reasonably short period following slaughter. Totey *et al.* (1992) in their studies on IVM, IVF and IVC of follicular oocytes of buffalos obtained the ovaries within 2-3 hr of slaughter and transported to the laboratory at 25-30°C in normal saline. Another source of ovaries is surgical removal of ovaries by laparotomy. In their studies on maturation of follicular oocytes from ovaries of immature goats, Song *et al.* (1985) removed the ovaries immediately after laparotomy, kept in saline at 35°C and transported within 30 min to the laboratory.

In some earlier studies, oocytes were collected by aspirating follicles by means of a needle attached to a syringe (Song *et al.*, 1985; Parrish *et al.*, 1988; Younis *et al.*, 1991; Shamsuddin *et al.*, 1993). The aspirated follicular contents were placed in a sterile watchglass, and the oocytes were removed with a small-bore pipette under a dissecting microscope. In his studies with porcine oocytes, McGaughey (1978) tore the follicle with the tip of a sterile 18-gauge needle. As the follicle was torn, the

ovaries were shaken in the medium to allow the released oocytes to fall onto the floor of the watchglass. Another technique on collection of oocytes is slicing the surface of the ovaries. The ovaries were then flushed with the washing medium using a syringe followed by washing the ovaries in a small beaker. Slicing of ovaries is then repeated before another flushing.

Recently, Pawshe *et al.* (1994) compared three methods of recovery of goat oocytes for IVM-IVF namely, slicing, aspiration and puncturing. They concluded that the recovery of oocytes through the slicing method was simpler and more efficient compared to the aspiration and puncturing methods.

2.6.1. Classification of Oocytes

Classification of oocytes varies, depending on the researchers. McGaughey (1978) classified porcine oocytes into three categories, depending on their general appearance and the density of granulosa cells surrounding them. They are classified as "good" if the oocytes are well rounded and are completely surrounded by a dense layer of granulosa cells, "fair" if they are round and contain a nearly complete layer of surrounding cells and as "poor" if they are either abnormally shaped or have a few or no surrounding granulosa cells. Oocytes of the first category usually

represent 25% of the total pool of oocytes harvested from small or medium-sized follicles and are considered to be the group most capable of maturation in vitro (McGaughey, 1978).

Song and Iritani (1985) and Song *et al.* (1985) has classified goats' oocytes into three categories based upon the presence of cumulus cells, that is 'disperse cumulus', 'compact cumulus' and 'without cumulus'. They found that the presence of cumulus cells has an important role on oocyte maturation, but is not necessary for sperm penetration. Shioya *et al.* (1988) in their IVF studies on cattle has classified the oocytes into 3 groups. Class A oocytes had compact and dense cumulus cell layers. Class B oocytes had compact but not dense cumulus cell layers; some were partially naked oocytes with compact cumulus cells. Other class B oocytes (B") were partially naked oocytes with thin cumulus cell layers or with small remnants of cumulus cells. Class C consisted of naked oocytes.

Leibfried and First (1979) classified bovine oocytes based on cumulus cells, cytoplasm and chromatin. On the basis of cumulus cells, they classified oocytes into four categories: group 1 oocytes were those with complete cumuli oophori, more than three layers thick and compact. Group 2 were those with partial cumulus cells, either not completely

surrounding the oocyte or less than three layers thick and compact. In group 3, expanded cumulus present, cellular investment showed expansion, and cumulus cells appearing in scattered clumps in matrix. Group 4 is nude oocyte, cellular investment not present, and the oocytes were only enclosed by zona pellucida.

De Loos *et al.* (1989) described four categories of oocytes based on the following criteria: category 1, compact multilayered cumulus investment, homogeneous ooplasm and total COC light and transparent; category 2, compact multilayered cumulus investment, homogenous ooplasm but with a coarse appearance, and a darker zone at the periphery of the oocyte and total COC slightly darker and less transparent; category 3, less compact cumulus investment, ooplasm irregular with dark clusters and total COC darker than categories 1 and 2; category 4, expanded cumulus investment, cumulus cells scattered in dark clumps in a jelly matrix, ooplasm irregular with dark clusters and total COC dark and irregular.

2.7. TISSUE CULTURE MEDIA AND FACTORS AFFECTING THEM

Culture media may influence the number and quality of embryos. Water is the single largest component of culture media and water quality has been shown to affect pregnancy rates in human IVF (Rinehart *et al.*, 1988). Glassware,

syringes, filters and other materials used to prepare culture media may be embryotoxic (Bavister and Andrews, 1988). There is a concensus that all contact materials used in IVF embryo culture and transfer should be toxicity tested.

El-Badrawi and Hafez (1982) noted that the appropriate culture media for IVF have several characteristics: supports the acrosomal reaction of spermatozoa, the fertilization process and provides adequate energy sources to maintain sperm motility and metabolism of oocytes. Culture media therefore, should be sufficient to support the oocytes for maturation (IVM), fertilization (IVF) and also for embryonic development (IVC).

The important difference of these three media, among others, is the the concentration and the presence or the absence of glucose. IVM medium requires a lower concentration of glucose compared to that of IVF medium, whereas that of IVC medium does not require it at all. The rationale for this will be explained in 2.7.3. IVC medium also, compared to IVM and IVF media, consists of taurine, the reason of which will be discussed in 2.7.3. To simulate the situation in vivo, the IVF and IVC media require somatic cell co-culture system for fertilization and pre-embryonic development (see Section 2.11). The use of lactate and pyruvate in either IVM, IVF or IVC media is a matter of opinion between workers (see Section 2.7.3). The importance of hormones such as LH

for the fertilization and development of rat oocytes has been demonstrated by Shalgi *et al.* (1979). Beside these, capacitation of sperm with specific reagents such as caffein (Aoyagi *et al.*, 1988), calcium ionophore (Byrd, 1981; Ben-Av *et al.*, 1988) or heparin (Parrish *et al.*, 1988) is important consideration for the success of IVF (see Section 2.9.1).

2.7.1. Inorganic Salt

It is generally accepted that the principal function of NaCl is to regulate the osmolarity of the culture medium. In their study on the development of preimplantation mouse embryo, Wales (1970) found that development occurred over a wide range of K^+ concentration (0.6 to 48 mM) but almost completely inhibited by the absence of K^+. The absence of Ca^{+2} in media also has been shown to inhibit cleavage and prevent the compaction of mouse morula. Normal development has occurred over a wide range (0.4 to 10 mM) of Ca^{+2} concentration (Ducibella and Anderson, 1975). The absence of PO_4^{-2}, Mg^{+2} and SO_4^{-2} in media has little effect on the development of mouse embryos to the blastocyst stage (Wales, 1970). The presence of HCO_3^- in culture medium is for the regulation of pH. Studies by Brinster (1972) showed that removal of HCO_3^- and CO_2 from the culture medium results in reduced development.

2.7.2. Osmolarity and pH

Work on laboratory animals suggests that 2-cell mouse embryos developed into blastocysts in media ranging in osmolarity from 0.2 to 0.354 OsM (Brinster, 1965) whereas that for the 2-cell rabbit embryos from 0.23 to 0.339 OsM (Naglee *et al.*, 1969). Wright and Bondioli (1981) have noted that the effect of osmolarity of the medium on embryo development is minimal. The pH of media and supplements commonly used for the culture of embryos ranged from 7.1 to 7.4 (Wright and O'Fallon, 1987) with the addition of BSA or sera likely to lower pH. Studies in mice (Brinster, 1965) and rabbit (Kane, 1974) indicated that development occurred over a wide range of pH values (6.0 to 7.8). Thus, it looked as if pH has a minimal effect on mouse embryo development in vitro. Bicarbonate is most often used to regulate pH although greater stability of pH is obtained when Hepes (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid) is added to the medium. However, inclusion of Hepes in the medium may reduce the proportion of embryos that develop to the blastocyst stage (Walker *et al.*, 1989).

Compared to other mammalian embryos, human embryo may be more sensitive to osmolarity and pH changes. Daya *et al.* (1987) noted that pH alteration has been associated with reduced fertilization rate in human. A decrease in pH ranging from 0.1 to 0.4 leads to fertilization failure, and

increasing pH above the physiological range leads to decrease embryo development (Boyers *et al.*, 1987). Compared to that in animals, the osmolarity range for normal development in human is narrower, and an increase in osmolarity leads to decrease fertilization and development (Fisch *et al.*, 1990b).

2.7.3. Energy Substrate

Like any other living cells, embryos require certain substrates during development. Porcine embryos, for example, require α-ketoglutarate, while lactate and pyruvate inhibit development (Davis and Day, 1978). The significance of this observation however, remains to be determined. The requirement for glucose was also found to be increased from 2-cell to blastocyst stage. This has been demonstrated in mouse (Wales, 1986), pig (Flood and Wiebold, 1988), sheep (Thompson *et al.*, 1991) and human (Wales *et al.*, 1987). Robl *et al.* (1991) has also shown that glucose improved development of cattle embryos after 8-cell stage. Kim *et al.* (1993) has also shown that adding 5.56 mM glucose 120 hr postinsemination improved development of bovine embryos to the blastocyst and expanded blastocyst stages. However, in early embryonic development, Takahashi and First (1992) and Kim *et al.* (1993) found that glucose was not only unnecessary but also could cause detrimental effects. Glucose was thought to increase the production of oxygen radicals which

were severely detrimental to early embryo development (Reiger, 1992).

Glutamine, on the other hand, is found to be a significant source of cellular energy in the embryos, as it is for many rapidly proliferating cells (Newsholme *et al.*, 1985). This is because glutamine metabolism can provide reducing equivalents for energy production and to counteract lipid peroxidation, under conditions where the metabolism of other substrates cannot (Reiger, 1992).

Lactate and pyruvate are capable of supporting development of one-cell bovine embryos (Takahashi and First, 1991). These workers also found that lactate played an important role in first cleavage while amino acids enhanced early development. Kim *et al.* (1993) found that lactate and pyruvate, alone or combined, supported the development of in vitro fertilized bovine embryos to the 8-cell stage, but pyruvate was required to support development to the morula stage. In their studies on development of bovine embryos in vitro, Rosenkrans *et al.* (1993) found that adding pyruvate to a medium containing lactate was not necessary for development of embryo to morula stage.

Embryo development can be improved by addition of hypotaurine and/or taurine in the medium as shown in pig (Reeds *et al.*, 1992) and hamster (Bavister *et al.*, 1991).

Reeds *et al.* (1992) found that a greater proportion of the morulae become blastocysts with the addition of both taurine and hypotaurine. These workers noted that the effects of taurine and/or hypotaurine observed may be due to, firstly, metabolic effects leading to a simple acceleration of in vitro development to the blastocyst stage, or secondly, a direct effect on the process of differentiation from the morula to blastocyst stage.

2.7.4. Amino Acids

Amino acids have been reported to be essential for in vitro development of early embryos in rabbits (Kane and Foote, 1970), hamsters (Bavister *et al.*, 1983a), rats (Zhang and Armstrong, 1990), pigs (Rosenkrans *et al.*, 1989) and cattle (Takahashi and First, 1992). It is not clear how amino acids improve in vitro embryonic development, but some amino acids such as glutamine may act as an energy source as mentioned earlier (Reiger, 1992). Supplementation of amino acids to culture medium may also increase the pool size of endogenous amino acids and *de novo* protein synthesis (Zhang and Armstrong, 1990). The rate of protein and RNA synthesis increase dramatically between the 16-cell and the blastocyst stages in cattle, and incorporation of methionine also increases progressively from the 16-cell stage (Frie *et al.*, 1989).

2.7.5. Trace Elements

Trace elements are not required for blastocyst formation in the mouse or in the rabbit (Kane and Foote, 1970). However, Kane (1978) has suggested that Analar grade salts that make up the major salt constituents of the medium may be contaminated with enough trace elements to supply the embryo's need.

2.7.6. Macromolecules

Many studies have shown that the growth and development of mammalian embryos are enhanced when a macromolecular component is present in culture medium. Bovine serum albumin (BSA), for example, has been shown to be necessary for blastocyst formation in the rabbit (Kane and Foote, 1970). Lindner *et al.* (1979) demonstrated a beneficial effect of increasing BSA concentration on premorulae ovine embryo development.

Besides BSA, various sera have been used as media supplements. Sera is heat-treated at 56°C for 30 min for the removal of compounds that have been shown to be toxic to embryos (Chang, 1949).

2.7.7. Gas Atmosphere

Two gaseous atmospheres have been routinely used for the culture of mammalian embryos, namely 5% CO_2 in air and the mixture of 5% CO_2, 5% O_2 and 90% N_2. The nitrogen component of gas mixture is considered to be inert. The 5% CO_2 component, in combination with 25 mM HCO_3^-, is used to regulate the pH at about 7.4. There is evidence that bovine (Tervit *et al.*, 1972), ovine (Tervit *et al.*,1972; Trounson and Moore, 1974; Wright *et al.*, 1978) and porcine (Wright, 1977) premorula stage embryo development is superior under a 5% as opposed to a 20% oxygen atmosphere. The oxygen tension within the female reproductive tract is approximately 5%; therefore in general, embryo development in vitro is considerably better under 5% oxygen than under air (Khurana and Wales, 1989).

In their studies with mice, however, Whitten (1971) suggested that the beneficial effect of 5% O_2 atmosphere was only slight and most pronounced between one- and two-cell stages. It has been suggested also that the optimal O_2 levels may vary, depending on embryo cell stage (Pabon, 1987). Therefore it might be advantageous to vary gas concentration over the course of incubation in order to attain optimal outcome.

2.7.8. Temperature

Most IVF in mammals is performed at 37°C regardless of the normal basal body temperature of the species (cat, 38.6°C; cow, 38.5°C; hamster, 38°C; horse, 37°C; human, 37°C; mouse, 37.8°C; rabbit, 39.4 to 39.6°C; rat, 38.0 to 38.2°C; sheep, 39.1°C) (Lavy *et al.*, 1988). However, Eng *et al.* (1986) found that the optimal fertilization occurs at body temperature rather than at an arbitrary 37°C. Altering oocytes temperature resulted in maximal cumulus expansion between 35 to 39°C, but not at 41°C. Therefore optimal temperature for oocytes and zygotes development for different species remains a question.

2.8. MATURATION OF OOCYTES IN VITRO

The availability of viable, developmentally competent oocytes is critical for the progress of the development of in vitro maturation, in vitro fertilization, embryo culture and related reproductive technologies in mammals. In mammals, primary oocytes reach the diplotene stage of meiosis at approximately the time of birth (Brambell, 1956; Zuckerman, 1960). The germinal material of the oocytes is then arranged within the vesicular nucleus, the dictyate stage. The oocytes remain in the arrested state until a few hours before ovulation. However, the primary oocytes are capable of resuming meiotic maturation spontaneously when removed from their follicles and cultured in vitro (Chang, 1955).

Mammalian oocytes from small antral follicles can be matured in vitro up to the stage where they can be fertilized but their average subsequent developmental capacity is limited. This natural phenomenon in mammals has greatly facilitated the use of in vitro matured oocytes for fertilization and embryo production.

Before any oocyte can be expected to be able to mature in vitro, it should be visualised as being normal. This includes the presence of cumulus cells surrounding the zona pellucida, absence of cracked zona pellucida, and absence of vesicles in the cytoplasm. The presence of more and compact layers of cumulus cells is considered the better. A good oocyte also appears golden in colour and have granulated appearance in the cytoplasm. The size of oocyte is also important for the attainment of maturation. De Smedt *et al.* (1992) showed that 86% of goat oocytes from follicles 2 to 6 mm in diameter progressed to metaphase II, whereas only 24% oocytes from follicles 1 to 2 mm attained that stage. A good oocyte also has cytoplasm which fills the entire part of vitelline space.

2.8.1. Criteria For Matured Oocytes

Normal development after fertilization requires that the oocytes have first attained nuclear and cytoplasmic maturity. Oocyte shed before maturity may be deficient in

materials or mechanisms needed for fertilization and development, while mature oocyte retained in the follicle may suffer loss or degeneration of these materials or mechanisms. It is now clear that the nuclear maturation is not the only event occurring during maturation because in mouse, the extrafollicular oocytes can complete all aspects of its maturation in vitro, as shown by the presence of 15-day old living foetuses (Cross and Brinster, 1970) and the birth of young (Mukherjee, 1972) after complete maturation and fertilization in vitro before transfer to foster mother.

2.8.1.1. Cumulus Cells Expansion

In animals there exists a sequential relationship between cumulus cell expansion and oocyte maturation. The degree of dispersion of the cumulus, corona cell layers also correlates well with nuclear events in immature human oocytes (Testart *et al.*, 1983). This in fact becomes a rationale for the categorization of human oocytes as immature on the basis of very tight corona cell layers and equally dense or absent cumulus cell layers (Dandekar *et al.*, 1991). Regarding the time of expansion, in bovine, for example, cumulus cells started to expand after 12 hr of incubation in vitro when all oocytes were in metaphase I (Shamsuddin *et al.*, 1993). The degree of expansion increased up to 18 hr of incubation in vitro and remained steady thereafter (Shamsuddin *et al.*, 1993).

Evaluation of these morphological criteria could however, differ between observers and the diagnosis of maturity may not be uniform (Dandekar *et al.* 1991). Therefore it is important that in all studies all oocytes should be evaluated by one individual.

Cumulus expansion has been shown to be induced in vitro by FSH (Thibault, 1972; Salustri *et al.*, 1989), via a mechanism that appeared to be mediated by cAMP (Dekel and Kraicer, 1978). Epidermal growth factor (EGF) is also a potent stimulator of cumulus expansion (Downs *et al.*, 1988). However study in mice showed that separation of the oocytes from the cumulus cells impaired the ability of the cumulus cells to synthesize hyaluronic acid and undergo cumulus expansion in vitro, indicating that these oocytes secrete a specific, developmentally regulated cumulus expansion-enabling factor that allow cumulus cells to undergo expansion in response to FSH, cAMP or EGF (Buccione *et al.*, 1990). Recently, Vanderhyden (1993) had further shown that a factor(s) secreted by mouse and rat oocytes is necessary for the cumulus cells to undergo expansion in response to FSH, EGF or cAMP. Pig oocytes can secrete a cumulus expansion-enabling factor, but expansion of pig oocyte complexes does not depend upon this factor.

2.8.1.2. Nuclear Maturation And its Control

The final nuclear maturation of the oocyte leading to a haploid gamete occurs during the last few hours before ovulation. This contrasts with maturation of spermatozoa which occurs weeks before ejaculation. This difference in timing of nuclear maturation is illustrative of the different life spans and cellular strategies of the two gametes (Sirard *et al.*, 1992). During spermatogenesis, the chromosomes become highly condensed, thereby preventing new RNA synthesis. This phenomenon reduces the cell's lifespan but the resulting spermatozoon is a very small cell with reduced metabolism is being supported by the epididymis. The oocytes, on the other hand, provide all the necessary metabolites to early zygote until the new embryonic genome becomes activated and takes over control of development. The size of oocyte necessitates active metabolism. Therefore, it is important that the immature oocytes maintain an active nucleus and RNA and protein synthesis, almost until the last minute. This explains the need for a specific signal to trigger maturation in oocytes, but not in spermatozoa.

Nuclear maturation occurs spontaneously in vitro when oocytes are removed from follicle and cultured in a proper medium (Chang, 1955). Two major events are involved in this process. First, the cumulus-oocyte complex (COC) is removed

from the influence of the follicular micro-environment (follicular fluid). Second, physical contact with mural granulosa cells is ruptured, terminating intercellular communication via the gap junctions. This chemicophysical stimulation of the oocyte causes condensation of the chromatin and the breakdown of the nuclear membrane (germinal vesicle), leading to metaphase II and a second artificial arrest in the cycle (Edwards, 1965). To explain this phenomenon, it was postulated that the follicle produces an inhibitory factor preventing meiotic resumption, since in foetal gonads of females, meiotic arrest is associated with the presence of somatic cells forming the primordial follicle (Wassarman, 1988a).

Numerous studies have been carried out to isolate the follicular fluid fractions or components that inhibit spontaneous maturation of cumulus-oocyte complexes in vitro. In their studies on inhibition of oocytes maturation in porcine, Tsafriri and Channing (1975) and Tsafriri *et al.* (1976) found the presence of low molecular weight fractions in the follicular fluid. Sato and Koide (1984) found the presence of small peptides from bovine granular cells and these peptides can influence meiotic resumption. Stimulation of cAMP-dependent protein kinase (PKA) has proven as the principal factor controlling meiotic resumption in the mice and the rats although the exact mechanism is still un-

known (Downs *et al.*, 1989). Recently Sobajima *et al.* (1993) investigated the role played by mitogen-activated protein kinase (MAP kinase) in the cascade of protein kinase activation in mouse oocytes. They found that MAP kinase is involved in the regulation of meiotic maturation, and that the activation of MAP kinase requires protein synthesis and is inhibited by the protein phosphate during meiotic maturation in mouse oocytes. In the mouse, purines are high candidates for natural meiotic inhibitors since they are present sufficiently in murine follicular fluid (Downs *et al.*, 1985). Later, Downs *et al.* (1989) found that purines act by inhibiting the enzyme phosphodiesterase.

From the above discussion it appears that multiple follicular factors combine to create environment in which the germinal vesicle is maintained. It is thought that the sensitivity of the oocytes to maturation inhibitors is modulated during folliculogenesis (Sirard and First, 1988).

A protein could also be involved in meiotic control. Sato and Koide (1984) have isolated a small peptide (< 10 KD) from bovine follicular fluid and found that this protein could inhibit meiotic resumption of mouse oocytes in culture. Sirard *et al.* (1992) found the association between the mural granulosa cells and cumulus cells in preventing the nuclear maturation.

2.8.1.3. First Polar Body

The presence of first polar body (Pb1) in the in vitro cultured oocytes suggests the change of the genetic apparatus from its dictyate state to completion of the first maturation division. Similar criteria for maturation have been adopted by Laufer *et al.* (1984) and Wahid *et al.* (1990). In their studies on goat IVF, Younis *et al.* (1991) identified matured oocytes on the basis of expanded cumulus cells and / or Pb1. In rabbit, guinea pig and hamster, the first polar body normally remains as a stable structure in the perivitelline space until the first cleavage (Bedford, 1971). In rats and mice, the first polar body disappears soon after its extrusion, and in the mousedeer it appears only as a faint cytoplasmic globule (Marston and Chang, 1966).

It appears then, that the presence of the first polar body as a criterion for maturation depends on the species as well as the inherent biologic variability of the oocytes. As such the presence of first polar body alone might not be sufficient for identifying matured oocytes.

2.8.1.4. Cytoplasmic Ultrastructural Changes

At present ultrastructural studies on maturing oocytes of farm animals appear to be limited to sheep (Cran *et al.*

1980), cattle (Fleming and Saacke, 1972; Kruip *et al.* 1983; Hyttel *et al.* 1986) and pig (Norberg, 1973). Study on sheep oocytes during antral follicle development by Cran *et al.* (1980) showed that the development of sheep oocytes may be divided into three phases based on the size of growing follicles. In the smallest follicle (0.2-0.4 mm diameter), the golgi apparatus was present in a peripheral position, the endoplasmic reticulum was distended and mitochondria were intimately associated with it. Processes from surrounding cumulus cells were in contact with the oolemma having slender villi, increasing the oocyte surface area 5-fold. In these and all subsequent follicles, the oocyte cytoplasm contained numerous 30 nm particles or vesicles. In follicles of about 2.0 mm in diameter the mitochondria became located in a peripheral band, interior to which were numerous large vesicles, and the villi became shorter and thicker. In follicles of more than 2.0 mm in diameter, the mitochondrial band was dispersed and the cortical granules rested close to the oolemma.

In their studies on bovine oocytes during final maturation in vivo, Kruip *et al.* (1983) found that the mitochondria dispersed, and the majority of the organelles became located towards the centre of the cell. The relatively organelle-free cortical region contained cortical granules immediately adjacent to the plasma membrane together with

aggregates of tubular smooth endoplasmic reticulum (SER).

Development of goat oocytes in vitro has been studied (Kim *et al.*, 1984; De Smedt *et al.*, 1992) but no fine structural basis for the evaluation of such oocytes has been demonstrated. Such studies on this animal will indicate the interspecies cytoplasmic ultrastructural characteristics compared with other known farm animals.

2.8.1.5. Chromosomal Configuration Changes

One of the criteria of the maturing oocytes is the change in the configuration of chromosome during different stages of meiosis. Reports on this aspect, particularly in goat species is lacking. Deb and Goswami (1990) who studied on the cytogenetic analysis of goat oocytes cultured in vitro found that the percentage of matured oocytes was maximum after 32 hr of culture and thereafter the percentage of oocytes with abnormal chromosomal configuration increased, causing a decrease in the number of matured oocytes. Deb and Goswami (1990) also found that soon after liberation from the follicles, majority of the oocytes were at germinal vesicle stage, but this was decreased gradually with increase in culture time indicating the continuation of the meiosis process. The percentage of diakinesis, metaphase I (MI) and metaphase II (MII) increased over the increasing period up to 32 hr. About 20-40% of the oocytes

were at MI stage. Their finding also showed that the percentage of oocytes which attained MII stage was in the range of 10.5 to 18.2% and appeared after culture for 28 hr and beyond. The maturation rate reported by these workers was comparable to that reported by Song *et al.* (1985), although the maturation time was more prolonged in the former (32 hr) than in the latter studies (25 to 35 hr). Deb and Goswami (1990) pointed out the difference as due to the difference in the treatment of ovaries used in both studies i. e. while they used untreated animals of varying age and probably of low reproduction rate, Song *et al.* (1985) used gonadotrophin-treated goat. Resumption of meiosis in pig has been shown to be induced after injecting gonadotrophins (Hunter and Polge, 1966).

2.9. PREPARATION OF SPERM FOR IN VITRO FERTILIZATION

2.9.1. Sperm Capacitation

Although mammalian spermatozoa complete their morphological and biochemical maturation in the epididymis, they remain unable to penetrate oocytes. They need further changes, capacitation, events which occur normally within the female reproductive tract, but now can take place in vitro (Bedford, 1970, 1974). Procedures used to capacitate spermatozoa in vitro vary considerably, depending on the species. Hamster epididymal spermatozoa, for example, become

capacitated after incubation for a few hours in a culture medium containing albumin (Yanagimachi and Chang, 1964; Bavister, 1973). Mouse epididymal spermatozoa were capacitated in vitro by incubation in culture medium for 15 min (Toyoda *et al.*, 1971). Other methods include employing high ionic strength (Brackett *et al.*, 1982; Bondioli and Wright, 1983), bovine follicular fluid (Fukui *et al.*, 1983; Sanbuissho and Threlfall, 1989), caffeine (Aoyagi *et al.*, 1988), calcium ionophore (Byrd, 1981; Ben-Av *et al.*, 1988) and heparin (Parrish *et al.*, 1988). It has been observed that heparin-like activity in the female reproductive tract may play a role in sperm capacitation and fertilization. Parrish *et al.* (1988) showed that glycosaminoglycans (GAG), including heparin, can induce acrosome reaction in bovine spermatozoa and improve both the frequency and quality of fertilization in vitro. However, fertilization frequency achieved with semen from different bulls and with different heparin concentrations have been variable in the above studies.

Despite the availability of various techniques to capacitate the sperm, there is no specific test for capacitation, and fertilization is apparently the common parameter by which capacitation may be judged. Assessment of the state of capacitation of sperm is best made by IVF, where no contribution can be made by oocyte donor. One field of study which can be used to asses capacitation is the ultramorphology of the sperm. This is based on the study by Parrish *et*

al. (1988) that the addition of heparin into bovine spermatozoa induces acrosome reaction.

2.9.2. Sperm Acrosomal Reaction

The acrosomal reaction consists of morphological changes with multiple fusions between the outer acrosomal membrane and the overlying sperm plasmalemma. The membrane changes lead to the loss of the plasma membrane and outer acrosomal membranes over much of the surface of the sperm head, leaving the inner acrosomal membrane exposed. Only acrosome reacted sperm can fuse with the oolemma. The reaction causes the release of several enzymes from the acrosome, including hyaluronidase. A specific trypsin-like enzyme released during this process was extracted, identified and called acrosin (Srivastava *et al.*, 1965).

2.9.3. Sperm Concentration

In in vivo fertilization, of the millions of spermatozoa ejaculated into the vagina, only a very small number reach the site of fertilization. There is quantitative and qualitative selection as sperm penetrate the cervical mucus, the uterine lumen, the uterotubal junction, the isthmus and the ampullary-isthmic junction to reach the normal site of fertilization. The selection of sperm in vitro is arbitrary and random. The required sperm concentrations for IVF range

from $1x10^3$ to $1x10^7$ sperm/ml with an average of about $1x10^6$ sperm/ml (El-Badrawi and Hafez, 1982).

2.9.4. Sperm Characteristics as Indicator of Fertilization Potential

Various sperm characteristics have been suggested as indicators of fertilization potential: sperm motility (Gerris and Khan, 1987), qualitative motility (Talbert *et al.*, 1987), amplitude of lateral sperm head displacement (Jeulin *et al.*, 1986), swim-up migration capacity (Fisch *et al.*, 1990a) and sperm morphology (Mahadevan and Trounson, 1984; Kruger *et al.*, 1986; Jeulin *et al.*, 1986). Using ultramorphological technique, Mashiach *et al.* (1992) demonstrated that the ultrastructural morphology of the sperm head and not the tail component is a key parameter for assessing the sperm fertilizing competence in vitro. However, contradictory conclusions have been drawn by Glazerman and Bartoov (1986) regarding the relationship between sperm tail ultramorphological features and fertilization in vivo. This difference may arise from the fact that in vitro, unlike in vivo conditions, the sperm cells are placed in the immediate vicinity of the oocytes. Therefore, it is possible that in culture, effective sperm motility is not as crucial as in situ (Mashiach *et al.*, 1992). Mashiach *et al.* (1992) have also found that the hyperelongated head, acrosome deficiency and acrosome damage were encountered more

frequently in the nonfertilizing group of patients. Whether such finding can be found in goat remains to be investigated.

2.10. FERTILIZATION OF OOCYTES IN VITRO

Fertilization is a complicated, programmed sequence of events, which begin with fusion of a sperm and an oocyte and culminates in the formation of embryo. Technically it is not possible to study the progress of fertilization within the mammalian oviduct. However, with the technique of in vitro fertilization (IVF), such a study becomes possible. IVF procedure enables researchers to study not only the early, rapid events of fertilization, but also the later stages of development preimplantation embryo.

Research has now clarified not only the basic events of mammalian fertilization but also the molecular mechanisms underlying those events. Bleil and Wassarman (1980a,b) have identified and characterized three different molecules in the zona pellucida of mouse oocytes, zp1, zp2 and zp3. They found that the zp3 is the one which functions in binding of sperm and induction of acrosome reaction. The binding of sperm to the zona pellucida is followed by the acrosome reaction in the bound sperm. The acrosome is an organelle rich in digestive enzymes that lies in the anterior region of the sperm head, just under the plasma membrane

encasing the head. During the reaction, the front two-thirds or so of the plasma membrane fuses with the outer membrane of the acrosome. The fusion results in the formation of small vesicles that consists of the sperm's plasma membrane and the outer chromosomal membrane (Bleil and Wassarman, 1983). These vesicles are eventually sloughed off, exposing the zona pellucida to the inner acrosomal membrane and the acrosomal enzymes. Abetted by the enzymes, acrosin, glycosidases (Dean, 1992) which digest the zona pellucida, the acrosome reacted sperm then bore through the outer coat. One sperm reaches the perivitelline space and fuses with the membrane, fertilizing the oocyte. By this way, the genetic material of the male mingles with that of female, initiating development of the resulting embryo (Wassarman, 1988a). This triggers a series of reactions including the exocytosis of cortical granules. By this, the enzyme that is being secreted by cortical granules change the glycoprotein molecules of zona pellucida and this is responsible for the block to polyspermy (Wassarman, 1988b).

2.10.1. Criteria Of Fertilization

The best criterion of fertilization is that of implantation and continued foetal development following transfer of experimental embryos. However, occasionally this type of procedure is not possible. The following criteria are

important in establishing the occurrence of fertilization of single-cell embryo.

2.10.1.1. Presence of Fertilizing Sperm Tail

The fertilizing sperm tail can be identified in one-celled embryo, and can sometimes be found in the one-blastomere stage after cleavage (Bedford, 1971). Yanagimachi and Chang (1964) showed sperm tails in fresh hamster eggs, which were compressed between cover-glass and slide and viewed with a phase-contrast microscope. In rabbits, hours after entrance of the sperm into the oocyte, the tail becomes separated from the male nucleus and begins to split. The two fragments formed should not be mistaken for two sperm tails, which are seen only in cases of polyspermy (Bedford, 1971). The presence of sperm remnant in the fertilizing cow oocytes has been shown by Brackett *et al.* (1980) using the electron microscopic preparation. Shioya *et al.* (1989) in their studies on cattle and Huneau and Crozet (1989) on sheep also used the presence of sperm tails as one of the identifying criteria for fertilization. Besides in rabbits, it is not clear regarding the timing of the sperm tail separation. Indeed, in oocytes which have a dense ooplasm, as in the pig, detection of the fertilizing tail presents a greater problem (Hancock, 1961).

2.10.1.2. Swelling of Sperm Heads in Ooplasm

This parameter may be used during the first hour after sperm entry. The sperm head begins to swell soon after penetration into the vitellus, this often occurs first in the posterior half of the sperm nucleus. In the early stage, the fertilizing head becomes slightly larger than those lying outside the vitellus and its outline appears less distinct.

2.10.1.3. Male And Female Pronuclei

The time of appearance of pronuclei varies with different species. In laboratory animals this occurs 4 or 5 hr after sperm penetration, whereas in sheep, cow and pig about eight hours later (Bedford, 1971). In goats such information is yet to be documented. Regarding the gender of either pronucleus, the male pronucleus in mouse and rat, for example, is larger than that of female. Whether this disparity is also true for other species remains to be investigated.

Abnormal numbers of pronuclei are sometimes seen. This could be due to polyspermy, failure of extrusion of the second polar body or fragmentation of the female pronucleus prior to syngamy. Oocytes displaying only one pronucleus may arise as a result of spontaneous activation with or without

extrusion of the second polar body, or by failure of development of the male pronucleus after sperm penetration.

2.10.1.4. Second Polar Body

The presence of the second polar body (Pb2) is another indication of fertilization. It is extruded from the vitellus 1 or 2 hr after fertilization; this is approximately coincident with the swelling of the fertilizing sperm head (Odor and Blandau, 1951). In the rabbit, the first polar body (Pb1) appears as a number of scattered particulate fragments, whereas the Pb2 displays a somewhat dense chromatin mass which is often moon-shaped (Bedford, 1971). In the mouse and hamster, the Pb1 and Pb2 may be distinguished from each other since the latter tends to form a compact nucleus (Bedford, 1971).

2.10.1.5. Presence of Sperm in the Zona Pellucida or the Perivitelline Space

Fertilized oocytes commonly show presence of sperm on the surface of, or within the zona pellucida. Perivitelline sperm is a normal feature of fertilized oocytes in rabbits, rats, ferrets, guinea pigs and mouse (Bedford, 1971). However, the existence of sperm in the perivitelline space, though highly indicative, cannot be taken as unequivocal evidence of fertilization.

2.10.1.6. Activation of the Oocytes

If the oocytes remain unfertilized, the process of aging often accompanied by development of a coarse appearance of the ooplasm in which floccules appear. This contrasts with the appearance of the fertilized (activated) oocytes, whose ooplasm presents a bland uniform appearance (Bedford, 1971). It is believed that the cortical granules at the periphery of the vitellus break down and disappear soon after the oocytes become activated by penetration of sperm (Szollosi, 1967). This change probably ensures the block to polyspermy (Wassarman, 1988b). Thus, the presence or absence of cortical granules is a useful indicator in electron microscope studies of early fertilization. The presence of intact granules indicates that the penetration of the oocytes has not yet occurred, albeit sperm may be found within the zona of the oocytes (Bedford, 1971).

The metaphase spindle lies tangential to the surface of the freshly ovulated oocyte, and its rotation to a radial position is one of the first signs of activation in some species. Such rotation may occur spontaneously, and in some species may be the normal orientation of the second maturation spindle. Thus, rotation of spindle alone cannot be considered criteria of activation and fertilization.

2.10.1.7. Ultrastructural Changes in Fertilizing Oocytes

The importance of this criteria until recently seems to be overlooked. This could be due to the sufficiency of defining fertilized oocytes by using the above mentioned criteria via light microscopy. After fertilization, there is no apparent change in the ultrastructure of the hamsters zona pellucida (Meyenhofer *et al.*, 1977), but its ability to bind sperm is lost (Gwatkin, *et al.*, 1973; Wassarman, 1988b). Following the fusion of sperm with the egg's plasma membrane, cortical reaction occurs whereby enzymes in the cortical granules is released into the zona pellucida. This then alters the zp3 molecule, transforming it into an impenetrable barrier to sperm as a guard against polyspermy (Wassarman, 1988b). Ultrastructural changes in organelles of cattle embryo have been studied by Brackett *et al.* (1980). They found the presence of sperm remnants within ooplasm and disappearance of cortical granules and appearance of centrioles at the 8-cell stage. The changes in the organelles of the goat embryo remains to be investigated.

2.10.1.8. Cleavage

The result of fertilization between sperm and oocyte is the formation of blastomere, or cleavage. The initiation of cleavage varies between species of animals as summarised in Table 2.1.

Table 2.1: The initiation of cleavage among mammalian species (modified after Bedford, 1971)

Species	Initiation of cleavage (hr)
Rabbit	12 hr after penetration
Rat	15 to 17 hr after ovulation
Mouse	15 to 17 hr after ovulation
Hamster	15 to 17 hr after ovulation
Gerbil	22 to 24 hr after ovulation
Sheep	16 to 18 hr after sperm entry
Cattle	20 to 24 hr after penetration
Sow	20 to 24 hr after mating
Man	30 hr after fertilization

A similar study in goat embryos either through in vivo or in vitro fertilization is until now lacking. Cleavage rate also varies with the species (Table 2.2).

Although cleavage at the 2- and 4-cell stages can be used with confidence as an indication of fertilization in mammalian species, careful assessment as regard to time/development relationship should be made which show a tendency for parthenogenesis (Bedford, 1971). Activation of rabbit oocytes may be achieved by parthenogenetic agents but spontaneous cleavage in vivo is rare, and occurs long after the expected time of the first cleavage (Bedford, 1971). The spindle in unfertilized rabbit oocytes may sometimes remain for up to 38 hr before fragmention. In the ferret, the second maturation spindle may persist for 64 hr or more, but spontaneous cleavage ultimately occur (Chang, 1957).

2.10.2. Present Status of IVF Studies

Effort to develop IVF technique began as early as 19th century when Schenk (1878) attempted such effort on rabbit and guinea pig oocytes. In 1891 Heape reported the first successful transfer of a normally fertilized rabbit embryo. However, little progress was made with IVF until Austin (1951) and Chang (1951) reported the necessity for capacitation of spermatozoa.

Table 2.2: Cleavage rate of embryos among mammalian species

ecies	Stage	Cleavage rate (hr)	Authors (year)
use	8-cell	47 hr after ovulation	Austin (1961)
	early blastocyst	63 hr after ovulation	
t	8-cell	60 hr after ovulation	Austin (1961)
	early blastocyst	80 hr after ovulation	
bbit	8-cell	20 hr after sperm penetration	Gregory (1930)
	early blastocyst	96 hr after penetration	
nster	8-cell	60 hr after ovulation	Austin (1961)
	early blastocyst	80 hr after ovulation	
ttle	8-cell	48 hr after fertilization	Utsumi *et al.* (1991)
t	2-cell	26 to 30 hr after insemination	Hanada (1985)
	8-cell	72 to 96 hr after mating	Hancock (1961)
	4-cell	40 to 50 hr after fertilization	Sadler (1990)

During the last two decades, significant progress has been made and successful IVF with varying degrees of consistency has been reported in more than 20 mammalian species (Niwa and Iritani, 1985). The first successful IVF in some mammals are summarized in Table 2.3.

2.10.2.1. Laboratory Animals

Recent advances in IVF in laboratory and farm animals have been widely reviewed by Niwa and Iritani (1985). In laboratory animals, IVF has been successfully done in mice (Brinster and Bigger, 1965; Whittingham, 1968; Toyoda and Takasugi, 1982; Thadani, 1982; Suzuki and Toyoda, 1986), rabbits (Chang, 1959; Bedford and Chang, 1962; Brackette and Williams, 1965 , Brackett and Williams, 1968; Brackett and Oliphant, 1975; Niwa *et al.*, 1983) and hamster (Yanagimachi and Chang, 1961; 1964; Barros and Austin, 1967; Bavister, 1969). Hamster became the first mammalian species with which IVF was first achieved using in vitro capacitated sperm (Leibfried and Bavister, 1982). Studies on IVF in rat have produced a successful birth (Toyoda and Chang, 1974; Kaplan and Kraicer, 1978; Gaddum-Rosse *et al.*, 1984; Shalgi *et al.*, 1979). The birth of young through IVF in Chinese hamster (Pickworth and Chang, 1969; Yanagamachi *et al.*, 1983) has yet to be reported. For Mongolian gerbil, the only report on IVF in this species was by Noske (1972).

Table 2.3: First successful IVF in some mammals

Species	Authors (year)
Rabbit	Chang (1959)
Sheep	Thibault and Dauzier (1961)
Hamster	Yanagimachi and Chang (1964)
Mice	Whittingham (1968)
Chinese hamster	Pickworth and Chang (1969)
Pig	Harms and Smidt (1970)
Mongolian gerbil	Noske (1972)
Rat	Toyoda and Chang (1974)
Dog	Mahi and Yanagimachi (1976)
Cattle	Iritani and Niwa (1977)
Man	Steptoe and Edwards (1978)
Guinea pig	Fleming and Yanagimachi (1982)
Cat	Niwa *et al.* (1985)
Goat	Hanada (1985)
Horse	Del Campo *et al.* (1990); Zhang *et al.* (1990)

Clearly, further studies on IVF in this species is warranted. IVF in guinea pig has been investigated (Fleming and Yanagimachi, 1982) but the birth of young awaits further research.

2.10.2.2. Domestic Animals.

Two domestic animals had so far been investigated thoroughly, namely cat and dog.

2.10.2.2.1 Cat

Studies in cat was initiated by Niwa *et al.*(1985) who introduced diluted sperm collected from cauda epididymal duct into 0.4 ml medium containing superovulated oocytes and found that 90-100% of the oocytes were penetrated 0.5 to 5 hr after insemination. Further development of the fertilized oocytes were however, not reported. Johnston *et al.* (1991a) then reported not only the successful fertilization of oocytes in vitro, but also followed by in vitro development of embryos to morula. However, they found that most of the embryos exhibit a developmental block and are unable to advance to blastocyst. Johnston *et al.* (1991b) has further shown that fertilization and development of cat zygotes were not that sensitive to temperature and gas composition. Hitherto, birth of young after IVF-ET in this species remains to be reported.

2.10.2.2.2. Dog

In their studies on IVF in dogs, Mahi and Yanagimachi (1976) found that immature and cultured oocytes were penetrated by washed ejaculated sperm capacitated for 7 hr in BWW medium (Biggers *et al.*, 1971) 11-24 hr after insemination. Later, Mahi and Yanagimachi (1978) found the same result using sperm capacitated for 7 hr in a canine-capacitation medium. Until now there is no report on the birth of young through IVF-ET.

2.10.2.3. Wild Animals

The continued, accelerated loss in global biodiversity has led to emergence of conservation biology, a discipline defining and understanding the many factors influencing ecosystem and species survival. The generation of embryos, either in vivo or in vitro, has clear benefits for conservation biology. Unfortunately, embryo technologies have not as yet contributed to practical conservation of rare wild species although there is encouraging progress.

IVF systems have been developed for rhesus monkeys (Bavister *et al.*, 1983b; Boatman *et al.*, 1986; Morgan *et al.* 1990), squirrel monkeys (Dukelow *et al.*, 1983), cynomolgus monkeys (Balmaceda *et al.*,1984), baboons (Irsigler *et*

al.,1984) and chimpanzee (Gould, 1983) with varying degrees of success. Wilton *et al.* (1993) in their study on IVF-ET in mamoset monkey (*Callithrix jacchus*) have demonstrated that the fertilization rate in this species was highest in oocytes preincubated for 21-29 hr compared with those for 2-5 hr or 9-11 hr.

There is only one report on IVM and IVF in red deer (Fukui *et al.*, 1991). These workers found that red deer oocytes attained maturity between 16-24 hr, and after IVF the embryo cleaved to 2- to 8-cells. No further study, particularly on the birth of kid has ever been reported.

In vitro fertilization in other wild species has also been done, namely pumas (Miller *et al.*, 1990), tiger (Donoghue *et al.*, 1990 and cheetah (Donoghue *et al.*, 1991). In one of this species (tiger), IVF technique has successfully produced a live young (Donoghue *et al.*, 1990).

2.10.2.4. IVF in human

The first successful human birth, a baby girl, conceived by means of embryo transplantation after in vitro fertilization occurred in England (Steptoe and Edwards, 1978). Two months later, a second birth of another baby girl, resulting from a similar procedure but involving an

additional technological step, cryo-preservation, took place in Calcutta, India (Tayaranan, 1978). Soupart (1980) recorded the birth of a third baby, a boy in 1979 as a result of IVF-ET procedure performed again by Steptoe and Edwards. Soupart (1980) stated, from a strictly academic viewpoint, the birth of a male offspring following IVF-ET rules out the possibility of accidental activation of the human ovum, possibly due to extracorporeal manipulation, whereas the birth of female offspring does not.

At present, there are several teams in different parts of the world practicing IVF-ET and other assisted reproductive techniques. In the United States for example, human IVF research had been very active in the early 1970s (Jacobson *et al.*, 1970 Soupart and Strong, 1974). In Australia also, assisted reproductive techniques has been reported (de Kretzer *et al.*, 1973; Matthews *et al.*, 1979; Yovich *et al.*, 1984; Hughes *et al.*, 1990). However, the procedure had raised ethical concern and led to a temporary standstill (Soupart, 1980).

The present status of the ethics concerning human IVF-ET and gamete intrafallopian tube (GIFT) is well documented in the guidelines for the new reproductive techniques by the Ethic Committee of the American Fertility Society (Medical Research International and Reproductive Technology, 1990). It is stated that on the basis of experience so far, IVF

procedure involves no greater risk neither to the child nor to the parents as compared to natural conception. Therefore IVF is ethically acceptable and GIFT may be more acceptable among certain cultural and religious groups as the fertilization occurs at the natural site in vivo.

The IVF-ET and GIFT procedures are becoming more and more successful and achieving recognition from the community. The Medical Research International and Reproductive Technology (1990) reported that in the centres of the United States of America, the overall successful live delivery rates of IVF-ET procedures was 12%. Maturation studies on oocytes obtained from unstimulated ovaries has shown varying results. Immature oocytes incubated with follicular fluid (FF) showed a maturation rate of 55.8% compared to 35.9% with foetal cord serum (FCS) (Cha *et al.*, 1991). When these oocytes were inseminated, 81.0% of the FF group were fertilized as opposed to 31.6% in FCS group (Cha *et al.*, 1991). Implantation rate for human embryos range from 8 to 28% after coculture in simple balanced solution and tissue culture media, and from 18 to 53% after coculture with feeder layer cells (Trounson *et al.*, 1994). In view of the varying degrees of succsess, more studies are required to enhance our knowledge in this area of development.

2.10.2.5. Farm Animals

It is surprising that IVF has proved more difficult to accomplish in farm animals than in human. Nevertheless, in vitro fertilization and embryo transfer (IVF-ET) of common farm animals has been accomplished at varying degree of success. Currently, extensive studies have been done in cattle, to a lesser extent in sheep, but there are still problems in pig and horse, whereas in goats the information is lacking.

2.10.2.5.1. Cattle

The first IVF in cattle was shown by Iritani and Niwa (1977) (Table 2.3). Brackett *et al.* (1980) found that oocytes recovered near the expected time of ovulation from follicles or oviducts could be penetrated by ejaculated sperm treated with hypertonic (380 mOsm/L) medium and developed to 2- to 4-cells. Two years later, Brackett *et al.* (1982) reported the birth of the first bull calf after IVF when the preincubation of sperm was modified for 45 min to 5 hr in isotonic medium after hypertonic treatment. Parrish *et al.* (1986) later showed the importance of heparin in IVF. Since then, Garcia *et al.* (1986) successfully investigated IVF in this species and also transfer the embryos into recipient mother.

Various factors have been shown to affect IVF in cattle. Ball *et al.* (1983) indicated that; 1) sperm exposure to inomycin or preincubation at high sperm was not necessary for IVF, 2) the presence of hypotaurine and epinephrine during fertilization did not increase penetration rate, 3) maturation of oocytes in the presence of FSH or cAMP resulted in higher fertilization rate, and 4) the presence of cumulus cells was not necessary for penetration of oocytes. The fourth finding was however, in disagreement with that of Goto *et al.* (1989) who found that embryos produced in cell-free culture were of poorer quality than those produced in co-culture.

Other research studies have shown encouraging results in cattle IVF. Bovine twins resulting from in vitro fertilization has been reported (Brackett *et al.*, 1984). Two years later, Parrish *et al.* (1986) reported a succesful IVF in cattle using frozen-thawed semen. The research has gone one step further when Fayrer-Hosken *et al.* (1989) reported the success of laparoscopic oviductal catheterization and transfer of in vitro matured and in vitro fertilized embryos that resulted in pregnancy.

Among farm animals, it appears that cattle have been most extensively studied. This could be due to the fact that this animal is most utilized for human consumption, either for meat or dairy products. Another reason is that techni-

cally it is easier to accomplish IVF-ET procedure in this animal, despite the fact that the embryos and sperm are susceptible to freezing. Despite this, however, the success rate in IVM-IVF among workers was still variable. For example, using bovine oviductal cells as coculture, Xu *et al.* (1992) found that 18 to 31% of embryos developed to morulae and blastocyst, whereas Trounsen *et al.* (1994) using granulosa or oviductal epithelial cells in TCM 199 + 19% FCS obtained 43 to 53%.

2.10.2.5.2. Sheep

Work on IVF in sheep was pioneered by Thibault and Dauzier (1961). Then Bondioli and Wright (1980) reported that 3-15 % of superovulated oocytes were penetrated by sperm which had been preincubated for 3 hr in a medium containing 3% BSA or 20% inactivated lamb serum. Further studies have been performed on the effect of medium concentration at insemination (Huneau and Crozet, 1989) and the difference in the penetrability of the spermatozoa from individual rams and among individual ejaculates from the same ram (Fukui *et al.*, 1988). IVF of sheep oocytes matured in vivo showed that 82.6% (range: 67 - 100%) were penetrated, and after ET, 50% of the recipients ewes were pregnant, with 43% of them gave birth to healthy lambs (Cognie *et al.*, 1991).

Sheep is relatively easy but slightly more difficult compared to cattle to accomplish fertilization through in vitro. This is reflected by the lesser extensive studies on this animal compared to cattle. Walker *et al.* (1992) showed that sheep embryos are relatively insensitive to change in the composition of media and that embryos can develop to blastocysts at rates equal to or higher than those obtained in vivo. In particular, sheep embryos can develop in simple media without co-culture (Walker *et al.*, 1992).

2.10.2.5.3. Pig

The first successful IVF in pigs has been reported by Harms and Smidt (1970), then followed by Iritani *et al.* (1978). Since then, Nagai *et al.* (1984) and Davis (1978; 1985) has also carried out IVF study on this species. Later, Yoshida (1987) successfully fertilized follicular and tubal oocytes and subsequently transferred the embryos into the oviduct of a recipient, yielding four piglets. At present the success rate of IVF in pigs is about 78%, and in IVC the development to 2- to 4-cell stage is 24% (Yoshida *et al.*, 1993).

One of the important problems in pig IVF is the high rate of polyspermic fertilization (Nagai *et al.*, 1984; Nagai and Moor, 1990). Some suggestion has been put forward to

solve this problem. Cheng *et al.* (1986) found that the rate of polyspermy increased when the exposure of oocytes to spermatozoa was longer than 8 hr Coy *et al.* (1993) suggested that co-incubation time of 4 hr is optimum for pig IVF. Nagai (1994) showed that boar spermatozoa have been shown to be capacitated without preincubation if the fertilization medium contains caffein and high concentrations of Ca^{2+}, but the incidence of polyspermy in IVM-IVF was still high (60%-100%). To prevent polyspermy, Nagai (1994) co-cultured the system with oviductal cells, preincubated with porcine follicular fluid, or added oligopeptides in the fertilization medium and found that the effect was significant but the rate of polyspermy was still high (32%-58%). Embryo transfer is also difficult in pigs because embryos remain viable out of body for only 24-48 hr. Beside this, there is a low rate of male pronucleus formation after IVF (Motlik and Fulka, 1974; Nagai *et al.* 1984; Mattioli *et al.*, 1988). To make matters worse, everything has to be done surgically, due to peculiar nature of porcine uterus -- it is about a metre long with two enormous horns. In porcine, pregnancy is only maintained when a minimum of 2 to 4 embryos implanted and at least 25% of the uterine surface is covered by embryonic tissue (Polge *et al.* 1966; Webel *et al.*, 1970).

2.10.2.5.4. Horse

Research in horses has been slow due to inability to collect large numbers of equine oocytes (Vogelsang *et al.*, 1988). The lack of success when attempting to superovulate mares with exogenous gonadotrophin (Squires *et al.*, 1986) remains one major obstacle to the production of large numbers of oocytes and embryos. The exact culture time and medium requirements for maturation of equine oocytes are still unknown. Maturation has been shown to occur between 15 and 48 hr after collection of oocytes via follicular aspiration (Desjardins *et al.*, 1985; Willis *et al.*, 1991). On medium, recently Shabpareh *et al.* (1993) showed that equine oocytes matured much slower in Ham's F10 than TCM-199 medium. Perhaps Ham's F10 is deficient in some nutrients that are needed for oocyte maturation or contains a substance that inhibits oocyte maturation (Shabpareh, 1993). Vines (1987) has also noted that stallion semen freezes badly. IVF of in vitro matured equine oocytes has been reported but cleavage rates were low (Del Campo *et al.*, 1990; Zhang *et al.*, 1990). Only one foal has been produced from IVF; however, that birth resulted from an oocyte that was matured in vivo (Palmer, 1991).

2.10.2.5.5. Goat

There has been no systematic studies on IVF in this species (Niwa and Iritani, 1985). Kim (1981) preincubated epididymal sperm from 5-10 hr in a m-KRB medium or in isolated uterus from an oestrus sow and doe, and then cultured the sperm with in vitro matured zona-free goat oocytes. He found that 8 out of 10 oocytes were penetrated. However, no penetration of zona-intact oocytes was observed. Hanada (1985) treated ejaculated sperm with 0.5 uM Ca^{2+} ionophore A 23187, cultured a portion of the sperm with oocytes which were recovered near the expected time of ovulation from follicles or oviducts, in a modified Tyrode's solution supplemented with 2mM caffeine and 3 mg BSA/ml, and then continued to culture the oocytes in Ham's F10 or TCM 199 medium with 20% inactivated goat sperm from 6 hr after insemination. They transferred twelve 2-cell embryos, which were obtained 26-30 hr after insemination, to 5 recipient females and found that one recipient became pregnant after receiving 3 embryos and a healthy male kid was delivered 144 days post-transfer. Song and Iritani (1985) found that about 50% of oocytes in compact cumulus, which had been collected from the ovaries of immature goat treated with gonadotrophins and cultured for 25 hr, were penetrated by ejaculated sperm once washed and preincubated for 6 hr in a m-KRB medium at a concentration of $4\text{-}5x10^8$ cells/ml.

The oocytes used by these workers (Kim, 1981; Hanada, 1985; Song and Iritani, 1985) and also by Jufen *et al.* (1991) were retrieved from superovuloated goats. Younis *et al.* (1991, 1992) reported a succesful IVF and pregnancy using non-superovulated and superovulated goats, but they were later aborted. These observations suggest that more basic and practical research on IVF-ET in this animal is required.

In their studies on IVM-IVF in goat oocytes, De Smedt *et al.* (1992) found that 86% of the oocytes from follicles 2 to 6 mm in diameter achieved meiotic maturation, whereas the oocytes from follicles 1 to 2 mm in diameter only 24%. Recently in their studies on IVMFC in goats, Pawshe *et al.* (1994) co-cultured the oocytes with goat oviductal cells in C. Rosenkrans 1 amino acid medium (CR 1 aa) supplemented with 10 ng/ml epidermal growth factor, 5 µg/ml insulin, 5 µg/ml transferrin and 0.05 µg/ml selenite and found that the rate of IVMFC was not significantly different among the three different methods of oocyte recovery, namely, aspiration, puncturing and slicing.

2.11. THE IMPORTANCE OF CO-CULTURE SYSTEM

The biochemical requirement for normal, in vitro development of early preimplantation embryos in mammalian species

have not been defined (Rexroad, 1989). To overcome the in vitro developmental block of cultured mammalian embryo, the technique of somatic cell co-culture has been employed. Methods for follicular oocyte IVM-IVF (Critser *et al.*,1986; Xu *et al.*, 1987), followed by in vitro co-culture with oviductal cells (Goto *et al.*, 1988; Eyestone and First, 1989; Iwasaki and Nakahara, 1990; Goto *et al.*, 1994), trophoblastic vesicles (Camous *et al.*, 1984; Heyman *et al.*, 1987), a trophoblastic cell strain (Scodras *et al.*, 1990), cumulus cells (Goto *et al.*, 1988; Chian and Niwa, 1994), embryonic chicken cells (Blakewood *et al.*, 1988), uterine cells (Voelkel *et al.*, 1985), fibroblast cells (Wiemer *et al.*, 1989) and a transformed monkey-kidney cell line (VERO; Menezo *et al.*, 1990) have been established. The oviductal cell co-culture system has been used for early embryos in bovine (Eyestone and First, 1989; Pollard *et al.*, 1989), pigs (White *et al.*, 1989), sheep (Gondolfi and Moor, 1987), goats (Sakkas *et al.*, 1989), horses (Ball and Altschul, 1990) and human (Sathananthan *et al.*, 1990).

Although these co-culture systems do not add our knowledge on the exact requirements for embryo development and metabolism, all previous studies suggested that embryos produced in cell-free culture were of poorer quality than those produced in co-culture. What has been speculated so far is that the somatic cells used for co-culture may produce unknown, embryo growth promoting factor (s) and/or

delete embryo-toxic factor(s) from the culture medium (Eyestone and First, 1989).