CHAPTER 2

LITERATURE REVIEW

2.1 Oogenesis and oocyte maturation

The primary oocyte stage of gamete differentiation in most female mammals is reached during fetal life after proliferation of primordial germ cells and oogonia with subsequent entry into meiosis and transformation of the gamete into the primary oocyte (Baker, 1972). The life history of the female gamete is diagrammatically shown in Figure 1. The oocyte, arrested in prophase of meiosis I, is entrapped by follicle cells that form a squamous, single-layered epithelium around individual oocytes; and the total population of such units found in the ovarian cortex, termed the primordial follicle, form the resting pool of gametogenic material that must last throughout an animal's reproductive life history (Hirshfield, 1992). At various times throughout the female animal's life, cohorts of primordial follicles begin a developmental and differentiative period of growth, which will result in production of the mature, preovulatory follicle under the appropriate hormonal conditions in vivo (Hirshfield, 1991). Entry of primordial follicles into the growth phase has been observed in fetuses and prepuberal animals, throughout menstrual and estrus cycles, and even during pregnancy (Erickson, 1966), but conditions appropriate for producing the mature follicles in vivo with and oocytes ready for fertilization are found only in reproductively mature animals during specific stages of the reproductive cycle and during the breeding season in those species responsive to seasonal influences

(Donoghue et al., 1993). Growing follicles not attaining dominance, in other words not selected for ovulation, will eventually become atretic and be lost from the ovarian population. It is these follicles and the enclosed oocyte on which much effort has been directed toward perfecting protocols for in vitro maturation, since they form a large pool of genetic material research, or tissue banking. This material is more abundant than the few follicles that attain dominance, and the timing for recovery of this material is much less critical than that for the limited number of in vivo matured oocytes in the preovulatory follicles.

During growth and differentiation of the follicle unit that has left the resting pool for primordial follicles, the somatic component begins to replicate and forms a single layer of columnar or cuboidal cells around the primary oocyte which has also begun to increase in size (Hirshfield, 1991). This morphological stage is identified as the primary follicle. A schematic of oocyte growth and folliculogenesis is shown in Figure 2. After the follicle unit has begun growth and differentiation the oocyte's nonliving extracellular coat, the zona pellucida, is first observed to form. The zona pellucida is composed of several glycoproteins that modulate later sperm-egg interactions at fertilization such as species specificity, sperm binding, the acrosome reaction, and the zona mediated block to polyspermy. In at least one species, mice, secretion of the zona can be attributed to the oocyte. Proliferation of the follicle cells continues until a multi-layered epithelium is formed, intercellular associations are established, creating a unique environment aroud the oocyte, and an outer layer of squamous cells forming the thecal layer is acquired.

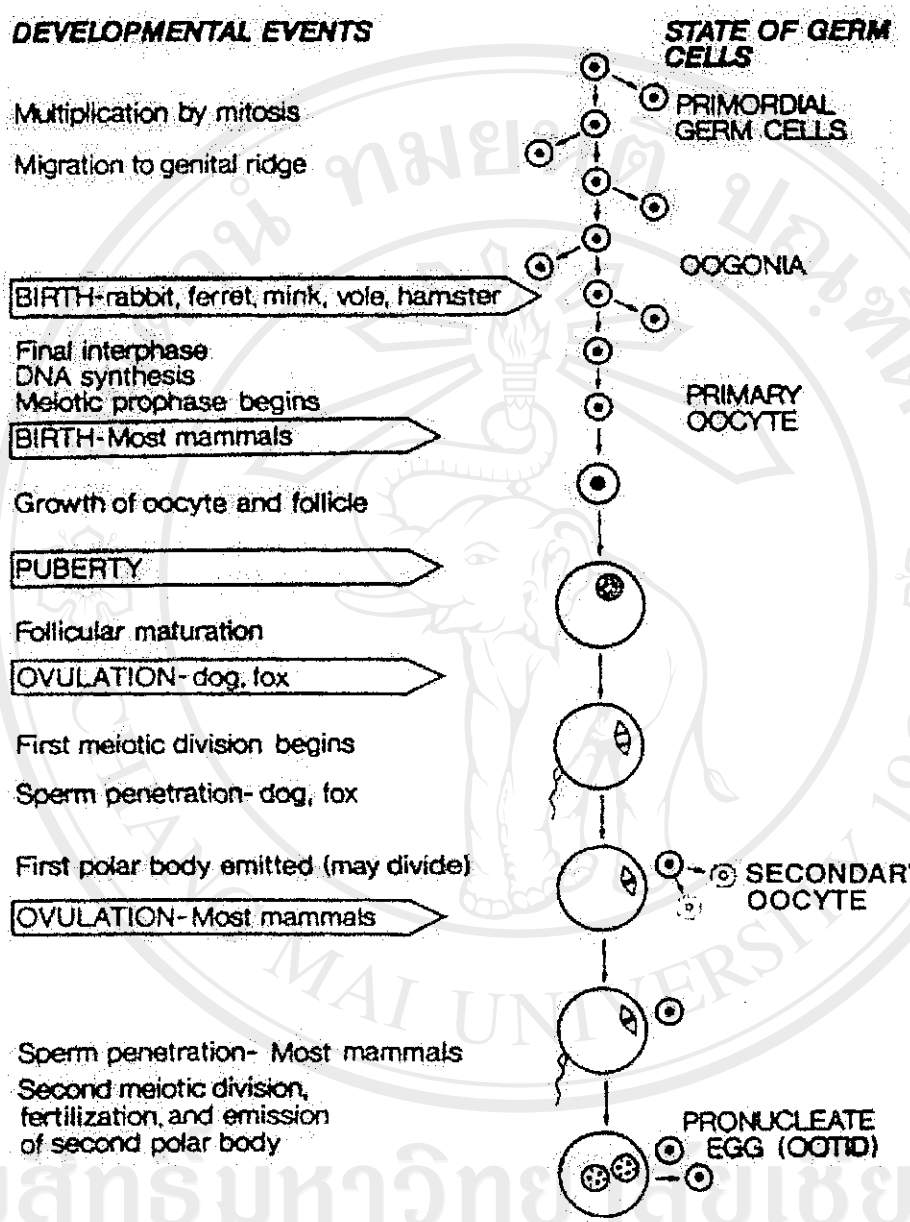

FIGURE 1. Generalized diagram of oogenesis in mammals. The life cycle of female gametes from embryonic stages through to reproductive maturity is represented. (Baker, 1972)

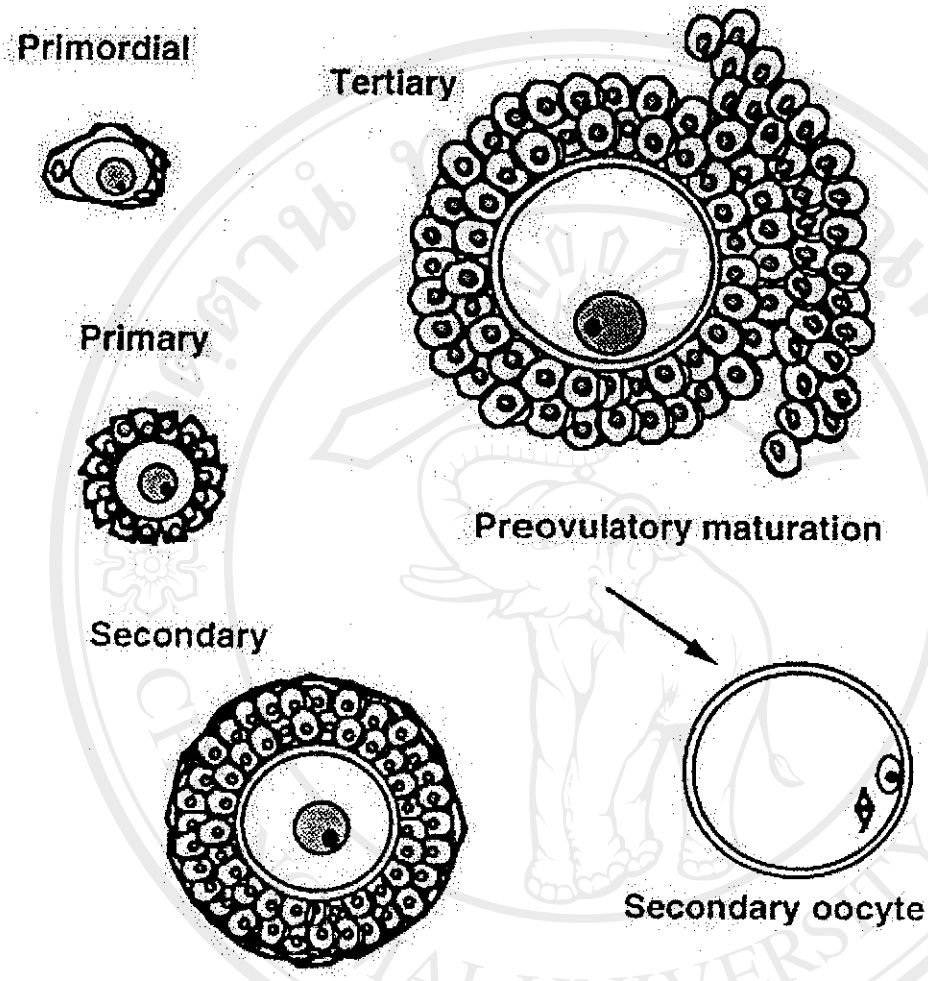

FIGURE 2. Generalized diagram of folliculogenesis in mammals. Growth and differentiation of the follicle unit starting from the non-growing pool of primordial follicles, entering the growth phase and ending with the oocyte-cumulus complex undergoing meiosis I in the tertiary follicle prior to ovulation (Leibfried-Rutledge et al., 1997).

In this secondary follicle, the primary oocyte continues to grow. After continued proliferation of both types of follicle cells and division of theca into interna and externa, the follicle now begins to form a fluid-filled lumen in which the oocyte remains attached to the cells, now termed granulosa, at one side of the follicle wall. In the tertiary or antral follicle of most mammals, the oocyte remains surrounded by a hillock of cells called "cumulus oophorus", which maintains cellular associations with granulosa, cumulus cells themselves, and with oocyte. Those cells immediately surrounding the oocyte that have villi interdigitating with those of the oocyte plasmalemma are the corona radiata cells. In some mammals, the oocyte has now attained its maximum diameter and thus has completed growth; found in short-cycle species such as rodents (Iwamatsu and Yanagimachi, 1975), while in long-cycle species such as domestic animals and primates, growth of the oocyte continues to occur (Motlik and Fulka, 1986). Proliferation of the follicle cells continues in the tertiary follicle, as does fluid secretion into the lumen, resulting in an increasingly larger follicle, almost up to the time of ovulation. Many literatures provide details of the hormonal requirements for follicular growth, components and mechanism for antral fluid formation, and functions of the follicle cells (Hirshfield, 1991; Findlay, 1993; Donoghue et al., 1993).

During follicular growth and formation, the primary oocyte which is still in meiotic arrest, also undergoes a large increase in volume, begins a very active synthetic phase, stockpiles large stores of both protein and mRNA which is very stable, and also acquires what has been termed meiotic competence (Leibfried-Rutledge et al., 1989). Part of a histological section taken from the ovary of an adult hamster is illustrated in Figure 3; showing the dramatic growth of the oocyte once it

has left the non-growing pool of primary oocytes. Unlike oocytes of lower vertebrates or invertebrates where a hormonal signal for induction of meiotic maturation (i.e., resumption and completion of meiosis I) is necessary, mammalian ooyctes removed from antral follicles and placed into culture spontaneously undergo meiotic maturation. Acquisition of meiotic competence coincides with attainment of maximum diameter and antrum formation in the follicles of rodents (Iwamatsu and Yanagimachi, 1975). In long-cycle species, meiotic competence is not acquired until later during growth of the antral follilcle. Conditions necessary for acquisition of competence in vivo have been studied in rats and involve the presence of FSH and estrogen. At the time that meiotic competence is attained, the oocytes decrease synthetic processes and dismantle the interphase array of microtubules, the nucleolus morphology becomes indicative of very low levels of mRNA production, and chromatin begins condensing in the oocyte nucleus or germinal vesicle (Albertini, 1992). The growth phase of the oocyte through to meiotic competence is highly reminiscent of the transversal of G2 phase in somatic cells. At the point at which the oocyte becomes able to resume and complete meiosis I, the oocyte has stored a genetic program that will later be expressed during fertilization and early preimplantation embryonic development. There are many excellent reviews concerning mammalian ooycte development and meiotic regulation in the literature (Leibfried-Rutledge et al., 1989; Moor and Gandolfi, 1987; Parrish et al., 1992 and Downs, 1993).

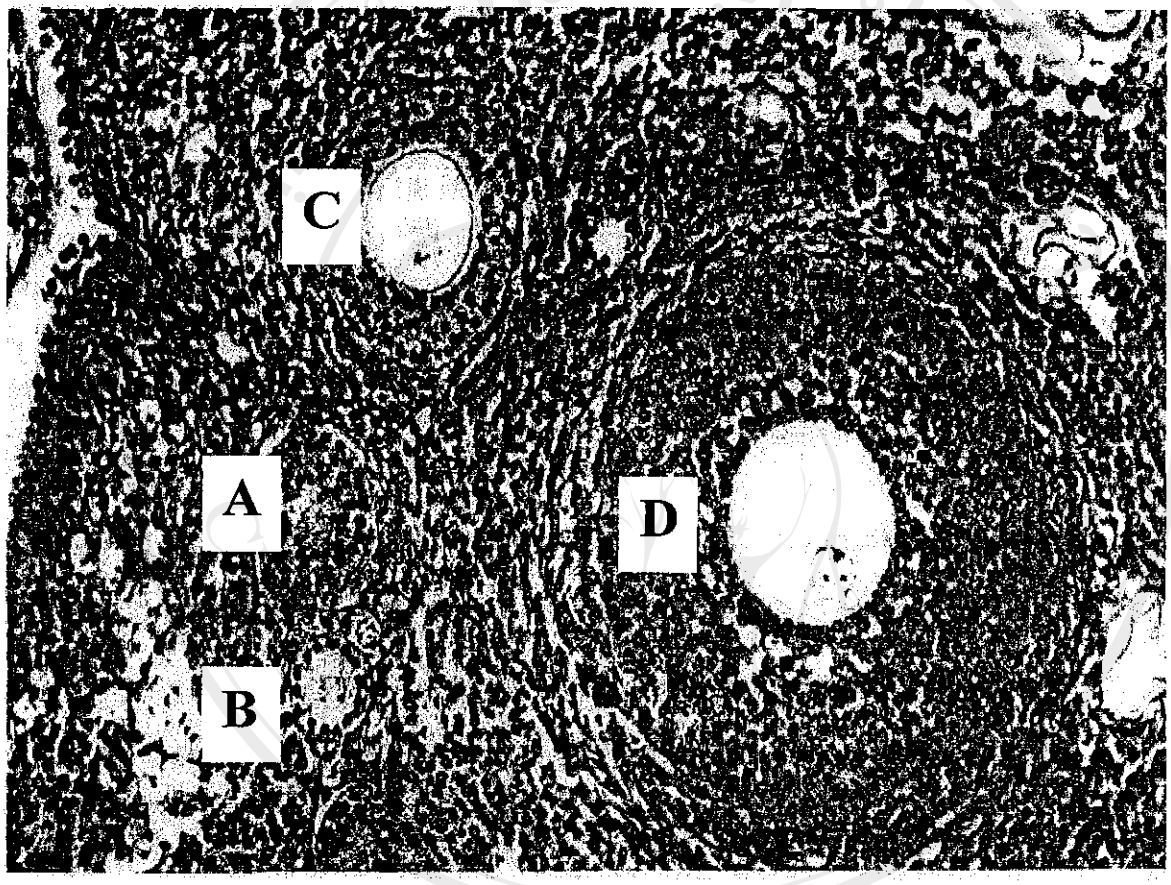

FIGURE 3. Histological section of an adult hamster ovary. Examples of primordial (A), primary (B), secondary (C) and early tertiary follicle (D) are shown (Leibfried-Rutledge et al., 1997).

2.2 Source of oocytes for embryo production

Primary oocytes from tertiary follicles are most frequently recovered postmortem from slaughterhouse, surgically with the aid of a laparoscope or most recently via ultrasound-guided follicular aspiration. Circumstances, species, value of the donor animal, and relative abundance or scarcity of the genetic material and in some situations, policy and ethical considerations, are some of the factors that will determine which source of cells will be used in tissue culture to the point at which the genetic material can best be stored or banked. (Leibfried-Rutledge et al, 1997)

Meiotically competent oocytes from antral follicles are commonly obtained postmortem in domestic animal species quite easily by collecting ovaries at an abattoir. In cattle, transport of ovaries in a simple physiological saline is compatible with production of live offspring after recovery and maturation of the oocytes from antral follicles (Sirard et al., 1988). A few report existed concerning the most appropriate temperature at which to transport the ovaries prior to recovery of the oocytes (Sekine et al., 1992), but on the whole, little attention has been paid to the thermodynamics of tissue cooling, rewarming, etc., in obtaining the most successful result afterwards. Exposure of excised ovaries containing primary oocytes or the oocytes themselves to cooler temperatures impairs the gamete's ability to undergo meiotic maturation in vitro and hence negates success of any further chances for producing live offspring (Moor and Crosby, 1985; Pickering et al, 1990). Length of time that ovaries can be held prior to recovery of oocytes has also been dealing with temperature for transport. Ovaries of elite cattle obtained postmortem can be transported and those oocytes can be used to produce calves after oocyte maturation, fertilization, and embryo culture in vitro (Gordon 1994).

A variety of methods have been used to recover oocytes from postmortem ovarian tissue in domestic species (Gordon, 1994). Aspiration of antral follicles with hypodermic needles and a syringe or vacuum pump is common. The variety in morphological appearances seen in cumulus-oocyte complexes aspirated from antral follicles 2-6 mm in diameter visible on the surface of ovaries taken from adult cows at slaughter. The oocytes have been made to develop rating criteria associated with selecting into a transferable blastocyst after IVF. These selection systems base on morphology of the oocyte and surrounding cumulus investment. the systems. Slicing of the ovarian cortex with sharp blades or dissection of individual follicles with rupture of the follicle wall to release the enclosed oocyte have also been used to obtain oocytes in domestic animals. Gordon (1994) has summarized some of the reported efficiencies and expectations for different methods of obtaining oocytes from follicles of domestic animals. Slicing of the ovarian tissue or dissection of all follicles including those buried in the ovarian cortex will release more oocytes than aspiration or dissection of follicles presented on the ovarian surface. Differences in developmental competence has been observed for oocytes taken from peripheral antral follicles vs those deeper in the ovarian cortex in cattle, implying that location in the ovary may indicate different populations of follicles containing oocyte with different developmental competence (Arlotto et al, 1996). The primary oocytes in antral follicles located deeper in the ovarian cortex of adult cows tend to have smaller diameters on the average and a decreased potential to complete the first reduction division are located below the surface and rise toward the germinal epithelium at later stages of development and differentiation of the follicular unit (Spicer et al, 1987). Other reports indicated that oocytes from the two follicle populations did not differ in

the various endpoints observed after in vitro protocols (Hamano and Kuwayama, 1993).

Although oocytes obtained postmortem is useful in many situations, it does not allow for choice of genetics that may often be desired nor is it the best way to utilize scarce living resources. Surgical means of recovering oocytes have been utilized in specific situations via laparotomy or standing flank incisions depending on the species and facilities available. Large antral follicles may be aspirated or the ovary excised. Ovaries of larger domestic species may also be removed via salpingectomy using a vaginal approach, but this affect reproductive usefulness of the animal. Secondary oocytes matured in the follicle, ovulated secondary oocytes, and even early embryos can be recovered in this manner. Since surgical intervention is complicate and expensive. Laparoscopy can also be used successfully in some domestic animals to recover oocytes from antral follicles (Lambert et al., 1983) and again has resulted in birth of live offspring after IVF or transfer back to the oviducts for fertilization and development (cattle: Brackett et al., 1982; Sirard and Lambert, 1986; Lambert et al., 1986; Armstrong et al., 1992; swine: Cheng et al., 1986; sheep: Crozet et al., 1987). Again, secondary oocytes or those that have begun maturation in the follicles are the most frequent stage recovered using laparoscopy in domestic animals. In small ruminants such as sheep, laparoscopic folliculocentesis can be used to recover oocytes for use in IVM (Baldassarre et al., 19960). If this is done after a hormonal regime to induce increasing follicular development and to obtain control of the estrous cycle, a single animal can increase its total contribution to the genetic pool dramatically.

Santl et al. (1998) compared the methods between laparoscopy and OPU that they found both to be equally efficacious for the successful recovery of oocytes from

living animals and for in vitro production of bovine embryos. However, the OPU procedure is less traumatic to the vagina and especially the fornix than laparoscopic technique, which is confirmed by the fact that OPU could be performed successfully in all donors throughout the entire experiment, whereas laparoscopy was sometimes difficult or impossible to perform. Further, for laparoscopy, practical experience is required in order to avoid detachment of the parietal peritoneum from the vaginal wall or damage the abdominal organs.

2.3 Ultrasound guided transvaginal ovum pick-up (OPU)

The method for bovine oocyte retrieval by OPU has been successfully developed at the University of Utrecht, Netherland (Pieterse et al., 1988). Adequate restraint and sedation of the animals are very important. Drugs have to be administered to provide sedation and relaxation of the intestines so that rectal manipulation of the ovaries is possible over a longer period of time. An epidural anaesthesia is given to prevent abdominal straining. The cow is restrained to permit as little movement possible during puncturing. After emptying the rectum, the vulva and perineum are cleaned thoroughly and the transducer is brought into the vagina along side the cervix by the operator. The ovaries are then placed against the head of the transducer by rectal palpation. The follicles can be clearly visualized on the monitor and were then positioned so that the puncture line on the monitor transected the follicle about to be punctured. An assistant inserts the needle through the company-made equipment to be handled by only one person. The fluid is collected in warm test tubes (between 35 °C and 38 °C) and examined in the laboratory for the presence of oocytes.

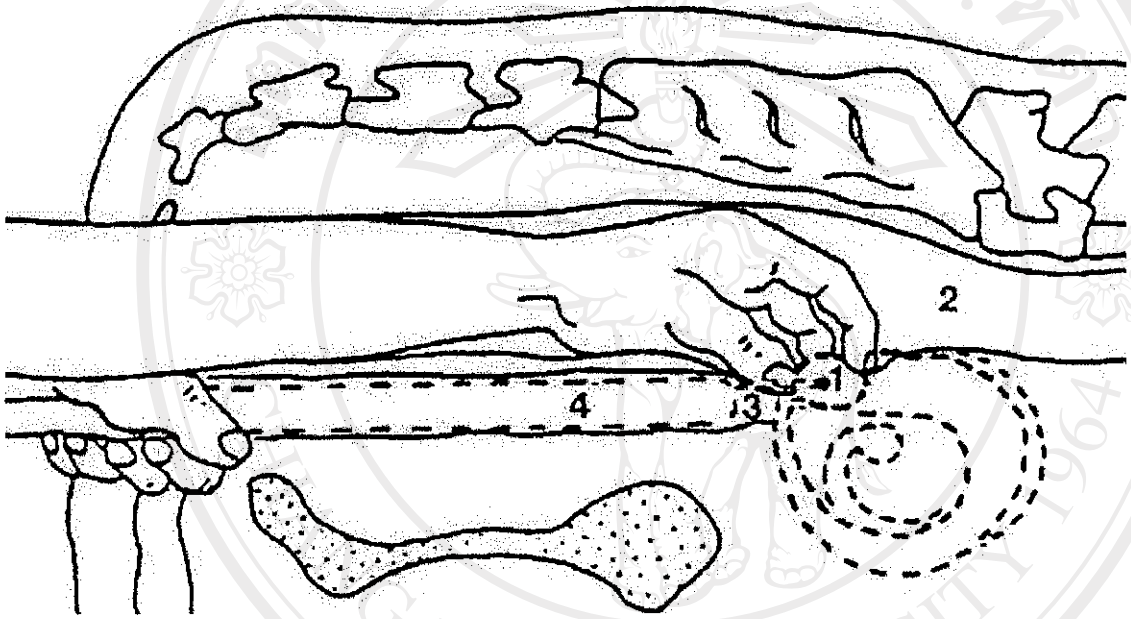

FIGURE 4. Diagram of transvaginal ultrasound-guided follicular aspiration in cattle. Ovary (1) is positioned against the transducer by rectal manipulation (2) while the ultrasound transducer containing the needle guide and aspiration needle (4) is placed against the anterior portion of the vagina beside the cervix (3) (Kruip et al., 1991).

The recent introduction of OPU technique coupled with in vitro embryo production in cattle breeding scheme aims to produce large numbers of offspring from females of high genetic value. The final goal is to increase the intensity of selection on the female line combined or not with multiple ovulation and embryo transfer (MOET; Kruip et al., 1994). The technique being applied very recently to domestic animals is that of ultrasound-guided, follicular aspiration (Pieterse et al., 1988,1991; Kruip et al., 1991). Positioning of the ovary in relation to the ultrasound transducer and puncture needle during transvaginal ultrasound-guided follicular aspiration in cattle is shown in Figure 4. This is currently being used on a commercial basis in cattle to recover primary oocytes from antral follicles that will be matured, fertilized, and cultured to the blastocyst stage using in vitro procedures (Looney et al., 1994; Hasler et al., 1995). It can be done on an individual animal repeatedly over an extended period of time without damage to the ovary or subsequent reproductive impairment. Unlike conventional methods for increasing fecundity in cattle such as nonsurgical embryo recovery normally used in conjunction with superovulation, no gonadotropin is required and the response does not decrease. Older animals, pregnant animals (Figure 5), prepuberal animals, or animals with clinical infertility may also be utilized as donor animals (Kruip et al., 1994; Meintjes et al., 1995; Duby et al., 1996) using this method for follicular retrieval of immature oocytes with ensuring production of live offspring after IVM-IVF-IVC and transfer to recipients. OPU can be successfully applied in Holstein Friesian heifers from 6 months of age onwards, but is less efficient than those of adults (Rick et al., 1996). Nevertheless, blastocysts can be obtained from cattle as early as 6 months of age, which should allow the generation interval to be shortened as a major factor in animal breeding strategies.

When OPU is employed in live cattle, there may be a need to refine evacuation techniques so that the punctured follicle is flushed with medium after the initial recovery attempt (Kruip et al., 1991). However, oocyte recovery could presumably fail because the immature oocyte remains firmly bound to follicular cells.

At the moment, the entire system commonly used for the in vitro production of cattle embryos has been successfully applied to the other species. Systems for producing bovine embryos in vitro have actually worked at some levels of efficiency for a number of non-domestic ungulates. This approach of applying successful strategies developed from an abundant member of a family to members where material is more difficult to obtain is being used in a number of exotics and endangered species and should be borne in mind when attacking a new species of interest (Wildt et al., 1986).

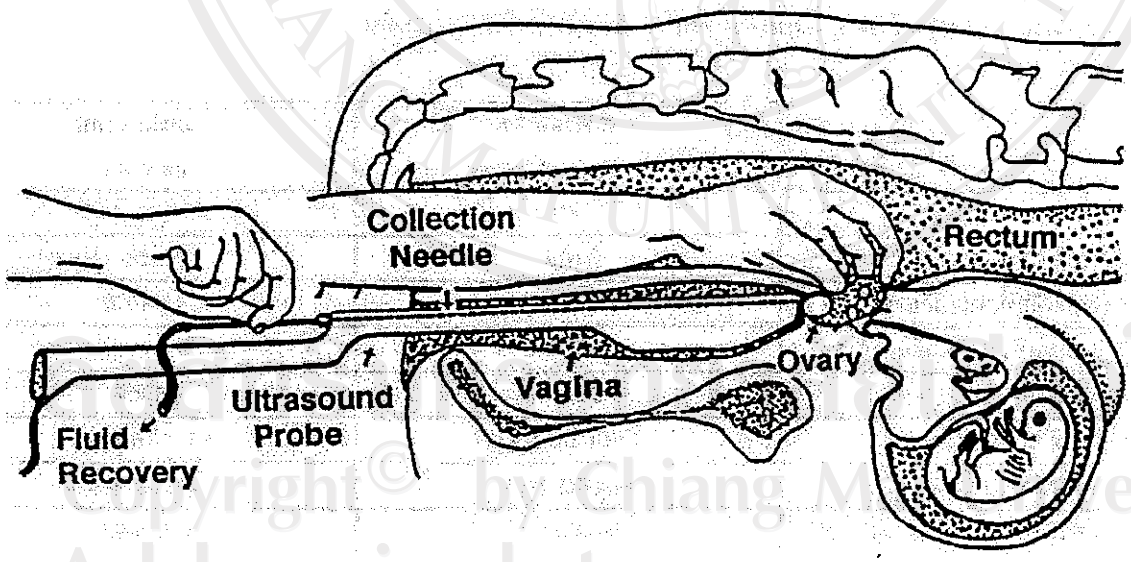

FIGURE 5. Diagram of transvaginal ultrasound-guided follicular aspiration in pregnancy cows up to the third and fourth month of gestation (Meintjes et al., 1995).

2.4 Non-ultrasound guided transvaginal ovum pick-up (n-OPU)

Several studies have developed new OPU devices that are practical and economical for routine use in oocyte retrieval. To optimize the OPU technique and to increase the recovery rate of oocyte, it is important to reconsider those procedure. The important parts of the system are vacuum pressure that especially noticeable between 70 to 130 mmHg and type of needles that are used (Bols et al., 1995 and 1996). Attention should be paid at the point of the needle, which has to be as sharp as possible in order to pass the vaginal wall easily and to minimize damage to the ovaries. Long non-disposable needles become dull after being used in two or three OPU sessions and need resharpening frequently; however, a resharpened needle never regains its initial sharpness. The use of a disposable needle eliminates this problem by replacing a dull needle with a new one. Furthermore, disposable needles are very inexpensive and because of their small dead volume, they are very well suited for collection of small amounts of follicular fluid from individual follicles. A study of Bols et al (1996) shows that the length of the needle bevel also has a significant effect on oocyte recovery; i.e., the short bevelled needle resulting in a higher oocyte recovery rate. However, the disadvantages of disposable needles are that it requires a more complex needle guidance system to permit changing of the needle, and its implications concerning the type of ultrasonographic scanner used. Another possible cause of low oocyte yield is that COCs may be left behind in the needle tubing system; thus, it is necessary to rinse the tubing and system frequently since; this system consists of several junctions and cavities in which the COCs can be trapped. The gain in numbers of oocytes by rinsing follicles is probably offset by the loss of COCs due to the complexity of the tubing system (Bols et al., 1995). Therefore, new

systems should be of simple construction to permit easy changing of the needles, tubing system and to prevent loss of oocytes.

A simple method of transvaginal follicular aspiration method has also been developed for collection of oocytes from live donors under field situations. That does not require ultrasound visualization of the ovary (Hill, 1995) and both reducing the initial outlay for equipment and allowing application to smaller animals such as young or prepuberal heifers. This technique may be well suited for many ungulates.

With this on-farm technique, all cows maintained regular estrous cycles during the aspiration periods. The cows were slaughtered with in 14 days of the last puncture session with no adhesions detectable. In another study, it was also found that if the cows were subjected to superovulation immediately following the last puncture session, normal responses existed (Hill, 1995).

2.5 Hormonal stimulation of follicular development

An increase in the quality and quantity of the COCs retrieved with the transvaginal OPU technique in combination with more economical approach can only be achieved after a profound study on the different aspects involved. Apart from biological factors such as hormonal pretreatment of the animals prior to puncture, there are more technical aspects dealing with the most reliable way to reach the follicles and retrieve the present oocytes

Differences in protocols for ovarian stimulation and in the type of donors (breed, age) do exist. For donors from 5 months onwards, there are conflicting reports on the effect of pretreatment with gonadotropins on oocyte collection and embryo development. Some authors have shown a beneficial effect (Paul et al., 1995) even on

pregnant donors (Meintjes et al., 1995) while others have not found any difference compared with controls (Fry et al., 1994; Fry et al., 1998). Other authors have shown an age-dependent mechanism whereby hormonal stimulation is beneficial only in young donors up to 3 months or up to 9 to 10 months (Presicce et al., 1997). Despite these conflicting data on the effect of hormonal pretreatment, several studies demonstrated (Steeves et al., 1999) that the developmental capacity of the oocytes increases with the age of the donor; with puberty being the relevant turning point.

Some studies prefer to combine superovulation with OPU and to recover the oocytes before the onset of estrus (Bousquet et al., 1999; Guyader et al., 1997) but the procedure can be repeated at best every two weeks. To increase the mean number of blastocyst per donor, Ooe et al. (1997) used FSH treatment before transvaginal oocyte collection. These authors also found the ovarian response in terms of number of aspirated follicles, recovered oocytes and blastocysts per donor and session were significantly lower if the cow were treated on Day 8 through 14 of their estrous cycle. On the other hand, FSH application on Day 1 or removal of the dominant follicle 24 h before superstimulation result in the increased mean numbers of blastocysts from 0.7 to 1.5 per cow and session. The average yield of embryos can be higher per OPU but the total number over a period of time is lower than those of the twice weekly collection (Galli et al., 2001). But two studies (Bungartz et al., 1995; Goodhand et al., 1996) have found that the results of a twice-weekly aspiration schedule could not be improved upon by the additional administration of FSH, possibly because the increased frequency of aspiration resulted in donors having elevated endogenous levels of FSH by themselves.

Sirard et al.(1999) found that both FSH treatment (constant or decreasing dosages) with n-OPU resulted in an increased number of follicles, oocytes and the embryo yield per cow. Since prostaglandins were not used, the FSH treatment could be repeated on a weekly basis to maximize retrieved oocytes and in vitro embryo production from valuable animals. The difference observed in the embryo yield between the two treatments could be a consequence of early differentiation of the oocyte in a follicle subjected to decreasing amounts of FSH (decreasing FSH) as compared with a follicle receiving nearly maximal growth stimulation (constant FSH). Blondin et al. (1996) demonstrated that superstimulation alone (multiple injections of FSH-P over 4 days) without coasting period did not effective in increasing the developmental competence of bovine oocytes. Lonergan et al. (1994) used a 3-days FSH treatment in cattle prior to their slaughter to evaluate the quality of the resulting oocytes. Although, there was an increasing in the number of follicles greater than 6 mm., the developmental rate of the enclosed oocytes was not found to be superior as compared with that for the untreated animals (Bousquest et al., 1995). The results indicate a possible lack of differentiation of oocytes obtained from actively growing follicle.

The study of Kim et al. (2001) showed that removal of the dominant follicle before superstimulation had a beneficial effect on superovulatory response and embryo production. Maintenance of the dominant follicle and atresia of subordinate follicles may be mediated by negative feedback at the pituitary level and/ or by direct action of intraovarian factors at the ovarian level (Findlay, 1993; Law et al., 1992; Lussier et al., 1994). Thus, it has been shown that superstimulation treatment in the presence of a dominant follicle resulted in reduced ovarian responses in dairy heifers

(Grasso et al., 1989; Rouillier et al., 1996). Therefore, removal of the dominant follicle might abolish the inhibition to recruitment and stimulate a pool of follicle responsive to superstimulation with gonadotrophin. A significant effect of dominant removal before superstimulation on follicular growth was detected on the increased number of medium follicles between Day 1 and 2 ($P< 0.001$); and then on the increased number of large follicles ($P< 0.01$) between Days 3 and 4 in the dominant follicle removal (DFR) group than control group, respectively. This data indicated that follicular growth during superstimulation was earlier in the DFR group. It appears that removal of the dominant follicle might initiate follicular growth, which was then further stimulated by the superstimulatory FSH injections (Guilbault et al., 1991 and Wolfsdorf et al., 1997). However, this results support the fact that the removal of the dominant follicle before FSH treatment likely initiated a new wave of follicular development caused by rise in FSH and that superstimulatory FSH injections stimulated further follicular growth during superstimulation treatment (Kim et al., 2001).

2.6 Non hormonal stimulation and collection program

Different schedules can be used for oocyte recovery while allowing the system to adapt to different practical situations. Twice weekly collection (usually Monday and Thursday) allows the maximum oocytes recovery with suitable quality for embryo production in a given time interval. Once weekly collection, however, results in the recovery of a expanded and atretic oocytes (Garcia et al., 1998). This is because in the twice weekly collection no dominant follicle develops, if all visible follicles are aspirated. In most once weekly collections, a dominant follicle is present at each

successive collection that causes the regression and degeneration of the subordinate follicles. In the once weekly collection schedule, the donors also can come into estrus cycle while this is not the case for the twice-weekly schedule.

The OPU technique has been proved to be a reliable tool for investigating follicular recruitment in cattle. Using twice-weekly ovum pick-up the frequency of follicular wave increases. The duration has changed from the normal 7-9 days to 3-4 days, therefore the follicular wave has been uncoupled from the estrous cycle because ovulation is inhibited. The results also support the notion that growing follicle(s) inhibits the growth of subordinate follicles (Ginther et al., 1989) either by means of a local or systemic feed back, or both. As soon as the larger follicles are removed a new follicular wave is initiated. This is similar to the findings of Adams et al. (1993), who reported an advance in the start of the second follicular wave surge after the removal of a dominant follicle on either Day 3 or Day 5 of estrous cycle. Repeated removal of all follicles > 2 mm. from the bovine ovary by ovum pick-up twice weekly induces an artificially increasing in the number of follicular waves over the 6 months. of the experiment (Boni et al., 1997). A 3-day interval between puncture session is enough to obtain a maximum number of newly recruited follicles. With a longer interval the number of follicles does not increase; however, the size of follicles does increase. Since preovulatory growth and ovulation is prevented by this procedure, it strongly suggests a linkage between estrous cycle and follicular waves. The highly estimated repeatability of follicular recruitment also indicates that the number of follicles present in the ovary is previously regulated for each individual animal. The good predictability of follicular recruitment on the basis of the first 4 or 6 puncture sessions, represents the characterization of individual animals on their potential for

follicular recruitment. This makes ovum pick-up a reliable tool for research into the genetic background of follicular recruitment as well as for studying new approaches of manipulating follicular growth by environmental or pharmacological methods.

In non-stimulated cows and calves, neither high-frequency (twice a week) nor prolonged-period (2 month) puntures (Kruip et al., 1994, Majerus et al., 1999) affected reproductive physiology of the donors and those undergone OPU showing no alteration in estrous cycles or pregnancy rate.

2.7 In vitro oocyte maturation

Meiotically competent, primary oocytes from antral follicles are currently being utilized in a number of scenarios in both commercial and research settings to provide material for IVF and the IVP of embryos (Gordon, 1994; Looney et al., 1994 and Hasler et al., 1995). Since meiotically competent primary oocytes of mammals spontaneously resume and complete the first meiotic division in culture and are convenient to obtain compared to earlier stages of the female gamete, the process of IVM has received much attention with the focus on producing a metaphase II-arrested, secondary oocyte capable of yielding live offspring after IVF. This has been slightly more difficult to achieve than first thought, due to the fact that although meiotic maturation takes place spontaneously and appears normal, other processes now referred to as cytoplasmic maturation appears to be more demanding as to the preferred in vitro environment for complete maturation of oocyte (Leibfried-Rutledge et al., 1989). The actual processes involved in cytoplasmic maturation are as yet not defined and the only good assay for this endpoint is to fertilize the IVM oocyte and to evaluate development. There are competencies other than meiosis that the oocyte

acquires to be fully competent, and these appear to be attained in a stepwise fashion (with meiotic ability apparent first) and are associated with the oocyte's stored genetic program. Once the oocytes become progressively able to undergo normal fertilization, cleavage which must involve ability to transition to mitotic cell cycles begins. Then blastocyst forms, and if possible, initiation and maintenance of pregnancy follows. In the process of cellular maturation competently, primary oocyte involves the ability to release its stored genetic program that allows complete expression of the various oocyte competencies. Rearrangement of cytoplasmic organelles also occurs during meiotic maturation of the oocyte (Van Blerkom et al., 1990).

Despite the fact that in vitro maturation yielding a fertilizable oocyte resulted in a new individual is still a rather understand, success has been achieved in many species. These range from laboratory species, domestic animals, and primates (Sirard et al., 1988; Trounson et al., 1994). With the obvious reliance on IVM as central to many assisted reproductive technologies and research endeavors, further discussion of oocyte maturation will focus on the meiotically competent primary oocyte taken from tertiary follicles of mammals. This will be followed by sections concerning its utilization in IVF and embryo culture. For each species of interest, one must keep in mind that there are many variations on this main goal that can be utilized to overcome when a specific part of the process has not yet been brought to maturity. Here the matured oocyte can be placed back into the oviduct for fertilization and initial development after which the embryos are recovered from the uterus and transferred to recipient animals. For cattle, prior to development of a wide choice of in vitro embryo culture systems, biological incubators such as rabbits and sheep were used to culture zygote resulting from IVM/IVF oocytes up to blastocyst stage which there was

compatible with the transfer to a recipient or cryopreservation (Lawson et al., 1972). Thus many in-vivo and in-vitro techniques are available which can be joined in wide variety of combinations to maximize the use of female gamete

The goal of culturing primary oocytes that are meiotically competent through completion of the first reduction division to yield secondary oocytes capable of development after fertilization are the same for all species of interest. Oocytes that have been cultured for various periods of time then fixed (Gordon, 1994) are shown in Figure 6. This figure illustrates the various stages of meiotic maturation, or meiosis I, which must be completed successfully prior to continuing IVF. The protocols utilized for specific species that support meiotic maturation might vary in details due to individual differences in reproductive characteristics. Culture systems found successful in one species can be applied to other species, as in the case of domestic cattle. There are aspects of culture that may also be very species dependent. Choice of antibiotics or macromolecular supplements for example, are much more sensitive considerations in humans, where anaphylactic shock, disease transmission, or ethical considerations are involved, than in domestic animals. In domestic animals and exotics, source of animal material used as media supplements or co-culture tissue needs consideration with respect to animal health regulations, especially if IVP embryos will be used for export or used for transfer to recipients.

The following discussion of culture systems will avoid some basic techniques such as quality and storage of reagents, water source, and source of disposables (Leibfried-Rutledge et al.,1997).

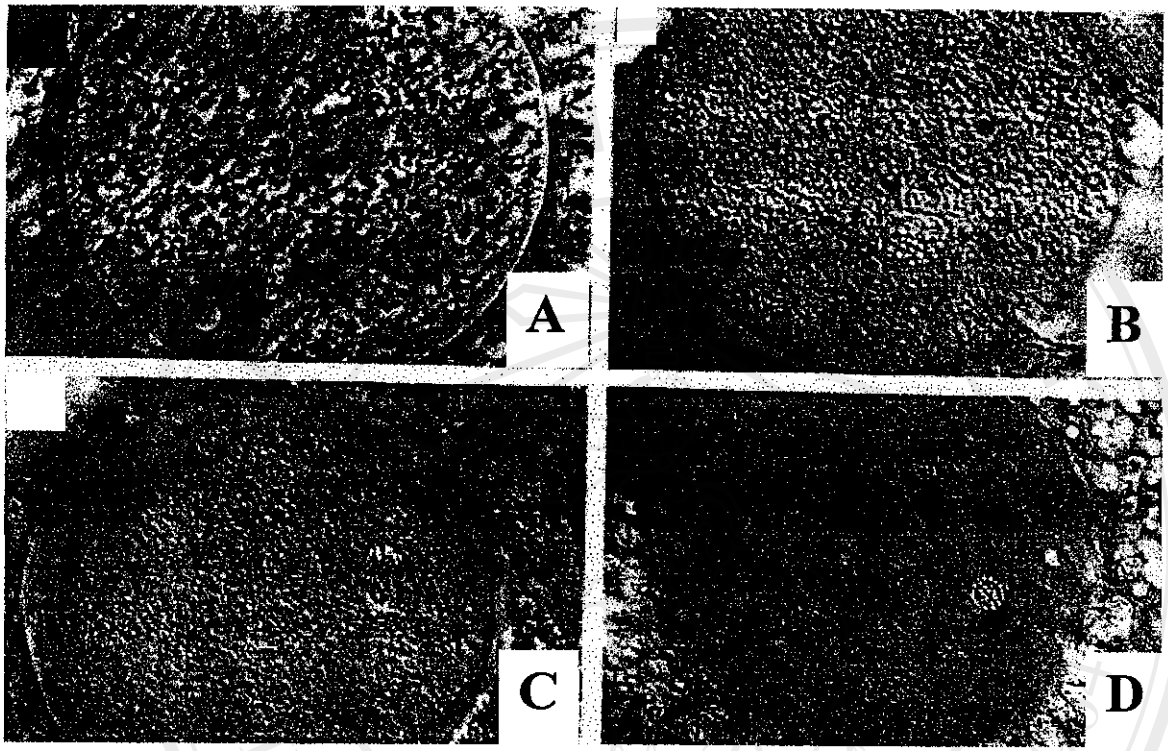

FIGURE 6. The stages of meiosis I in mammalian oocytes. Whole mounts of oocytes were fixed and cleared at various times after the start of culture for in vitro maturation and then photographed using Nomarski optics. (A) Bovine primary oocyte before culture showing the germinal vesicle. (B) Bovine oocyte at metaphase I. (C) Bovine oocyte at anaphase I. (D) Bovine oocyte at metaphase II after expulsion of the first polar body (Gordon, 1994).

2.7.1 Choice of media

There are two basic types of media to be considered. Simple balanced salt solutions that can be made up easily in the laboratory comprise one category, although they have become a little more complex than that first used to develop mouse one-cell embryos to the blastocyst stage outside the tubal environment (Whitten and Biggers, 1968). More complex media usually obtained from commercial sources make up the second category (categories reprised by Thompson, 1996). The simple salt solutions are typified by Tyrodes' modifications (Bavister and Yanagimachi, 1977; Bavister et al., 1983), Brinster's (1969), CR1aa (Rosenkrans et al., 1993; Rosenkrans and First, 1994), Menezo's B2 (Menezo, 1976), SOF (Tervit et al., 1972), CZB (Chatot et al., 1989) and HECM (Schini and Bavister, 1988; Seshagiri and Bavister, 1990). Formulations of balanced salt solutions that are commonly used for in vitro technologies with various species are listed in Table 1 along with their major uses and species applications. TCM-199, Ham's F10, Eagle's basal medium, etc., examplify the class considered as more complete media. Despite the basic type of media, both categories rely on either bicarbonate or phosphate buffering systems with some of the more complete media coming with either buffering system depending on whether the balanced salts that provide osmolarity and electrolytes are supplied by Earle's or Hank's salts. The vast majority of tissue culture in gametes and embryos has been done in media that rely on the bicarbonate buffering system (Mahadevan et al., 1986), which in addition to functioning as a buffering system is a metabolic requirement for many cell types including preimplantation embryos (Brinster, 1972). Maintenance of both extracellular and intracellular pH is important for the production of viable gametes and embryos. A limited amount of work has been done on medium used for a

specific species that yields matured oocytes with the greatest chance of becoming blastocysts (Eppig et al., 1990; van de Sandt et al., 1990; Bavister et al., 1992; Rose and Bavister, 1992; Hawk and Wall, 1993). Work in mice, cattle, hamsters, and non-human primates indicates that species does affect choice of medium (Bavister et al., 1992; Gordon, 1994) and that the mouse may have limited value as a model for other species (Winston et al., 1991). Even within a species, different laboratories may have greater success with one medium than other laboratories. Many small techniques are probably associated with these differences so each laboratory must determine what medium and protocols work best for their uses. To provide some foreshadowing to the reader who has stayed with the chapter to this point, medium selected for maturation of the oocyte particular species may not be the same one selected for use in other phases of the reproductive process such as fertilization or embryo culture. Some effort has been made to determine if one medium will support maturation, fertilization, and embryo development for cattle (Nagao et al., 1995) as is done in mice, but more work is necessary to optimize each process before this can be done for many species.

Table 1. Culture media commonly used for gamete and embryo culture

Medium	References	Most common use	Species
BMOC	Brister, 1965, 1972	Ova/embryo culture	Mouse
BO	Brackett and Oliphant, 1975	Sperm capacitation	Rabbit, cow, sheep
CDM	Seidel et al., 1991	Embryo culture	Cow
CR1aa	Rosenkrans et al., 1993	Embryo culture	Cow
CZB	Chatot et al., 1989	Embryo culture	Mouse, cow

Medium	References	Most common use	Species
HECM	Schini and Bavister, 1988	Embryo culture	Hamster, cow
HTF	Quinn et al., 1985	Embryo culture	Human, mouse
KRB	Commercially available	Gamete/embryo culture, collection	Many species
KSOM	Lawitts and Biggers, 1992, 1993	Embryo culture	Mouse, cow
M16	Whittingham, 1971	Embryo culture	Mouse
Menezo's B2	Menezo, 1976	Gamete/embryo culture	Human, cow, sheep
PBS	Versions commercially available	Gamete/embryo collection	Many species
SOF	Tervit et al., 1972	Embryo culture	Sheep, cow
Sperm-TALP (BGM1)	Parrish et al., 1986a,1986b	Sperm processing and capacitation	Cow, other species
TALP	Bavister and Yanagimachi, 1977 Bavister et al., 1983	Fertilization	Hamster, cow, other domestic
TL-hepes	Bavister et al., 1983	Oocyte/embryo handling, collection	Hamster, cow, other domestic

Processing reproductive tissue is normally done at atmospheric conditions so both simple culture media and more complex media have been modified by reducing bicarbonate levels, addition of synthetic buffers such as Hepes, or switching to phosphate buffer saline (PBS) alone. This is to maintain a relatively constant pH, which would not be possible using a bicarbonate-buffered medium in air. Medium having a high phosphate concentration is detrimental to sperm motility and early development of murine embryos (Quinn and Wales, 1973) and one of the present authors believes that prolonged holding of gametes or embryos in similar formations may not be compatible with maintaining optimum viability of these cells. As a reminder, media such as TCM-199 with Hepes buffer with the normal level of sodium bicarbonate for Earle's salts (commercially available) or Tyrode's medium modified for use with bovine sperm (Parrish et al., 1986b) will produce a drift in pH over time under atmospheric conditions, presenting uncontrolled changes in pH. These will be slower than in media using a bicarbonate-based buffering system only, but they do occur. Choice of media not only for final culture, but for processing material ahead of time under ambient atmospheric conditions should be selected with care.

When choosing media for processing or culture of tissue, considerations must be given to the osmolarity of the medium selected. Although only a limited amount of work has been done on effects of osmolarity with regard to oocyte maturation or fertilization, a greater amount of work has been compiled concerning effects on early embryo development beginning almost from the inception of in vitro tissue maturation as applied to preimplantation mammalian embryos (Whitten, 1957) and continuing to the present (Brinster 1965; Lawitts and Biggers, 1992, 1993; Biggers et al., 1993). The effects of osmolarity are tightly linked to that of media constituents,

particularly the ratios of various electrolytes, and also the gaseous atmosphere used for culture with a specific media. Introduction of molecules such as bovine serum albumin (BSA) can also shift osmolarity if products are not sufficiently purified. Monitoring osmolarity is one means of detecting possible drift in media quality.

Vigorous monitoring of all media components is another critical quality control measure necessary for success components degrade spontaneously during storage of media (Schiewe et al., 1990). Diluted and aliquated proteins even stored at low temperatures will also loose their biological activity after a period of several months as cautioned by sources such as the National Institutes of Health gonadotropin program. Some deliberation must be given to each component in regard to how it should be stored (most chemical companies indicate this on the container) and whether it can be stored as a diluted stock or should be diluted and added to media just prior to use with tissue. Media once prepared for in vitro manipulations will also deteriorate during storage (Stewart-Savage and Bavister, 1988). Length of storage for the water used to make up media can also affect the success of in vitro embryo production (Nagao et al., 1995). Quality control is essential.

There have been many media additives tested to determine if they improve tissue maturation of reproductive cells in culture. These range from anti-oxidants, chelators of heavy metals, vitamins, amino acids, co-factors, antibiotics, and on into countless other factors, most recently including growth factors and cytokines. It is suggested that the excellent references cited in the literature be consulted for the many possible modifications of culture media that have been tested and found useful in laboratories around the world to find the system that works most effectively for a particular laboratory, species, or cell type.

2.7.2 Gaseous atmosphere

Media considerations have been discussed slightly in regard to selecting media for processing of tissue and gametes vs choice of media for final culture of oocyte. Also the buffering system in the medium selected for maturation will dictate the CO_2 concentration required for culture. Oxygen and Nitrogen are the other two gases normally considered when choosing the atmosphere for culture. Nitrogen is fairly inert in most mammalian culture systems and is used to make up the balance of the incubator atmosphere when ambient air is not selected for use during culture. Oxygen at concentrations found in atmospheric conditions may be toxic to many cell types such as embryonic cells and so is frequently reduced to comprise 10% or less of the constituents of the gas atmosphere. O_2 concentrations in oviduct and uterus is known for a species and is consistently less than 10% (Fisher and Bavister, 1993; Leese,1991). Despite the early awareness of oxygen toxicity to the early embryo (Whitten, 1957; Brinster, 1965), a high proportion of the laboratories involved in the in vitro production of embryos continue to use a gas phase with constituents, osmolarity, and atmospheric components are exceedingly complex. Studying changes in any one of these factors must be considered in relation to the others. This fact is often forgotten and may affect conclusions of experiments designed to study just one aspect of total culture environment.

Elevated O_2 content of atmospheric air may lead to increased formation of radicals which damage intracellular processes. In fact the generation of free radicals in culture media in general is a topic receiving considerable attention in efforts to develop improved conditions for in vitro maturation of mammalian embryos (Nagao

et al., 1994). This focus is just beginning to be addressed for oocyte maturation and deserves more attention in the future. Perhaps, it has been held for too long that the oocyte's surrounding cumulus cells lend protection from the potential damage by free radicals. These cells are necessary in some fashion to the oocyte for final maturation as determined from in vitro studies concerning developmental competence of oocytes matured with or without surrounding cumulus (Staigmiller and Moor, 1984; Leibfried-Rutledge et al., 1989; Canipari, 1994). Follicle cell derivatives are a major route for the empty of metabolites and precursor molecules via gap junctions between cumulus and oocyte (Schultz, 1986b). Along with proteins from body fluids, they also appear to prevent a phenomenon referred to as zona hardening with impairs subsequent attempts to fertilize the in vitro matured oocyte. Cumulus cells may even have a positive inductive role in mammalian ooycte maturation, and in mediating the gonadotropin effects on the oocyte (Dekel, 1988). In vitro maturation of mammalian oocytes must actually be considered as an interaction of several types of tissue, both gametogenic and somatic.

2.7.3 Macromolecular supplements

Macromolecular supplements used in tissue culture perform two basic roles: one is to provide a fixed source of nitrogen; the other is to reduce stickiness of the cell surfaces to make handling and manipulation easier. As mentioned previously, blood serum used as macromolecular supplement during in vitro oocyte maturation prevents zona hardening, possibly by a blood component (Schroeder et al., 1990). A major amount of buffering capacity is also supplied by proteinaceous supplements such as found in body fluids. Two basic groups of supplements are utilized. Body fluids and

their products are one type. This includes fluids such as blood serum or products isolated from serum such as BSA. Culture systems utilizing these tissue-derived supplements are often termed undefined systems, since even purified components from them contain contaminants so amounts of specific substances and composition are not completely known. Different lots of blood serum from bovine fetuses and even lots of supposedly purified components such as BSA or human serum albumin (HAS; Ashwood-Smith et al., 1989) have differing abilities to support successful outcomes after tissue culture. Variability in frequency of development to the blastocyst stage, rate of hatching, and cell number of the resulting product after culture of rabbit and hamster embryos in different lots of BSA has been reported. Variability in lots of blood serum or its components and whether this is due to differing amounts of actual stimulatory factors or toxic effects of contaminants is not known for most reproductive cell types being cultured. This fact must be kept in mind when performing studies to determine the most appropriate tissue-derived supplement to use with various cell types. Several different lots or sources of a particular supplement should be planned into the experimental design so that the investigator is actually testing or characterizing the effect of the component rather than one lot on the desired cell type. Fetal calf serum (FCS) compared to BSA has been observed to improve viability of both hamster and bovine cumulus cells and their expansion (Leibfried-Rutledge et al., 1986) during maturation of the oocyte while supporting a higher frequency of oocytes that complete meiosis I (Cross and Brinster, 1970; Shea et al., 1976; Leibfried-Rutledge et al., 1986). Gonadotropin-induced expansion of cumulus cells requires a serum component that is not supplied by BSA (Eppig, 1979, 1980). Choice of supplement will depend on the final purpose of the tissue culture. Media

conditioned by cell secretions also can be included in the group of tissue-derived supplements. This type of macromolecular supplementation is most often encountered in embryonic culture.

The second group of macromolecular supplements is comprised of large synthetic polymers or defined serum replacements. Polyvinylpyrrolidone (Cholewa and Whitten, 1970) and polyvinyl alcohol (Bavister, 1981) are examples of polymers that are often used in current culture systems when a completely defined environment is desired to study cell phenomena. Since these are both synthetic polymers they can be brought in a number of chain lengths or molecular weights. Not all give equal results for tissue purposes. These substances from some sources may also have to be cleaned up by dialysis or chromatography prior to use in culture. These are characterized by having defined amounts of highly purified known components, such as defined percentages of albumin and globulin. On the whole serum replacements have not been frequently utilized for gamete or embryo culture, with the human system being the most notable exception.

The defined culture system can be utilized successfully (Menezo et al., 1984; Caro and trounson, 1986; Schini and Bavister, 1990) as demonstrated by production of normal offspring and is useful for several reasons. First, when transfer to recipients, or international transport is a feature of the use scenario, than considerations for control of disease vectors is simplified. Analysis of the parental genotypes is easier as is verification of an embryo's health status more readily achieved. The less defined culture systems are workable in this setting but take more documentation to provide supporting health certifications. The defined culture environment is also a useful model for studying specific cellular processes such as energy metabolism or amino

acid metabolism where total control of these substances in the culture media is required for the most definitive results (Gardner, 1994; Bavister, 1995). Unfortunately, except for a very few species demonstration of normal offspring resulting from completely defined systems, or rigorous testing of defined vs those using blood serums or its less pure components has not been accomplished although they have been used to produce blastocysts in a fair number of species. The investigator must be sure that the totally defined environment is not distorting the outcomes and forcing cellular processes down alternative pathways, and this may often be difficult to determine. The objective for using in vitro technologies will dictate whether a defined or less defined culture system is chosen. With greater attention being paid to the potential for inducing perturbations to normal developmental mechanisms with in vitro manipulations (Willadsen et al., 1991; Walker et al., 1992, 1996; Seamark and Robinson, 1995), we as researchers, clinicians, agriculturists or environmentalists are increasingly obliged to examine offspring produced both as the neonate and at stages of its future life history to verify normality of the product.

2.7.4 Energy substrates

Oocytes, as do all cell types, require sources of energy to maintain cell processes and hence viability. Oocytes of the cow and rhesus monkey show a similar preference (Rushmer and Brinster, 1973). More recent work extends this idea to support the notion that metabolic substrates may be capable of regulating meiotic maturation in vivo. As stated previously, current oocyte maturation systems must actually be considered as the culture of several different cell types since we are, on the

whole, unable to obtain a developmentally competent oocyte if it is matured without follicular companion cells. The cumulus cell appears able to convert a six-carbon sugar, such as glucose, into the three-carbon metabolite favored by the oocyte (Biggers et al., 1967). In cattle, addition of both glucose and pyruvate to the oocyte complex in culture results in the most successful oocyte maturation and development to the blastocyst stage after fertilization (Susko-Parrish et al., 1992). More blastocysts were obtained with glucose present during culture of the cumulus cells, while the oocyte showed a preference for pyruvate. Since glucose is thought to be inhibitory to development of early cleavage-stage embryos in some species (Schini and Bavister, 1988) and does hinder capacitation of sperm (Parrish et al., 1989b), it was thought that a similar principle might apply to the oocyte. On the whole, we know much more about the metabolic pathways and substrate perferences of sperm and embryos compared to those of the oocyte complex. The area of nutrient requirements needs more attention in the future. Reviews concerning substrate utilization throughout the various stages of maturation, fertilization, and development should be consulted when selecting culture medium for specific purposes and species where available (Bavister, 1990; Leese, 1991; Conaghan et al., 1993; Bavister, 1995; Barnett and Bavister, 1996).

2.7.5 Hormonal supplements

Preovulatory oocyte maturation *in situ* in the Graafian follicle is induced at a specific stage of the estrous or menstrual cycle in response to a specific hormonal milieu. Initial attempts to obtain offspring using oocytes matured in vitro tried to mimic this situation by supplementing culture medium with gonadotropins and

estrogen (Crosby et al., 1981; Staigmiller and Moor, 1984; Sirard et al., 1988). Since then, oocytes have been matured in vitro in media without hormonal supplement using blood serum or follicular fluid as protein supplement or in completely defined culture systems without hormonal supplementation. Many studies in a wide variety of species have been conducted to determine if gonadotropins and steroides are actually needed alone or in combination. From looking at the numerous variations in culture systems in use today, the literature at this point appears conflicting, with no definitive answer in sight. Very few studies have gone past blastocyst formation as an endpoint to assess the effect of hormones during oocyte maturation in culture. Complete developmental competence as affected by hormone treatment, in other words, has not been rigorously evaluated. In the majority of studies conducted to date, success rate for completing meiotic maturation does not appear to be affected by the presence of hormonal supplements although cumulus maturation is gonadotropin-dependent (Eppig, 1979, 1980). The expansion of the cumulus oophorous during in vitro maturation of cattle oocytes is shown in Figure 7. This is not anywhere near as extensive as that observed after in vivo maturation. Duration of time required for resumption and completion of meiosis I is changed in response to supplementation with hormones (Suss et al., 1988; Dominko and First, 1992). Gonadotropin-induced cumulus maturation uses cAMP as a second messenger and the increased levels of cAMP may be transiently inhibitory to the oocyte (Downs et al., 1988) so meiotic resumption is delayed. Events involved in regulating the time required for resumption and completion of meiosis I may have an impact on success in blastocyst formation, since the earliest maturing cattle oocytes appear to have the greatest success in developing to blastocysts after fertilization (Dominko and First, 1992). In some

studies, addition of hormonal supplements is associated with higher rates of fertilization or development to the blastocyst stage (Shalgi et al., 1979; Bracket et al., 1989). In vitro tissue maturation of the oocyte complex involves several types of differentiated cells and complete success of the process may depend on getting conditions "right" for all the cells involved. Cytoplasmic maturation of the oocyte has been thought to be at least partially responsive to steroids since the early days of extra-follicular culture of oocytes (Moor and Trounson, 1977; Moor et al., 1980) and so may need careful consideration as a component of media used for oocyte culture.

Although cytoplasmic maturation can be achieved without addition of supplementary gonadotroins or steroids as verified by production of blastocysts after IVF and in some species full-term offspring, consistency in achieving respectable rates of blastocyst formation after IVF is realized if hormonal supplements are added to the medium used for maturation. Since clearly defined increases in steroids and both FSH and LH are manifest prior to ovulation, it is not unreasonable to suppose that final oocyte maturation may be mediated at least indirectly by the changing hormonal content of the preovulatory follicle. Whether this is due to action of the hormones on their own receptors or cross-reaction with the individual receptors has not been clearly defined. Ovulation in vivo can be induced only by molecules with LH-like activity.

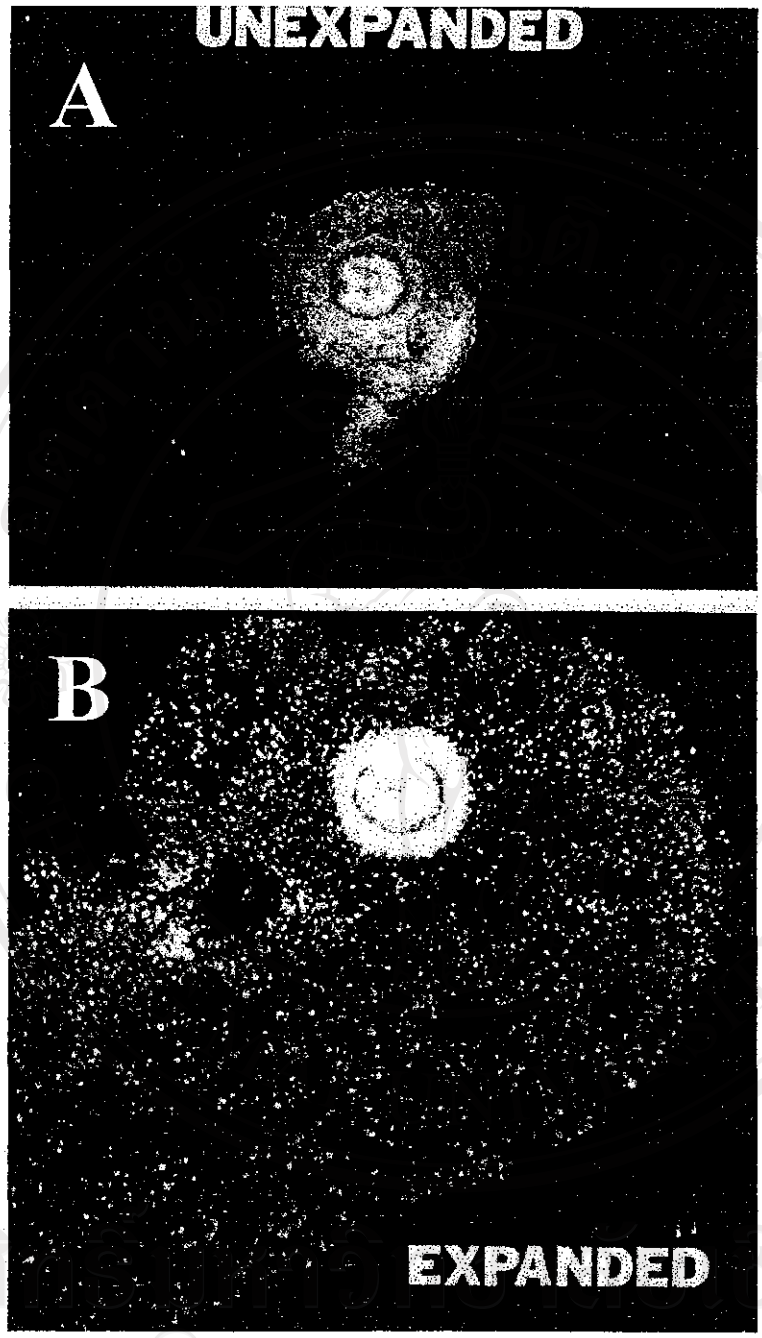

FIGURE 7. In vitro maturation of the cumulus investment. During culture of the primary oocyte the cumulus oophorus undergoes expansion, changing from a many layered epithelial like conformation (A) to a loose arrangement of cells entrapped in a sticky matrix (B).

2.7.6 Culture methods

Once a medium and its constituents have been selected and a gas atmosphere chosen, the method of culture for the particular cell type must be picked. Petri dishes, multi-well plates and chamber slides have all been frequently used as culture vessels. If disposable plastics are chosen, they should be tested for toxicity since not all sources yield the same success rates (Boone and Shapiro, 1990; Schiewe et al., 1990). The ratio of number of cells being cultured per volume of medium has become a topic of interest for both oocytes and embryos and is best determined for an individual laboratory since many subtle factors affect this variable. In the case of oocyte, the time required for maturation in vitro can actually be prolonged if not inhibited completely at too high a cell density (Leibfried-Rutledge et al., 1989). Microdroplets of medium are often utilized as described by Bavister (1981). This is very sparing of media and supplements. Depending on volume of culture media placed in containers, an oil overlay might be considered to prevent evaporation. The oil itself should be tested prior to use with critical material. Oil used for overlay is most often saturated with medium or saline. As described by Gordon (1994), the oil overlay reduces water evaporation when using small volumes of medium, provides some protection from microbial contamination, attenuates temperature and gas fluctuations and allows easier observation of cultures compared to other systems. The investigator should be aware that the oil overlay forms a two-phase separation system for hydrophobic substances. Since the oil is usually equilibrated with a water-based medium prior to use, the oil overlay will also allow diffusion of water-soluble compounds out of the medium. One can empirically demonstrate this for oneself by adding hyaluronidase to one of a pair of oil-overlaid microdrops containing oocytes having expanded cumulus.