Articles

Maturational and developmental competence of immature oocytes retrieved from bovine ovaries at different phases of folliculogenesis

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Abstract

Immature oocytes recovered from bovine ovaries were studied to determine if their maturational and developmental competence is affected by phase of folliculogenesis. Ovaries (a total of 39 pairs) were collected from a local abattoir. Following examination, each pair of ovaries was assigned to one of three groups, according to follicle size and with or without a corpus luteum: (i) early phase (n = 13 pairs): all follicles were ≤8 mm in diameter; (ii) late phase (n = 13 pairs): the largest follicle was ≥15 mm in diameter; (iii) luteal phase (n = 13 pairs): all follicles were ≤8 mm in diameter and there was a corpus luteum on one of the ovaries. All follicles were aspirated and cumulus–oocytes complexes (COC) were cultured in 1.0 ml maturation medium, TC-199 medium supplemented with 10% fetal bovine serum (FBS), 0.2 mmol pyruvate, and 75 mIU/ml FSH and LH (Humegon) at 38.5°C in a humidified atmosphere of 5% CO_2 and 95% air for 24 h. Following maturation *in vitro*, the oocytes were inseminated with frozen–thawed semen and cultured for further development *in vitro*. The mean number of oocytes retrieved from early phase ovaries was 32 ± 8.1 which was significantly higher ($P < 0.05$) than other phases (n = 20.1 ± 5.4 and 22.7 ± 6.9). The numbers of degenerated oocytes from early, late and luteal phase ovaries were not different (n = 1.6 ± 0.7, 1.8 ± 0.5 and 1.5 ± 0.5, respectively). The rates of oocyte maturation (84.4%, 89.2% and 83.6%) and fertilization (53.5%, 53.3% and 51.3%) were not significantly different across the three groups. In addition, there were no significant differences in the rates of cleavage (77.9%, 79.9% and 73.9%) and blastocyst formation (27.4%, 33.0% and 30.4%) in the three groups. These results indicate that the maturational and developmental competence of immature oocytes is not affected by the phase of folliculogenesis.

Keywords: *immature oocytes, in-vitro maturation, folliculogenesis, fertilization, development, bovine folliculogenesis*

Introduction

In monovular species, follicular development is characterized by selection of a dominant follicle destined to ovulate from a cohort of growing follicles, and initiation of atresia in those remaining in the cohort. Oocytes acquire meiotic competence after reaching their full size (Chuinard, 1971; Erickson and Sorenson, 1974). The acquisition of meiotic competence seems to correlate not only with the size of the oocytes but also with the size of the follicles (Quirk *et al.*, 1986; Stubbings *et*

al., 1990; Driancourt, 1991; Lonergan *et al.*, 1991; Pavlok *et al.*, 1992). In addition, developmental competence, as shown by the ability to undergo fertilization and develop to the blastocyst stage, is dependent on the sizes of the oocyte and the follicle (Eppig *et al.*, 1992; Lonergan *et al.*, 1994; Crozet *et al.*, 1995; Fair *et al.*, 1995), indicating RNA synthesis ceases as the oocyte diameter exceeded 110 µm (Fair *et al.*, 1996). In the animal industry, it is normal procedure to collect immature oocytes from small follicles that are 2–8 mm in diameter and then mature them *in vitro* to produce live offspring (Brackett and Zuelke, 1993). In-vitro maturation (IVM) of aspirated immature oocytes requires a period of about 24–48 h in culture, whereas maturation *in vivo* results when a dominant follicle is triggered by LH surge.

After a dominant follicle has been selected in a spontaneous ovulatory cycle, secondary follicles are generally thought to be atretic with only the dominant follicle possessing the appropriate hormonal milieu necessary to achieve oocyte maturity and ovulation. It is believed that development of a dominant follicle suppresses subordinate follicles and new growth of small follicles occurs only after the dominant follicle has ceased growing (Savio *et al.*, 1988; Sirois and Fortune, 1988). Less is known about how the dominant follicle detrimentally affects the oocytes from the small follicles in the ovaries. Although it seems that the developmental competence of bovine oocytes from small antral follicles is not adversely affected by the presence of a dominant follicle (Smith *et al.*, 1996), it is still not clear whether the developmental potential of oocytes derived from the small follicles is affected by phase of folliculogenesis.

Conventional IVF treatments involve daily injections of gonadotrophins to induce multiple follicular development *in vivo*. Recovery of immature oocytes followed by IVM of these oocytes is a potentially useful treatment for infertile women. In comparison with conventional IVF treatment, the major benefits of IVM treatment include avoidance of side-effects by ovarian stimulation using gonadotrophins, less complicated treatment and lower cost. Recently, it has been reported that the maturational and developmental competence of immature oocytes retrieved from women with polycystic ovarian syndrome (PCOS) is improved by human chorionic gonadotrophin (HCG) priming before immature oocyte retrieval (Chian *et al.*, 1999a,b; 2000). In these studies, patients were excluded when development of a dominant follicle occurred by day 8 of the cycle. Therefore, IVM treatment may be limited to those women undergoing IVF who have polycystic ovarian syndrome with anovulatory cycles (Franks, 1989; Buckett *et al.*, 1999). Women with polycystic ovaries that have been seen under ultrasound scan but who have a normal menstrual cycle, or women with normal ovaries, usually produce a dominant follicle at the middle of cycle. Fertilization, embryo development and live births have been achieved following transfer of embryos produced from oocytes retrieved from secondary follicles in treatment cycles when there has been development of a dominant follicle in the ovaries (Paulson *et al.*, 1994). This indicates that oocyte viability is maintained in these secondary follicles in spite of the possibility that the dominant follicle may induce atresia and regression in them. It is not known whether embryos derived from secondary follicles have the same chance of implanting as those from dominant follicles.

The objective of the present study was to determine the maturational and developmental competence of immature oocytes retrieved from bovine ovaries at different phases of folliculogenesis.

Materials and methods

Experimental design

Ovaries from heifers or cows were collected from a local abattoir. All animals had begun oestrous cycles and so were classed as adult. The ovaries were removed within 30 min of slaughter and transported to the laboratory at 35°C in 0.9% NaCl aqueous solution. Each pair of ovaries was checked and then divided into three groups, according to the size of follicles (**Figure 1**). If any pair of ovaries appeared abnormal, it was discarded or used for a different experiment. A total of 39 pairs of ovaries were used in this experiment. (i) early phase (n = 13 pairs): all follicles were ≤8.0 mm in diameter; (ii) late phase (n = 13 pairs): the largest follicle was ≥15 mm in diameter on one side of ovaries; (iii) luteal phase (n = 13 pairs): all follicles were ≤8.0 mm in diameter and there was a corpus luteum on one of the ovaries. The cumulus–oocyte complexes (COC) were aspirated from all visible follicles (2–20 mm) with an 18 G needle, and pooled for maturation in culture.

In-vitro maturation of oocytes

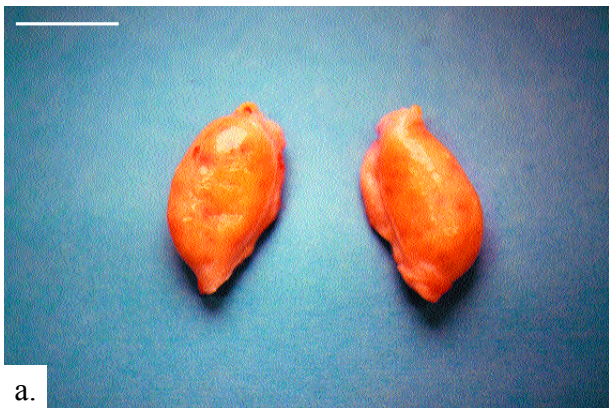
The COC were rapidly washed four times in HEPES buffered Tyrode's medium (TLH; Bavister *et al.*, 1983) supplemented with 10% heated (56°C, 30 min) fetal bovine serum (FBS), 0.25 mmol pyruvic acid (Sigma) and 50 µg/ml gentamycin (Sigma). After washing, 10 oocytes were cultured in 1 ml TC-199 medium, maturation medium, supplemented with 10% FBS, 75 mIU/ml FSH and LH (Humegon; Organon, Scarborough, ON, Canada), 0.25 mmol pyruvic acid (Sigma), and 75 µg/ml streptomycin sulphate (Sigma). The oocytes were incubated at 38.5°C under an atmosphere of 5% CO_2 and 95% air with high humidity. After 24 h of maturation, some oocytes were fixed to examine maturation rate and the remaining oocytes were used for in-vitro fertilization.

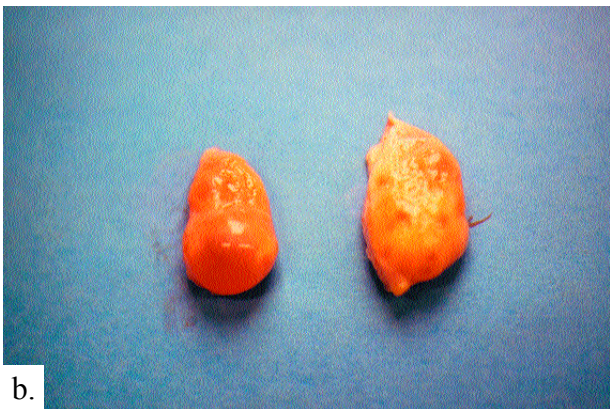
Sperm preparation and IVF

Frozen semen, pooled from five bulls, was donated by GENCOR, Guelph, Ontario. Straws of semen were thawed in a water bath (35°C) for 30 s and spermatozoa were subjected to the swim-up procedure as described by Parrish *et al.* (1986). Spermatozoa were then washed twice in modified Tyrode's albumin lactate medium (Sp-TALP), used for sperm culture, containing 6 mg/ml fatty acid-free BSA (Sigma), 10 mmol pyruvic acid, and 50 µg/ml gentamycin. Spermatozoa/oocytes were incubated in 50 µl drops of the fertilization medium, modified Tyrode's medium (mTALP; Parrish *et al.*, 1988) containing 2 µg/ml heparin, under mineral oil at 38.5°C in 5% CO_2 and 95% air with high humidity. A final sperm concentration of 1×10^6 sperm/ml was used for insemination and five oocytes were placed in each 50 µl drop.

Embryo Developmental Culture

Following 18 h of insemination, some oocytes were fixed for examining fertilization rate and the remaining oocytes were





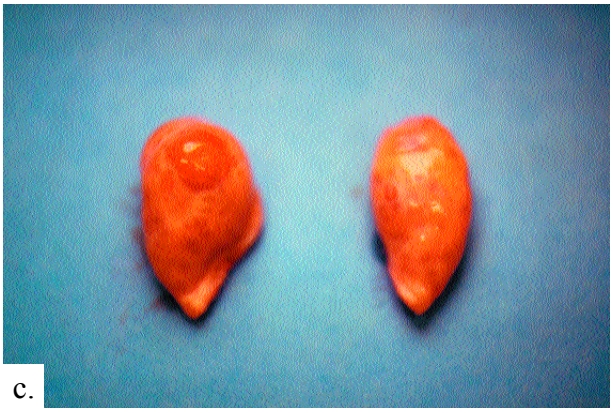


Figure 1. Each pair of ovaries was checked and placed into one of three groups. (a) Early phase: all follicles were ≤8.0 mm in diameter; (b) late phase: the largest follicle on one of the ovaries was ≥15 mm in diameter; (c) luteal phase: all follicles were ≤8.0 mm in diameter and there was a corpus luteum on one ovary. Scale bar indicates 2.54 cm.

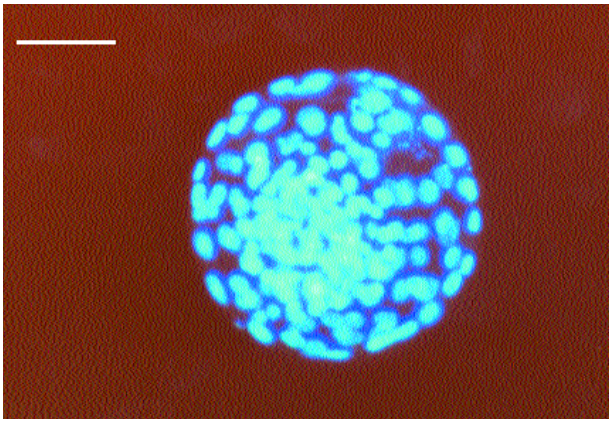


Figure 2. A blastocyst observed under fluorescence microscopy after staining with Hoechst 33342 glycerol solution to count the number of nuclei. The blastocyst was produced from a small follicle retrieved from an ovary presented with a dominant follicle. Scale bar indicates 40 µm.

washed three times with TLH and then transferred to 50 µl droplets of development medium (BECM: Bovine Embryo Culture Medium; Lim *et al.*, 1994) supplemented with 3 mg/ml BSA (fatty acid-free, Sigma) and 1 µg/ml gentamycin under mineral oil. The culture medium was changed at 24-h intervals until 120 h after insemination and then the embryos were transferred to 50 µl droplets of BECM supplemented with 10% FBS and 0.25 mmol pyruvic acid (Sigma) for further developmental culture.

Fixation of oocytes

At 24 h of maturation and at 18 h of insemination, the oocytes were mounted on slides with coverslips, fixed with acetic acid/ethanol (1:3) solution for at least 24 h. The oocytes were then stained with 1% orcein dissolved in 45% acetic acid solution and examined for evidence of fertilization. Fertilization was identified by observing two pronuclei with an accompanying sperm tail in the cytoplasm. Oocytes with two pronuclei and a clear second polar body but without a sperm tail were also considered to have been fertilized. Oocytes with a female pronucleus and a decondensed sperm head were considered abnormal fertilization. Oocytes with three pronuclei (two sperm) or more were considered polyspermic.

At the end of developmental culture (192 h), embryos were fixed with 1.25% glutaraldehyde in phosphate-buffered saline solution (PBS) containing 25 µl Triton X-100 and then stained with Hoechst 33342 glycerol solution (0.01 mg Hoechst in 1 ml PBS + 9 ml glycerol) to examine the number of nuclei. Embryos with more than 64 nuclei were considered to be blastocysts (**Figure 2**).

Statistical analysis

The numbers of immature oocytes, maturation, fertilization and embryo cleavage rates as well as blastocyst formation rates retrieved from each pair of ovaries at early, late and luteal phases of folliculogenesis were analysed by one-way analyses of variance. When analyses revealed significance, the groups were compared using the Student-Newman-Keuls' test (Steel and Torrie, 1980).

Results

The mean numbers of oocytes retrieved from each pair of ovaries at early, late and luteal phases of folliculogenesis were 32.0 ± 8.1, 20.1 ± 5.4 and 22.7 ± 6.9, respectively (**Figure 3**). The mean numbers of oocytes from early phase compared with late and luteal phases of folliculogenesis were different ($P < 0.05$). However, the average numbers of degenerated oocytes retrieved

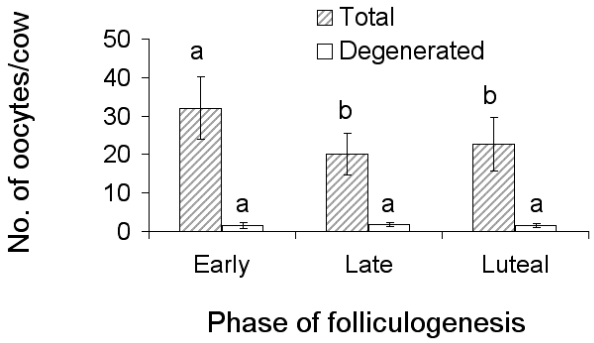


Figure 3. The mean numbers of oocytes retrieved from each pair of ovaries at different phases of folliculogenesis. A total of 13 pairs of ovaries was used in each group. Total = the mean number of oocytes collected; Degenerated = the mean number of oocytes degenerated. ab = Within 'total' or 'degenerated' categories, means with different letters differed significantly ($P < 0.05$).

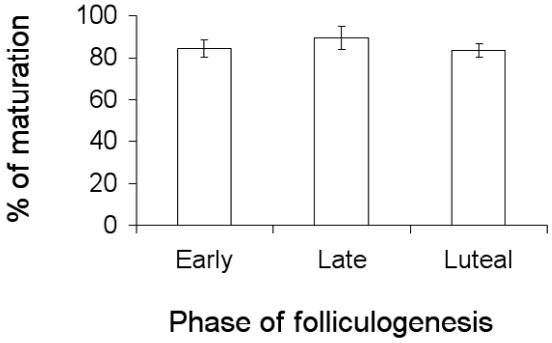


Figure 4. Maturation *in vitro* of bovine oocytes retrieved from ovaries at different phases of folliculogenesis ($n = 224$ oocytes with four repeats). There were no significant differences among the three groups.

Table 1. Fertilization *in vitro* of bovine oocytes retrieved from ovaries at different phases of folliculogenesis.

Follicular phase	*Oocytes inseminated (n)*	*No. oocytes penetrated (%) Total*	*Fertilization*	*Abnormalities*
Early	71	59 (83.1)	38 (53.5)	21 (29.6)
Late	75	64 (85.3)	40 (53.3)	24 (32.0)
Luteal	76	65 (85.5)	39 (51.3)	26 (34.2)

There were no differences among groups within columns.

Table 2. Early embryonic development of bovine oocytes retrieved from ovaries at different phases of folliculogenesis.

Follicular phase	*Oocytes inseminated (n)*	*No. oocytes cleaved (%)*	*No. embryos developed to 8–32 cell (%)*	*Blastocyst (%)*
Early	258	201 (77.9)	134 (66.7)	55 (27.4)
Late	144	115 (79.9)	78 (67.8)	38 (33.0)
Luteal	138	102 (73.9)	65 (63.7)	31 (30.4)

There were no differences among groups within columns.

from each pair of ovaries were 1.6 ± 0.7, 1.8 ± 0.5 and 1.5 ± 0.5, respectively. There were no differences among the groups. As shown in **Figure 4**, maturation rates of oocytes retrieved from early, late and luteal phases of folliculogenesis were 84.4% (n = 65/77), 89.2% (n = 66/74) and 83.6% (n = 61/73), respectively, and there were no differences among each phase.

The total penetration rates were not different when the oocytes retrieved from early (83.1%), late (85.3%) and luteal (85.5%) phases of folliculogenesis were compared (**Table 1**). In addition, normal fertilization rates were not different when oocytes were retrieved from early (53.5%), late (53.3%) and luteal (51.3%) phases of folliculogenesis. As shown in **Table 2**, there were no differences in the embryonic cleavage rates (77.9%, 79.9% and 73.9%, respectively). The rates of blastocyst formation were 27.4%, 33.0% and 30.4% with oocytes retrieved from the early, late and luteal phases of folliculogenesis, respectively, and there were no differences among the groups.

Discussion

This study demonstrates that the developmental competence of bovine oocytes from small antral follicles is not affected either by the presence of a dominant follicle or by the phase of folliculogenesis. In general, it has been believed that a dominant follicle suppresses subordinate follicles and initiates atresia of the small follicles. In normal ovulatory ovaries, follicular fluid oestradiol concentration correlates well with follicular health and maturity, because the mature granulosa cells produce more oestradiol. Binding of FSH to granulosa cells of antral and preovulatory follicles increases aromatase activity and, subsequently, increases LH-stimulated follicular 17α-hydroxylase cytochrome P-450 activity and messenger RNA content. Binding of FSH to the granulosa cells also increases the cyclic adenosine monophosphate-dependent protein kinase type-II and enhances the production of oestradiol. Higher concentrations of oestradiol are associated with healthy follicles. In addition, there is evidence to support

the hypothesis that levels of progesterone in follicular fluid are closely associated with oocyte maturity (Seibel *et al.*, 1989). The maturation of granulosa cells is associated with stimulation of the phosphatidylinositol pathway, involving the mobilization of intracellular Ca^{2+} and increase in protein kinase C, which together stimulate a reduction in progesterone. The changes in intrafollicular oestradiol concentrations may influence the oocyte indirectly via the granulosa and cumulus cells. Therefore, the atresia of follicles may be associated with a shift from oestradiol to progesterone production by the granulosa cells, which is reflected in the ratio of progesterone to oestradiol (Ireland and Roche, 1983; Grimes *et al.*, 1987; Kreiner *et al.*, 1987; Spicer *et al.*, 1987). However, it has been reported that follicular steroid concentrations do not differ in small follicles taken in the presence versus the absence of a dominant oestradiol-active follicle, and that there are no differences in the developmental capacity of the oocytes (Smith *et al.*, 1996). It seems that a dominant follicle does not affect the steroid concentrations of the small follicles. These results suggest that the appearance of a dominant follicle may not trigger atresia of the small follicles in the ovaries. Assey *et al.* (1994) suggested that early follicular atresia might occur in the small follicles around the time of the LH surge following an ultrastructural study of the oocytes. However, the exact timing of the initiation of atresia in small follicles during folliculogenesis needs further clarification.

It has been reported that the total number of immature oocytes retrieved from each pair of ovaries are different during phases of folliculogenesis (Stringfellow *et al.*, 1993; Smith *et al.*, 1996). In the present study, it was also found that the mean numbers of immature oocytes were higher in the early phase as compared with late and luteal phases of folliculogenesis. However, there were no differences in the numbers of degenerated oocytes retrieved from the ovaries during different phases. It appears that morphological atresia may occur infrequently during folliculogenesis because the numbers of atretic oocytes detected by morphology were low. Although maturation rates were at variance with immature oocytes derived from different types of follicles (Moor and Trounson, 1977), the use of defined morphological criteria does not improve oocyte maturation and fertilization *in vitro* (De Loos *et al.*, 1989). The acquisition of meiotic competence seems to correlate not only with the size of the oocytes and follicles but also with the morphology and transcriptional activity of the immature oocyte nuclei and nucleoli (Motlik *et al.*, 1984; Crozet *et al.*, 1986). Similar maturational and developmental potential was demonstrated when immature oocytes were retrieved from >2–8 mm follicles (Pavlok *et al.*, 1992). In the present study, similar rates of oocyte maturation were achieved in each phase of folliculogenesis. It may be that all immature oocytes were collected from ≥2 mm in diameter. This observation indicates that overall, immature oocytes could possess the meiotic maturational competence of they are collected from follicles that have reached a certain size.

Each individual anovulatory follicle disappears by entering the degenerative process of atresia, which must be considered a normal process allowing the ovary to produce a specific number of ovulated mature oocytes. There is evidence that oocytes from slightly atretic follicles may have a higher competence for embryo production due to that the same initial process occurred in the dominant follicle (Hyttel *et al.*, 1997). There are several waves of folliculogenesis during each oestrous cycle in which a group of antral follicles begin to grow simultaneously and such groupings emerge at regular intervals (Savio *et al.*, 1988; Sirois and Fortune, 1988; Guilbault *et al.*, 1993). Although the evidence has been presented that the presence of a dominant follicle decreases ovulation rate and the number of transferable embryos produced *in vivo* (Guilbault *et al.*, 1991; Bungartz and Niemann, 1994), the data presented in this study indicate that the competence of fertilization and development of oocytes is not affected by phases of folliculogenesis. Furthermore, it has been reported that one healthy baby was born from immature oocytes retrieved from an egg donor at the time of Caesarean section after which the oocytes were matured *in vitro* (Cha *et al.*, 1991). Hence, it is possible that waves of follicular development occur during pregnancy as well as during the menstrual cycle. Thornton *et al.* (1998) reported that immature human oocytes can be retrieved successfully from subordinate follicles during the mid-cycle aspiration of the dominant follicle and that these immature oocytes contribute to the overall pregnancy success of unstimulated IVF cycles. This indicates that immature oocytes derived from small follicles have maturational and developmental potential even in the presence of a dominant follicle. The result from our previous study also indicates that healthy infants can be produced from subordinate follicles even if a significant dominant follicle exists at the time of egg retrieval (Chian *et al.*, unpublished data). The results from this in-vitro model study support the notion that developmental competence of immature oocytes derived from small follicles is not adversely affected by phases of folliculogenesis regardless of the phase of the menstrual cycle or the period of pregnancy. In conclusion, the maturational and developmental competence of immature oocytes derived from the small antral follicles is not affected by the presence of a dominant follicle and phases of folliculogenesis.

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