Effects of Inhibition of Meiotic Resumption upon the Subsequent Development of Bovine Oocytes *In Vitro*

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Abstract. Immature bovine oocytes aspirated from small follicles (2–5 mm) are not all developmentally competent following maturation and fertilization. It is suspected that the follicles provide the proper signal to the oocyte during late folliculogenesis. To assess the effects of follicular components, steroids, and gonadotropins on subsequent development, it is important to maintain the oocyte in a state of maximum receptivity, at the germinal vesicle (GV) stage. This study evaluates the inhibitory effects of co-culture with hemi-sections follicles on the meiotic resumption and subsequent development of cumulus-enclosed primary oocytes *in vitro*. One or 2 follicular hemi-sections prepared by careful dissection of follicles (2–5 mm) were co-cultured for 15 h with groups of 10 oocytes in the presence of FSH, estradiol or both. Oocytes were fixed after incubation for 15 h or incubated for an additional 24 h in a conventional maturation medium (FSH-LH and estradiol) or a medium containing progesterone and LH to stimulate meiotic resumption. Co-culture with 1 or 2 hemi-sections resulted in 37 to 76% GV rates. Inhibition of spontaneous oocyte maturation due to the co-culture was influenced by neither estradiol nor FSH, and 57 to 97% of oocytes reached the metaphase II stage in the subsequent 24 h culture, but at a lower rate with estradiol alone. Successful embryonic development of these oocytes after successive treatments of *in vitro* maturation (24 h), fertilization (10 h) and culturing with oviduct cells (96 h) indicates that viability is maintained after co-incubation with hemi-sections.

Key words: Oocyte, Meiotic inhibition, Meiotic resumption, Developmental competence.

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Spontaneous maturation of mammalian oocytes after removing them from follicles [1] is an obstacle to long-term culture in many mammals. This transformation results from the removal of an inhibitory signal coming from the follicle [2, 3]. Much progress has been made in mice toward completely understanding the mechanism involved in nuclear maturation events [4–6]. In cattle, previous studies have shown that purines, cyclic adenosine monophosphate (cAMP) or phosphodiesterase inhibitors, such as isobutylmethylxanthine (IBMX), do not inhibit nuclear

maturation completely [7] unless a high amount of dibutyryl-cAMP or forskolin is used [8, 9]. Other factors reported to inhibit bovine oocyte resumption *in vitro* are protein synthesis inhibitors [10, 11], phosphorylation inhibitors such as 6-Dimethylaminopurine (6-DMAP) [12], protease inhibitors such as p-amino-benzamidine [13], and RNA synthesis inhibitors such as α -amanitine or sodium fluoride [13, 14]. In the bovine oocyte, new protein synthesis is not required for oocyte maturation as it is in the mouse oocyte. Purines or cAMP can modulate meiotic resumption [5, 6] in the bovine oocyte, but not in the mouse oocyte.

In cows biological materials from follicles have been examined to determine whether or not they

can stimulate the inhibitory signal observed *in vivo* in an attempt to modulate the nuclear maturation process. Monolayers of granulosa cells [15], granulosa cells in suspension [16], or follicular fluid (BFF) with or without cells [17] can partially inhibit bovine oocyte maturation. The variability of response reflects not only the heterogeneity of the oocyte population from small follicles but it also indicates a dynamic process and a possible interaction between the different follicular compartments [17]. Because dissected, un-ruptured follicles do not survive under normal culture conditions, half shells or follicular hemi-sections were used for culturing bovine [18] and porcine [19] oocytes with some success. However it still is not clear if this procedure works physiologically. To evaluate the different means of controlling meiotic resumption, it is important to verify if the inhibition is reversible and, to assess whether or not the oocytes can become embryos. Therefore, in this study, the first incubation was carried out in conditions known to inhibit nuclear maturation followed by a second incubation in non-inhibiting conditions. These oocytes underwent *in vitro* fertilization and further culturing *in vitro*. The results indicate that nuclear maturation of bovine oocytes can be arrested *in vitro* temporarily without significantly affecting further development *in vitro*.

Materials and Methods

Recovery of oocytes

Ovaries were removed from cycling or pregnant heifers or cows within 30 min after slaughter. They were transported to the laboratory in a 0.9% NaCl aqueous solution containing 100,000 IU/l penicillin, 100 mg/l streptomycin and 250 µg/l amphotericin B (Sigma, St-Louis, MO, USA) at approximately 35 C. The cumulus oocyte complexes (COC) were aspirated from 2 to 5 mm follicles with an 18 G needle, pooled, and selected as published previously [20]. The COC were rapidly washed once in Hepes buffered tyrode's medium (TLH) [21] supplemented with 10% fetal calf serum (heat-treated FCS, Flow, NY, USA), 0.2 mM pyruvic acid (Sigma) and 50 µg/ml gentamicin (Sigma).

Preparation of half-follicles

Ovaries were removed from cycling or pregnant heifers or cows within 30 min after slaughter. They

were transported to the laboratory wrapped in gauze humidified with a 0.9% NaCl aqueous solution containing 100,000 IU/l penicillin, 100 mg/l streptomycin and 250 µg/l amphotericin B (Sigma, St-Louis, MO, USA) at approximately 4 C. Follicles were dissected from the interstitial tissue and were selected according to size (2 to 5 mm), transparency, and a near-perfect spherical shape. The selected follicles were cut in halves with a scalpel blade before they were washed in a TCM-199 medium. Follicles with loosely attached granulosa layers were not used.

Culture of oocytes

In the first series of experiments, groups of 10 oocytes were co-cultured with 1 or 2 freshly prepared hemi-sections for 15 h in 50 µl droplets of maturation medium consisting of TCM-199 with Earle's salts (Gibco Lab., NY, USA) and bicarbonate (Sigma) supplemented with 10% fetal calf serum (heat-treated FCS, Flow laboratories, Virginia, USA), 0.2 mM pyruvic acid and 50 µg/ml gentamicin sulfate. The maturation media droplets covered with mineral oil (Aldrich Chemical, Milwaukee, USA), were initially exposed to the culture conditions for at least 2 h (38.5 C, 5% CO₂, 95% air atmosphere with 100% humidity). To evaluate *in vitro* maturation (control), cumulus-enclosed oocytes were cultured in the maturation medium supplemented with 0.5 µg/ml FSH (National Institute of Diabetes and Digestive and Kidney Diseases; NIDDK), 5 µg/ml LH (NIDDK) and 1 µg/ml estradiol (FLE) [20]. With either 1 or 2 hemi-sections, hormonal supplements were added either without hormones (no H), or with 1 µg/ml estradiol 17β (Sigma) (E), or with 1 µg/ml FSH (NIDDK) and estradiol (FE). Following the inhibitory culturing period for 15 h, the oocytes were either fixed or further cultured without the hemi-sections (non-inhibitory culturing) for 24 h in TCM-199 as described above, and supplemented with either 5 µg/ml LH (NIDDK) and 1 µg/ml progesterone (Sigma) (LP), or with 0.5 µg/ml FSH (NIDDK), 5 µg/ml LH (NIDDK) and 1 µg/ml estradiol (FLE) [20]. Some of the oocytes were then fixed for histologic examination, and the rest were submitted to the regular *in vitro* fertilization protocol followed by *in vitro* culture of zygotes to evaluate development potential. At least one group of 10 oocytes were used and distributed among all co-culture or chemical treatments for each replicate.

Fixation of oocytes and embryos

At the end of the incubation period, the oocytes were transferred into small centrifuge tubes and vortex-agitated for 5 min. Completely denuded oocytes were recovered under a stereomicroscope and transferred into small drops on glass slides. Vaseline and paraffin were used to maintain a coverslip in contact with the oocytes without excessive pressure. The coverslip was fixed with epoxy glue and the slide was immersed in a fixative solution (ethanol: acetic acid, 3:1) for a minimum of 24 h. The slides were stained with 1% aceto-orcein and examined with phase contrast at 100 $\times$ and 400 $\times$ magnification. Oocytes were classified as germinal vesicle (GV), intermediate (int) (from the germinal vesicle breakdown up to metaphase-I), mature (mat) (from anaphase-I to metaphase-II), or degenerated (if any abnormal change was noted in the cytoplasm or the chromatin). Significance of individual comparison was evaluated using the Chi-square analysis [21].

Fertilization of oocytes

In vitro fertilization took place in fertilization medium droplets (48 μ l) composed of modified Tyrode lactate medium [22]; 0.6% BSA fatty acid free, 0.2 mM pyruvic acid, 2 μ g/ml heparin (Sigma) and 50 μ g/ml gentamicin containing 5 COC previously washed (twice at 5 min each) in TLH. The fertilization media droplets were placed in sterile petri dishes under mineral oil and equilibrated for 2 h in culture conditions prior to use. Frozen-thawed semen from a pool of 5 bulls (Le Centre d'Insémination artificielle du Québec, CIAQ) were separated by progressive motility [23], and 2 μ l of sperm suspension (25 million per ml) were added directly into each droplet for a final concentration of 1 million spermatozoa per ml. The COC and sperm were co-incubated for 10 h. The zygotes were washed in TCM-199 as described previously and transferred to culture drops for development.

Culture of zygotes

In vitro embryo development was obtained by co-culturing the embryos with bovine oviductal cells that were prepared according a modified Eye-stone and First [24] protocol. Proestrous or estrous oviducts were transported on ice to the laboratory, washed 3 times in a 0.9% NaCl solution, and dissected from the ovaries and other tissues. Oviducts

were then washed 3 times in a NaCl solution. After each washing both extremities were trimmed. Finally, they were washed once in Hank's medium without calcium and magnesium (containing 5% FCS, penicillin, streptomycin and amphotericin B). Oviductal mucosal tissue was extracted by compressing the duct with a glass slide from the infundibulum toward the isthmus end. The mucosal tissue was transferred to a 50 ml conical tube with 20 ml Hank's medium [w/o Ca and Mg ?]-kag and washed twice at room temperature. Decanted tissue was broken using a 20 G needle and repeated aspirations. 50 μ l were incubated for 24 h at 38.5 C, 5% CO₂, 95% air atmosphere and 100% humidity in a 50 ml culture bottle with 10 ml of pre-equilibrated TCM-199, 10% FCS, 0.2 mM pyruvic acid and 50 μ g/ml gentamicin. After culture, oviductal tissue forming very small vesicles were chosen and 20 to 25 selected vesicles were put into each 50 μ l droplet of fresh medium. The fertilized oocytes were washed once for 2 h in 2 ml of the same medium before they were added to droplets containing vesicles. Cleavage rates were recorded after 1 day of culture (1.5 days after insemination) and 50 μ l of fresh medium was added to each droplet after 2 days of culture. After 4 days of culture (4.5 days after insemination), the embryos were fixed as described previously for oocytes [20] and the number of nuclei was counted. The significance of individual comparison was evaluated using the Chi-square analysis [21].

Results

Figure 1 illustrates the effects of 1 (A) or 2 hemisections (B) on the nuclear status of oocytes incubated in 3 different conditions. As a positive control, a group of oocytes was incubated in common culture conditions without additional follicular cells, but with FSH, LH and estradiol as published previously [20]. No significant differences were observed among oocytes co-incubated with 1 or 2 half-follicles. As expected, however, all the oocytes cultured in the control conditions resumed meiosis. The addition of FSH or estradiol did not diminish the ability of the follicular shells to inhibit meiotic maturation. To verify if the meiotic arrest was reversible in these conditions, the oocytes thus treated underwent a second period of maturation for 24 h. A maturation medium with 2

Fig. 1. Nuclear status of bovine cumulus-enclosed oocytes (2 to 5 mm) co-cultured for 15 h with 1 (graph A) or 2 (graph B) follicular hemi-sections in three different media: with no hormones (no H), with estradiol only (E), or with FSH and estradiol (FE). For control, a group of oocytes was cultured in inhibiting media with FSH, LH and estradiol but without any hemi-section. The oocytes were classified as germinal vesicle (GV), intermediary—from GVBD to metaphase I included (int), or as mature—anaphase 1 to metaphase II, (mat). Different letters (a, b) indicate significant difference among stages (Chi-Square $P < 0.05$).

different hormonal supplement, LH and progesterone (LP) or FSH, LH and estradiol (FLE), was used to evaluate the meiotic process re-initiation (Fig. 2). When cumulus-enclosed oocytes were cultured with one hemi-section for 24 h, the maturation rate was above 80% in all the treatments (Fig. 2A). When comparing the hormonal effect on the reversibility rate during the second culturing period (maturation), no significant difference in inducing maturation was observed between FLE and LP. When 2 hemi-sections were used in the first incubation, a significant ($P < 0.05$)

Fig. 2. Nuclear status of bovine cumulus-enclosed oocytes (2 to 5 mm) cultured in maturation media containing FSH, LH and estradiol (FLE) or LH and progesterone (LP), following an initial 15 h period with 1 (graph A) or 2 (graph B) follicular hemi-sections in three different inhibiting media: with no hormones (no H), with estradiol only (E), or with FSH and estradiol (FE). The oocytes were classified as germinal vesicle (GV), intermediary—from GVBD to metaphase I included (int), or as mature—anaphase 1 to metaphase II, (mat). Different letters (a, b) indicate significant difference among stages (Chi-Square $P < 0.05$).

effect of the estradiol-alone treatment was carried over into the second incubation period where the estradiol-treated oocytes showed a lower reversibility rate of 57 or 66% in FLE or LP medium than did the oocytes incubated without hormones during the first incubation (80% in FLE or 85% in LP medium).

Table 1 shows embryonic stages resulting from the various *in vitro* oocyte culture treatment levels. When 1 hemi-section was used during the co-culture period, a significant improvement ($P < 0.05$) in development rates was observed in 4 of 6 groups,

Table 1. *In vitro* culture for 4 days of oocytes following delayed maturation with 1 or 2 follicular hemi-sections and *in vitro* fertilization

With 1 hemi-sectioned follicle					
oocyte maturation* 0–15 h	15–39 h	no. oocytes	cleavage (%)	≥8 cells (%)	nuclei/embryo ≥8 cells
control	–	74	53	13 ^a	11.3 ± 4.4
no H	FLE	51	63	43 ^b	12.8 ± 5.1
E	FLE	38	58	26	12.7 ± 3.7
FE	FLE	40	57	38 ^b	12.5 ± 5.0
total		129	59	36	12.7 ± 4.7
no H	LP	46	53	26	10.9 ± 3.5
E	LP	42	51	29 ^b	10.1 ± 2.9
FE	LP	35	65	33 ^b	12.2 ± 3.7
total		123	56	30	11.1 ± 3.4
With 2 hemi-sectioned follicles					
oocyte maturation* 0–15 h	15–39 h	no. oocytes	cleavage (%)	≥8 cells (%)	nuclei/embryo ≥8 cells
control	–	31	56	13	10.5 ± 3.8
no H	FLE	59	46	17	10.3 ± 2.7
E	FLE	58	45	17	11.8 ± 5.9
FE	FLE	66	56	23	11.9 ± 2.8
total		183	49	19 ^a	11.3 ± 3.8
no H	LP	53	46	13	11.6 ± 3.0
E	LP	58	50	7	8.4 ± 0.9
FE	LP	69	59	16	10.0 ± 2.8
total		180	52	12 ^b	10.3 ± 2.8

*Developmental capacity of bovine cumulus-enclosed oocytes following a first maturation period 0–15 h and a second maturation period of 15–39 h. During the first maturation period, oocytes were co-cultured with 1 or 2 follicular hemi-sections in media with no hormones (no H), with estradiol (E), or with FSH and estradiol (FE). During the maturation period, oocytes were cultured without hemi-sections in maturation media containing FSH, LH and estradiol (FLE), or LH and progesterone (LP). a, b: different letters indicate a significant difference (Chi-square, $P < 0.05$).

but the cleavage rate was not significantly affected. The oocytes co-incubated with a hemi-section developed to 8 cells at a higher rate (34%, 86/252) than those incubated without a hemi-section (13%, 10/74). The number of nuclei per an embryo reaching the ≥8-cell stage was not significantly different among the treatment groups. With 2 hemi-sections, no significant difference was found in the cleavage and developmental rate to the 8-cell stage. Within these groups, FLE used in the second maturation period resulted in a significantly higher developmental rate to 8 cells than LP ($P < 0.05$), (19%

vs 12%). The developmental rates obtained in this laboratory using the regular 24-h maturation (without a co-incubation period for 15 h) are 54% ($n=108$) for cleavage and 38% for the 8-cell stage with 12.7 nuclei per embryo (results not shown).

The progression to the 8-cell or more advanced stage at day 4 was a good indicator of the capability of further development, and thus, the previous experiment was repeated by using 231 oocytes that had been co-incubated with a hemi-sectioned follicle, and the cell number 6 days after insemination was assessed. Addition of LH-progesterone dur-

ing the second maturation period (15 to 39 h) resulted in a significantly advanced embryonic development ($P < 0.05$). Numbers of nuclei obtained from embryos beyond the 16-cell stage were not different among the groups.

Discussion

The results of this study indicated that bovine cumulus-enclosed oocytes can be maintained in meiotic arrest *in vitro* for an extended period of time (15 h) by co-incubation with a section of the follicular wall. Even though many studies have been published regarding meiotic inhibition in cattle, very few have provided data about the capability of the oocyte for further development (reversibility). A thorough literature review revealed that this research is the first to show developmental potential in bovine oocytes following meiotic arrest *in vitro*. This study, demonstrated the ability of follicular hemi-sections to inhibit meiotic progression in a reversible manner, though the rate was not ideally high. It also demonstrated that developmental competence was maintained to some extent following a prolonged maturation period.

Furthermore, this research indicated that both

gonadotropins and steroid hormones in the maturation medium influenced the development potential of oocytes. The most preferable combination was the presence of FSH and estradiol during the first 15 h and the presence of LH and progesterone during the following 24 h (Table 2). The effect of this combination was possibly related to the 2 physiological conditions during follicular growth when oocytes are in meiotic arrest during follicular maturation and progesterone rises in response to the LH surge [25]. This was an important finding because controlling meiotic resumption affects the opportunity to acquire viable embryos from the immature follicles of valuable females at slaughter. This study showed that people can indeed oocyte developmental competence *in vitro* which is an important step to study embryo development in a culture environment.

Studying the events during the last few days before ovulation *in vitro*, requires a culture system where the oocyte remains in meiotic arrest. The experiments on oocyte maturation conditions in this study indicated that the follicle in and of itself did influence the capacity of oocytes to develop further [26]. In mice, meiotic resumption, fertilization and embryo development, are acquired step-wise [27, 28]. If this rule applies to cattle, a smaller number of oocytes would develop when

Table 2. Developmental stages of bovine oocytes, co-incubated with a follicular shell, matured, fertilized and cultured for 6 days *in vitro*

maturation*		no oo	cleavage	≥8 cells	≥16 cells	nuclei/embryo at
0–15 h	15–39 h		(%)	(%)	(%)	≥16-cells stages
control	-	120	53	15 ^a	7 ^a	32.0 ± 18.7
no H	FLE	45	58	18	13	40.7 ± 26.7
E	FLE	37	55	22	8	31.3 ± 13.6
FE	FLE	33	55	24	18	42.0 ± 19.8
total		115	56	21	13	39.3 ± 20.9
no H	LP	41	55	29	19	34.5 ± 20.4
E	LP	40	44	25	15	35.0 ± 17.5
FE	LP	35	59	28	23	39.2 ± 27.9
total		116	52	27 ^b	19 ^b	36.4 ± 21.8

*Developmental capacity of bovine cumulus-enclosed oocytes (2 to 5 mm) following the first maturation period of 15 h and a second maturation period of 24 h. During the inhibition period (0 to 15 h), oocytes were co-cultured with 1 follicular hemi-section in media with no hormones (no H), with estradiol only (E), or with FSH and estradiol (FE). During the maturation period (15 to 39 h), oocytes were cultured without hemi-sections in maturation media containing FSH, LH and estradiol (FLE), or LH and progesterone (LP). a, b: different letters indicate a significant difference in individual columns (Chi-square, $P < 0.05$).

they originate from smaller follicles. In mice, steroid hormones do not influence GVBD competence of oocytes from 10-day-old animals cultured for 12 days *in vitro* [29]. In large animals, meiotic competence is acquired at a certain follicular size (cow: >1.6 mm, pig: >2.2 mm) [30]. When bovine immature oocytes are recovered from follicles of different sizes, their ability for further development increases according to the follicular size: 0% (<2 mm), 20.9% (2 to 4 mm) and 27.7% (4 to 8 mm) [31]. To assess the effect of steroid hormones on the oocytes within the follicle, Moor *et al.* [32] used dissected follicles from superovulated sheep and evaluated pronuclear formation after treatments with specific steroidogenic enzyme inhibitors. Their results indicated the beneficial effect of testosterone and estradiol in the first 16 h of maturation. Using follicle-enclosed oocytes in culture for 24 h with various amounts of FSH, LH and estradiol, Moor and Trounson [33] showed a beneficial effect from different combinations of gonadotropins and estradiol. Their results agreed with the results of this study showing that steroid hormones did influence the developmental competence in the presence of hemi-sections. Recently, Sirotkin [34] found that progesterone stimulated re-initiation of meiosis at doses from 1 to 5 $\mu\text{g/ml}$ and that antiserum to progesterone had an opposite effect. In this study, the presence of progesterone did not influence meiotic resumption during the second

maturation period but it was beneficial to further development (Table 2).

In conclusion, the results of this study indicated that it is possible to maintain bovine oocytes at the germinal vesicle stage *in vitro* for a period to induce the final maturation of the cytoplasm or to influence the nuclei that modulates developmental competence. In single-ovulation species, ovulation is an exceptional event and most follicles undergo atresia. Several different hormones and growth factors may be involved in the final processes of follicular maturation and these are likely to be related with oocytes developmental competence. In order to fully understand such processes it was necessary to develop a culture system in which both follicles and oocytes can be maintained in relatively defined conditions. The method described here can be used to study changes in developmental competence during late folliculogenesis, since viability can be assessed in measurable forms. Relatively low developmental competence might be a reflection of the comparative insufficiency of the culture conditions used in this study.

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