Influence of Follicular Wall on Meiotic Resumption of Bovine Oocytes When Cultured Inside or Outside Hemi-Sections

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Abstract. We evaluated the importance of the attachment of the oocyte to the granulosa layer, using co-culture of follicular hemi-sections with groups of 10 COCs (cumulus-oocyte complexes) or 10 DOs (denuded oocytes) and oocytes cultured within their own half-follicle. Various products acting on protein phosphorylation (IBMX, PMA, H-7) and cellular differentiation (estrous serum, FSH, LH or estradiol) involved in follicular and nuclear maturation were also used to evaluate their effect on the capacity of the follicular wall to inhibit meiosis resumption. When oocyte were cultured within their own half-follicles, none of the treatments used in this study could significantly reverse the inhibitory action of the follicular wall on the meiotic resumption. When the contact between the oocyte and the hemi-section was ruptured (co-culture of COCs and DOs), oocytes responded differently according to whether they were surrounded or not with their cumulus cells, and inhibition of meiosis was lowered particularly with LH and PMA with COCs. These results show that the communication junctions between the oocyte and the follicle are important for the inhibitory action of the follicle and this contact between both overcome the effect of any treatment applied.

Key words: Bovine, Follicle, *In vitro*, Meiosis, Oocyte

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In mammals, including the cow, the oocyte is blocked at the dictyate stage until a specific signal, the preovulatory surge of luteinizing hormone (LH), allows it to resume meiosis leading to a second meiotic arrest, the metaphase II [1]. *In vitro*, the isolation of the cumulus-oocyte complex (COCs) from its follicular environment induce the spontaneous resumption of meiosis [2, 3] while the follicle-enclosed oocyte remains in germinal vesicle stage (GV) [4]. It is thought that this may be due to the privation, for the oocyte, of inhibiting substances secreted by the follicular complex [1, 2].

Granulosa cells are responsible for the synthesis of factors implicated in the control of meiosis [6–9]. In mouse, using porcine follicular fluid, hypoxanthine has been identified as the principal maturation inhibitor [2]. This substance has also been detected in bovine follicular fluid [10], which partially inhibits GVBD of bovine oocytes [11]. Other purines may have a role in this inhibition and a suggested way by which all those substances maintain the meiotic arrest would be the inhibition of phosphodiesterase activity in the oocyte [12]. It is not clear across species if the cAMP is provided by the oocyte itself, the cumulus cells or the granulosa cells [13] or if the granulosa cells secrete a factor acting extracellularly [12].

The inhibitory influence of pig granulosa cells led to two hypothesis: Tsafiri and Channing [6] and Meinecke and Meinecke-Tillmann [14] suggest that it is mediated by the follicular fluid; in opposite, Sato and Ishibashi [7] and Leibfried and First [15] believe that an intimate contact of the COC with the follicular wall is necessary for the efficiency of the message. While there is controversy in the pig, there are evidences, for the hamster and the cow, that a close contact between the oocyte and the granulosa cells is important. In hamster, using the culture of whole follicles, Racowsky and Baldwin [16] observed that follicular fluid delays the onset of GVBD while the meiotic arrest is persistently maintained by a close contact between the granulosa cells and the COC. Bovine oocytes present a dose response to follicular fluid and granulosa cells suspensions, which both inhibit meiotic resumption. Their action is greatly increased by their combination and the attachment of the cumulus-oophorus to the granulosa cells in monolayer or in suspension [8, 9, 17].

Culture of sections of follicular walls or whole follicles have been used in various species [rat: 13, 18; rabbit: 19; pig: 4, 14; sheep: 20; cow: 4, 9] but few studies deal with the control mechanism of meiotic inhibition. In this paper, known agonist and antagonist of protein kinases involved in the modulation of the cell cycle and hormone involved *in vivo* with the follicular maturation were used to evaluate the reaction of follicular hemi-section in terms of their capacity to block the meiotic resumption. To assess the role of cell to cell contact, fresh follicular hemi-sections were cultured with their own oocyte or co-cultured with selected cumulus-oocytes complexes.

Materials and Methods

Recovery of oocytes

Ovaries at various stages in the estrus cycle were obtained immediately after slaughter of cows and transported to the laboratory at approximately 35°C in a saline solution (NaCl 0.9% containing: penicillin 100,000 UI, streptomycin 100 mg and amphotericin B 250 µg per liter; Sigma Chemical Co., St-Louis, MO). Oocytes surrounded by cumulus cells were aspirated from follicles ranging between 2 to 5 mm in diameter with a 18G needle

and were selected on the basis of cumulus appearance and homogenous cytoplasm; denuded oocytes (DOs) were obtained by vortexing the cumulus-oocyte complexes (COCs) in their follicular fluid. Oocytes were washed once in a Tyrode medium with Hepes (TLH) containing pyruvic acid 0.2 mmol (Sigma), 10% fetal calf serum (FCS, Flow Laboratories, Mc Lean, VA) and gentamicin 50 µg/ml (Sigma).

Preparation of hemi-sections and culture of oocytes within or with a half-follicle

Follicles were chosen randomly from ovaries obtained at a slaughterhouse and transported on ice in bags containing gauze impregnated with the saline solution described above. Follicles from 2 to 15 mm in diameter were dissected, cut in halves with a scalpel blade in Hank's solution supplemented with gentamicin (50 µg/ml) and amphotericin B (25 µg/ml) (Sigma) and then washed once in culture medium. For the culture of oocytes within the follicle, halves in which the oocyte remained attached to the follicle were kept and distributed individually in wells from multiwell (24) plates (Corning, NY), each containing 1 ml of culture medium. Co-culture of oocytes with a hemi-section were done as follows: a half-follicle (3–4 mm) was cultured with 10 COCs or 10 DOs per drop of 50 µl culture medium covered with mineral oil (Aldrich Chemicals, WI). Treatments consisted of : a) TCM-199 culture medium with Earle's salts (Gibco, NY), glutamine, bicarbonate (Sigma), supplemented with 10% fetal calf serum (Flow), pyruvic acid 0.2 mmol and gentamicin 50 µg/ml (Medium A, control medium); b) Medium A containing 10% estrous bovine serum (extracted in the laboratory) instead of fetal calf serum (medium EBS); c) Medium A supplemented with o-LH 5 µg/ml (National Institute of Diabetes and Digestive and Kidney Diseases, NIDDKD) (medium LH); d) Medium A supplemented with o-FSH 0.5 µg/ml, o-LH 5 µg/ml (NIDDKD) and estradiol-17β (E₂) 1 µg/ml (Sigma) (medium with hormones, H); e) Medium D supplemented with 3-isobutyl-1-methylxanthine 0.1 mmol (IBMX) (Sigma) (medium H+IBMX); f) Medium A supplemented with IBMX 0.1 mmol (medium IBMX); g) Medium A supplemented with 10 µmol of the PKC activator phorbol 12-myristate 13-acetate (Sigma) (medium PMA); h) Medium A supplemented with 50 µmol of the PKC inhibitor

[1-(5-isoquinolinesulfonyl)-2-methyl-piperazine, di-HCl] or H-7 (Calbiochem Co., CA) (medium H-7). Culture media were pre-incubated for at least 2 h under maturation conditions (38.5 C, 5% CO₂ and water saturated air). The oocytes were incubated for 24 h, then fixed and stained with aceto-orcein for microscopic evaluation ($\times 400$) of meiotic status.

Culture of oocytes with a half-follicle from superovulated cows

Height heifers with normal estrous cycle were superovulated with 4 mg FSH-P (Shering, Kenilworth, NJ) twice a day, for a total of either 4 injections (n=4) or 6 injections (n=4) at mid-cycle. Animals were killed at a slaughterhouse and some ovaries from non-treated animals were also randomly chosen at the same time to serve as control. Ovaries were transported at approximately 35 C in bags containing gauze impregnated with saline solution (same as above). Follicles (5–7 mm diameter) were dissected and co-culture of a hemi-section and 10 COCs was made as previously described. Treatments studied here were: a) TCM-199 culture medium with Earle's salts (Gibco), glutamine, bicarbonate (Sigma), supplemented with 10% fetal calf serum (Flow), pyruvic acid 0.2 mmol and gentamicin 50 μ g/ml (medium –); and b) medium a supplemented with o-LH 5 μ g/ml (NIDDKD) (medium +).

Data analysis

Oocytes were classified in 3 groups based on their nuclear status: germinal vesicle (GV), inter-

mediate (GV breakdown, early condensation and metaphase I) or mature (anaphase I, telophase I and metaphase II). Degenerated oocytes and those that became detached from their hemi-section during maturation were not included in the evaluation after final analysis showing no treatment effects. The significance of individual comparisons was evaluated using Chi-Square analysis with continuity correction (Chi-Square corr.).

Results

Influence of the follicular wall on nuclear maturation of bovine oocytes cultured within their half-follicle

Oocytes cultured within their follicular hemi-section were not significantly stimulated to resume meiosis by the treatments experimented in this study (Table 1). Only the addition of gonadotropins, estradiol-17 β and IBMX (treatment H + IBMX) showed a significant difference with the H-7 group, as evidenced by the number of oocytes reaching MII stage (38.6 vs 12.9% respectively). The fraction of oocytes remaining at the germinal vesicle stage was not significantly decreased by LH alone and H + IBMX versus the usual hormonal complement in oocytes culture ($P < 0.08$, H).

Influence of the follicular wall on nuclear maturation of bovine cumulus-oocyte complexes

The use of EBS instead of FCS (control) had no significant effect at any stage of nuclear maturation (Table 2). The presence of both hormones

Table 1. Influence of follicle cell-oocyte communication on nuclear maturation of oocytes cultured within their follicular hemi-section¹⁾

Treatment	n ²⁾	Stage		
		GV(%)	intermediate(%)	mature(%)
control	104	47(45.2)	33(31.7)	24(23.1)
EBS	36	18(50.0)	8(22.2)	10(27.8)
LH	34	11(32.4)	13(38.2)	10(29.4)
H	48	26(54.2)	11(22.9)	11(22.9)
H+IBMX	44	17(38.6)	10(22.7)	17 ^a (38.6)
IBMX	52	27(51.9)	14(26.9)	11(21.2)
PMA	34	14(41.2)	9(26.5)	11(32.4)
H-7	31	17(54.8)	10(32.3)	4 ^b (12.9)

¹⁾Hemi-sections were incubated with the various agents for 24 h. Data are expressed as number of oocytes. ²⁾Number of stained oocytes. a and b (mat.) show differences between treatments (Chi-Square corr. $P < 0.05$).

Table 2. Effect of the presence of follicular wall on nuclear maturation of cumulus-oocyte complexes¹⁾

Treatment	n ²⁾	Stage		
		GV(%)	intermediate(%)	mature(%)
control	49	13 ^a (26.5)	19(38.8)	17 ^c (34.7)
EBS	40	13 ^a (32.5)	11(27.5)	16 ^{cd} (40.0)
LH	37	6 ^a (16.2)	8(21.6)	23 ^{df} (62.2)
H	39	14(35.9)	13(33.3)	12 ^{ce} (30.8)
H+IBMX	38	21 ^b (55.3)	12(31.6)	5 ^e (13.2)
IBMX	36	13(36.1)	9(25.0)	14 ^{cd} (38.9)
PMA	59	11 ^a (18.6)	14(23.7)	34 ^{df} (57.6)
H-7	36	6 ^a (16.7)	11(30.6)	19 ^{cd} (52.8)

¹⁾Cumulus-oocyte complexes were incubated in the presence of one hemi-section (3-4 mm diameter) with the various agents for 24 h. Data are expressed as number of oocytes of at least 3 replicates using 10 cumulus-oocyte complexes per assay. ²⁾Number of stained oocytes. a and b (GV), c, d, e and f (mat.) show differences between treatments (Chi-Square corr. P<0.05).

Table 3. Effect of the presence of follicular wall on nuclear maturation of denuded oocytes¹⁾

Treatment	n ²⁾	Stage		
		GV(%)	intermediate(%)	mature(%)
control	49	5(10.2)	19 ^{ac} (38.8)	25 ^e (51.0)
EBS	38	3(7.9)	12 ^{ac} (31.6)	23 ^e (60.5)
LH	44	5(11.4)	33 ^b (75.0)	6 ^f (13.6)
H	41	7(17.1)	16 ^{ac} (39.0)	18 ^{eg} (43.9)
H+IBMX	40	4(10.0)	17 ^{acd} (42.5)	19 ^{eh} (47.5)
IBMX	31	2(6.5)	21 ^{bd} (67.7)	8 ^{gh} (25.8)
PMA	58	8(13.8)	15 ^c (25.9)	35 ^e (60.3)
H-7	36	9(25.0)	19 ^{ab} (52.8)	8 ^{fg} (22.2)

¹⁾Denuded oocytes were incubated in the presence of one hemi-section (3-4 mm diameter) with the various agents for 24 h. Data are expressed as number of oocytes of at least 3 replicates using 10 denuded oocytes per assay. ²⁾Number of stained oocytes. a, b, c and d (int.), e, f, g and h (mat.) show differences between treatments (Chi-Square corr. P<0.05).

(FSH, LH, E₂) and phosphodiesterase inhibitor IBMX had a synergistic inhibiting action on oocyte maturation. While individually added in the medium, these compounds blocked 35.9% (hormones, H) and 36.1% (IBMX) of oocytes at GV stage compared to 55.3% when used together (H+IBMX). All other treatments were significantly less inhibitory than treatment H+IBMX. COCs co-cultured with LH or PMA matured more than other groups (62.2 and 57.6% oocytes reached MII stage vs 34.7% for the control COCs).

Influence of the follicular wall on nuclear maturation of bovine denuded oocyte

The substitution of FCS (control) by EBS had no significant effect on nuclear maturation of denuded

oocytes (Table 3). None of the treatments could efficiently block meiosis resumption of denuded oocytes. Perhaps, media with LH, IBMX or H-7 were able to delay the onset of GVBD compared to control DOs since more oocytes were at the intermediate stage (75.0, 67.7, 52.8 and 38.8% for LH, IBMX, H-7 and control oocytes, respectively).

Effect of bovine superovulation on the capacity of follicular wall to influence nuclear maturation of cumulus-oocyte complexes

Hemi-sections from superovulated animals allowed COCs to reached mature stage, while oocytes cultured with follicular wall from non-treated cows could not pass the intermediate stage (Fig. 1). The presence or absence of LH in the medium

Fig. 1. Effect of bovine superovulation on the capacity of follicular wall to influence nuclear maturation of cumulus-oocyte complexes. Animals received 0 (Control), 4 or 6 injections of FSH. 10 COCs per drop were co-cultured with 1 hemi-section (5-7 mm diameter) with (+) or without (-) 50 μ g O-LH/ml. Data are expressed as percentages of oocytes that stayed in GV configuration or reached the mature stage. Different letters above bars show significant differences between treatment, within the same stage (Chi-Square corr. $P < 0.05$). The total number of oocytes/group is shown below each histogram category.

was much less influential than the source (superovulated or not) of the follicle. The progression from 2 days of FSH treatment to 3 days (6 injections) simply enhanced the difference with control follicles.

Discussion

The inhibiting action of follicular cells is potentiated if cumulus cells are present as in pig [15] and if the oocyte is attached to the granulosa cells in bovine [8, 17]. Although experimental conditions were not exactly the same between experiments with oocytes cultured within or in co-culture with a half-follicle, comparison of results presented in Tables 1 and 2 confirm these later assumptions as in all treatments studied here, except when hormones and IBMX together and H-7 were present, the ability of the hemi-section to block nuclear maturation was similar, i.e. GV rates from oocytes

cultured within their follicular hemi-section > COCs co-cultured with a hemi-section > DOs co-cultured with a hemi-section.

There are differences in the ability of serum to support the maturation process of oocytes, depending on the source or the time in the cycle it is collected [21, 22]. The higher maturation supporting capacity of EBS compared to FCS demonstrated by Younis *et al.* [22] may be a consequence of high concentrations of gonadotropins, especially luteinizing hormone [23]. It would be expected, then, that oocytes cultured in the presence of both products, EBS or LH (with FCS as serum supplementation), would mature at higher rates than control oocytes. If this was the case with COCs cultured with LH, the addition of EBS in the medium did not improve the maturation capacity of oocytes (Table 2). The presence of a hemi-section may hide the beneficial effect of EBS on oocyte maturation on the culture of COCs alone, as the follicular

wall, like serum, provides substances required for oocyte growth and maturation. Surprising results are those obtained with LH stimulation on the co-culture of denuded oocytes and follicular wall. While COCs matured up to 62.2%, nuclear maturation of DOs was delayed so that 75.0% of them were at an intermediate stage after 24 h of culture and only 13.6% matured to the final stage. A similar observation was made by Ball [24] where oocytes with high levels of cAMP were blocked at the metaphase 1 stage. This suggests that the maturing signal originating from granulosa cells and induced by LH must be more complex than a simple cAMP rise.

Cyclic AMP is known to be involved at various levels in the meiosis control of many mammals [12, 25-30]. In cattle, the oocyte has an active adenylate cyclase and may produce enough cAMP to block the maturation process, but increasing the concentration of this nucleotide in the cumulus cells may overcome the inhibitory level of oocyte cAMP [25]. One action of gonadotropins is to stimulate the production of cAMP by follicular cells [31]. In our experiment, the addition of hormones (LH, FSH and E_2) in the culture medium did not change the capacity of the hemi-section to inhibit nuclear maturation of oocytes cultured within its half-follicle. COCs and DOs in co-culture with follicular walls were also not affected by this treatment compared to control oocytes, but the presence of FSH in the medium delayed the maturation stimulation by LH on COCs when compared to LH treatment (Table 1). This is in agreement with results obtained by Olson and Hubbard [32] with hamster COCs who demonstrated that FSH, in the first 2 h of culture, was inhibitory and then stimulatory, and that maturation action of LH was less apparent when co-present with FSH.

Phosphodiesterase inhibitor IBMX has also been used in culture to prevent oocyte maturation, but this inhibitory substance is effective only for a short time in bovine [11]. The presence of IBMX, gonadotropins and estradiol-17 β in the culture medium was the most potent inhibitory treatment in maturation of COCs co-cultured with a hemi-section, while it had not significant effect on maturation of DOs and oocytes cultured within their half-follicle. IBMX alone had more effect on denuded oocytes to delay their nuclear maturation. This indicates that IBMX may prevent the degradation of intra-oocyte cAMP. Bilodeau *et al.* [25] found that, con-

trary to an increase in the concentration of the nucleotide in the cumulus cells or in the oocyte where it has opposite effects, when cAMP is elevated in both, it has virtually no effect on oocyte maturation. Our results confirm this assumption as less oocytes from DOs reached the maturation phase (MII) when cultured in presence of IBMX than COCs.

Another way to control meiosis resumption is by the calcium-dependent protein kinase C, which have been widely studied in *Xenopus* [33-35]. The tumour promoter PMA stimulates PKC activity that, in turn, induces GVBD in hormone-free medium [33, 34]. Data presented here failed to prove such activation in bovine oocytes. The addition of PKC inhibitor H-7 has not resulted in an increase in the capacity of the hemi-section to inhibit nuclear maturation, although it may delay it compared to PKC-inducer treatment PMA when oocytes were free of their cumulus cells.

Injections of gonadotropins to the cow a few days before slaughtering increase the number of follicles that enter into the growth phase and are in the course of ovulation [36]. Follicles over 5 mm diameter are thus believed to be growing and more capable to assure the maturation of oocytes and to respond to the presence of LH. Results in this study confirm a part of this assumption as oocytes co-cultured with hemi-sections from superovulated cows reached the mature stage for about 30-37% of them, while those from unstimulated animals failed to go further than the intermediate stage. Injections of FSH *in vivo* must have initiated cellular reactions that ended in production of factors allowing the resumption of meiosis. The releasing effect of granulosa cells could not be increased by addition of LH in the culture media.

In conclusion, our results show that keeping intact intercellular communication between the oocyte and the follicular wall is among the most important factors to control the meiotic inhibition process. Once these junctions are interrupted, oocyte will respond to culture conditions according to the treatment applied.

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