Inhibitory Effect of Bovine Follicular Fluid on In Vitro Maturation of Bovine Oocytes

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ABSTRACT

To investigate its inhibitory proper­ties, bovine follicular fluid was collected at different stages of the estrous cycle from small, medium, and large follicles. Follicular fluids were incubated with germinal vesicle stage, bovine oocytes aspirated from small follicles. Nuclear maturation was determined at 24 h. Bo­vine follicular fluid inhibited the sponta­neous maturation of bovine oocytes. The inhibition was reversed when follic­ular fluid was removed from oocyte cul­tures by 24 h. Follicular fluid varied in its ability to inhibit germinal vesicle breakdown according to follicle size and estrual stage.

Follicular fluid from both small and medium follicles at estrus had the greatest ability to prevent germinal vesicle breakdown but became less potent postestrus. Follicular fluid from large follicles at estrus had less ability to in­hibit germinal vesicle breakdown than fluid from small and medium follicles. Strong germinal vesicle breakdown inhibiting activity was present in large, probably atretic follicles at late metestrus, early diestrus, and mid diestrus. However, follicular fluid from large fol­licles had less germinal vesicle break­down inhibiting activity at proestrus than follicular fluid from large follicles at late metestrus, early diestrus, and mid diestrus. This proestrual activity was similar to that in Graafian follicles at estrus. (Key words: germinal vesicle break­down, follicular fluid, bovine oocytes, oocyte maturation inhibitor)

Abbreviation key: COC = cumulus oocyte complexes, DAPI = 4,6-diamidino-2-phenylin­dole, FF = follicular fluid, GV = germinal vesicle, GVBD = germinal vesicle breakdown, MII = metaphase II, OMI = oocyte maturation inhibitor.

INTRODUCTION

The nuclei of mammalian oocytes enter meiosis during fetal life, but the meiotic proc­ess becomes arrested at late prophase [germinal vesicle (GV) stage] before or shortly after birth (7). The nuclei in these oocytes undergo spontaneous reinitiation of maturation in vitro on removal from their follicular en­vironment (18), whereas the nuclei in those remaining in follicular fluid (FF) remain at the dictyate GV stage (3, 18).

The mechanisms underlying GV breakdown (GVBD) in the bovine oocyte differ from those in amphibian and rodent oocytes (11). Despite abundant data supporting the original findings of Chang (3)—that mammalian oo­cytes do not undergo spontaneous GVBD in FF—several laboratories failed to detect inhibitory activity in porcine (14, 19, 20) and bovine (14, 19) FF. In addition, earlier data supporting the inhibitory activity of bovine FF or of extracts of granulosa cells on oocyte maturation were derived using immature ham­ster and mouse oocytes (8, 21).

The objectives of this study were to deter­mine whether bovine FF was inhibitory for bovine oocyte maturation in vitro and whether bovine FF collected at different stages of the
estrous cycle from small (2 to 4 mm), medium (5 to 9 mm), and large (10 to 20 mm) follicles varied in its ability to inhibit GVBD in bovine oocytes from small follicles.

**MATERIALS AND METHODS**

**Collection of Bovine Ovaries**

Bovine ovaries were collected from non-pregnant cows immediately after commercial slaughter. The stage of the estrous cycle for each pair was estimated using criteria outlined by Zemjanis (31). Ovaries were transported from abattoir to laboratory within 4 h, and their temperature was maintained at 30 to 33°C.

**Collection of FF**

The FF was aspirated from small (2 to 4 mm), medium (5 to 9 mm), and large (10 to 20 mm) bovine follicles with a syringe and 20-gauge needle, pooled according to size and stage of cycle, and centrifuged at 3850 x g for 15 min. The supernatant fluid was frozen at -20°C until used.

**Maturation Medium**

Maturation medium consisted of Medium 199 with Earle's salts (catalog number M3769; Sigma Chemical Co., St. Louis, MO), 2.2 g/L of NaHCO₃, and 100 mg/L of L-glutamine (Sigma). The medium was supplemented with 10 µg/ml of FSH (Burns-Biotec, Omaha, NE), 1.5 µg/ml of estradiol-17β (Sigma), 75 µg/ml of streptomycin, 100 IU/ml of penicillin-G, and 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY). The medium contained phenol red (.2%, 200 µl/100 ml) as a pH indicator. The medium was adjusted to pH 7.4 and sterilized by filtering through a .2-µm filter (Millipore, Bedford, MA).

**Oocyte Maturation**

Follicular contents were aspirated from 2- to 4-mm follicles without regard to estrual stage and placed in 15-ml conical tubes in a 35 to 37°C water bath. Oocytes possessing a full cumulus mass, unfragmented cytoplasm, and intact zonae were harvested from the lower part of each tube using a low power (20 to 30x) stereoscope. Cumulus-enclosed oocytes were washed thrice in specified FF or maturation medium before culture. Oocytes (10 oocytes per .5-ml droplet of FF or maturation medium) were placed under oil and incubated at 37°C in an atmosphere of 5% CO₂ in air for 24 h. Oocytes were then either analyzed for maturation stage or transferred to maturation medium for an additional 24 h to determine viability.

**Evaluation of Oocyte Nuclear Maturation**

After the designated incubation period, oocytes not exposed to FF were placed in hyaluronidase (3 mg/ml in .9% NaCl; Sigma) for 15 min and stripped of cumulus cells by drawing the cumulus-oocyte complexes in and out of a pulled pipet with a diameter only a few microns larger than the oocytes. Oocytes, incubated in FF, had a tightly adherent cumulus mass, the removal of which required exposure to .05% collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN) in maturation medium for 15 min. These oocytes were then transferred to 1 mM EDTA in .9% NaCl to stop the collagenase reaction. Cumulus cells were then stripped by pipet.

After removal of cumulus cells, oocytes were fixed in 3% glutaraldehyde in .9% NaCl for 15 min at 24°C, rinsed in .9% NaCl, and incubated in .001% 4,6-diamidino-2-phenylindole (DAPI; Sigma), a fluorescent stain specific for nuclear material, for 40 min at 37°C. The oocytes were then rinsed in .9% NaCl to remove DAPI particles, mounted on slides, and covered with coverslips. The oocytes were evaluated for stage of nuclear maturation at 400x using an inverted microscope equipped with epifluorescent illumination and filters giving maximum transmittance at 405 nm.

**Experimental Design and Statistical Analysis**

Experiment 1 was designed to document that bovine FF had oocyte maturation inhibitory activity. Cumulus oocyte complexes (COC) from small follicles were incubated under identical conditions in FF or M199 for 24 h before analysis for meiotic status. A chi-
TABLE 1. Effect of bovine follicular fluid (FF) or maturation medium (M199) on 24-h bovine oocyte maturation in vitro.¹

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Oocytes at meiotic stage</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GV (%)</td>
<td>MI (%)</td>
</tr>
<tr>
<td>FF</td>
<td>44.6 A</td>
<td>37.0 A</td>
</tr>
<tr>
<td>M199</td>
<td>12.4 B</td>
<td>28.0 B</td>
</tr>
</tbody>
</table>

A,B Superscripts denote differences within columns at $P < .05$.

¹GV = Germinal vesicle; MI = metaphase I; MII = metaphase II.

Experiment 1: In Vitro Effect of Bovine FF on Maturation

The nuclear maturation of the bovine oocytes cultured in vitro for 24 h in FF or in maturation medium is presented in Table 1. Follicular fluid inhibited ($P < .01$) the resumption of meiosis. Of the oocytes cultured in FF, 44.6% remained at the GV stage, and only 1.8% reached the metaphase II (MII) stage. Of the oocytes cultured in maturation medium, only 12.4% remained at the GV stage, and 46.2% matured to MII. In the bovine, the GV stage is present from 0 to 6.6 h after in vitro culture; GVBD occurs at 6.6 to 8.0 h (23). Thus, bovine FF inhibited bovine oocyte maturation. This finding is in agreement with other reports about FF collected from rabbit (3), sheep (12), cow (1, 8, 22), hamster (12), pig (6, 26, 30), and human (9, 12) ovaries. Others (14, 19) could not find this inhibitory activity in FF from cattle and pigs.

Experiment 2: Effect of Removal of Bovine FF

To rule out the possibility that the oocytes were irreversibly damaged by FF, it was replaced with maturation medium after 24 h. Replacement of FF with maturation medium (Table 2) completely reversed inhibition ($P < .05$) as the percentage of GV oocytes decreased from 46.7 to 13.4% and as MII oocytes increased from 1.7 to 25.1%. This finding agreed with data from pig oocytes (25) showing that removal of pig FF from in vitro cultures by 20 to 24 h after initiation of culture reversed the arrest of porcine oocyte maturation.

Experiment 3: Effect of Bovine FF from Small, Medium, or Large Follicles

Analysis of bovine FF collected from small, medium, or large follicles without accounting for day of the estrous cycle revealed no differences ($P > .05$) in ability of FF to inhibit the resumption of meiosis (Table 3). In contrast, Stone et al. (25) reported that the strongest
TABLE 2. Effect of removal of bovine follicular fluid (FF) after 24 h on subsequent maturation\(^1\) of oocytes in maturation medium (M199).

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Oocytes at meiotic stage</th>
<th>(n)</th>
<th>(%)</th>
<th>SE</th>
<th>(%)</th>
<th>SE</th>
<th>(%)</th>
<th>SE</th>
<th>(%)</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF (24 h)</td>
<td>GV MI MII Degenerate</td>
<td>218</td>
<td>46.7</td>
<td>6.1(^A)</td>
<td>34.2</td>
<td>3.8</td>
<td>1.7</td>
<td>1.0(^A)</td>
<td>17.5</td>
<td>3.4(^A)</td>
</tr>
<tr>
<td>FF (24 h) then M199 (24 h)</td>
<td>176</td>
<td>13.4</td>
<td>.8(^B)</td>
<td>34.2</td>
<td>1.6</td>
<td>25.1</td>
<td>1.0(^B)</td>
<td>27.7</td>
<td>1.8(^B)</td>
<td></td>
</tr>
<tr>
<td>M199 (48 h)</td>
<td>75</td>
<td>11.0</td>
<td>1.9(^B)</td>
<td>26.9</td>
<td>8.7</td>
<td>46.2</td>
<td>10.6(^B)</td>
<td>16.0</td>
<td>.1(^A)</td>
<td></td>
</tr>
</tbody>
</table>

\(^A,B\)Superscripts denote differences within columns at \(P < .05\).

\(^1\)GV = Germinal vesicle; MI = metaphase I; MII = metaphase II.

Inhibition of pig oocyte maturation occurred with pig FF from small follicles. The next strongest activity was in pig FF from medium follicles, whereas pig FF from large follicles had insignificant inhibitory activity. In addition, pig granulosa cells taken from small pig follicles were 10 times more potent in inhibiting pig oocyte maturation than those harvested from large follicles (26). Therefore, the underlying regulatory mechanisms of bovine oocyte maturation differs markedly from the porcine.

**Experiment 4: Effect of FF Collected at Different Stages of Estrous Cycle**

Bovine FF collected at different estrous stages and from specific follicular sizes varied \((P < .006)\) in its ability to block GVBD of bovine oocytes. Thus, the cross-classified statistical model that accounted for all combinations of estrous stage and follicle size was highly accurate in explaining GVBD.

Except at estrus, the GVBD inhibition activity of FF of the same estrous stage did not differ \((P > .05)\) with follicle size (Table 4). However, large follicles had less \((P < .05)\) inhibition activity at estrus than small or medium follicles at estrus.

The FF from small and medium follicles at estrus through mid diestrus had more \((P < .05)\) GVBD inhibition activity than at early proestrus. The FF from small and medium follicles at proestrus tended to be the least potent in this size range in preventing GVBD.

The FF from large follicles at estrus (Graafian) and early proestrus had the least \((P < .05)\) GVBD inhibition activity. Resumption of meiosis in vivo is triggered by LH (2, 13). In vitro, the addition of LH can overcome the inhibition of meiosis in the presence of FF (8, 28, 29). Luteinizing hormone may act either by eliminating the inhibitory signal, by blocking its transfer via the cumulus cell-oocyte junctions, or by terminating oocyte maturation in-

TABLE 3. Effect of bovine follicular fluid (FF) from small, medium, and large follicles and maturation medium (M199) on bovine oocyte maturation\(^1\) in vitro at 24 h.

<table>
<thead>
<tr>
<th>Culture medium(^2)</th>
<th>Oocytes at meiotic stage</th>
<th>(n)</th>
<th>(%)</th>
<th>SE</th>
<th>(%)</th>
<th>SE</th>
<th>(%)</th>
<th>SE</th>
<th>(%)</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF</td>
<td>GV MI MII Degenerate</td>
<td>378</td>
<td>44.5</td>
<td>3.3(^A)</td>
<td>34.2</td>
<td>3.1</td>
<td>1.4</td>
<td>.7(^A)</td>
<td>20.0</td>
<td>1.6</td>
</tr>
<tr>
<td>From small follicles</td>
<td>343</td>
<td>42.9</td>
<td>2.7(^A)</td>
<td>39.8</td>
<td>1.9</td>
<td>1.3</td>
<td>.7(^A)</td>
<td>16.0</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>From large follicles</td>
<td>352</td>
<td>44.3</td>
<td>4.0(^A)</td>
<td>37.0</td>
<td>2.1</td>
<td>2.7</td>
<td>.6(^A)</td>
<td>16.0</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>M199</td>
<td>114</td>
<td>12.4</td>
<td>4.2(^B)</td>
<td>28.0</td>
<td>2.9</td>
<td>46.2</td>
<td>5.3(^B)</td>
<td>12.6</td>
<td>4.3</td>
<td></td>
</tr>
</tbody>
</table>

\(^A,B\)Superscripts denote differences within columns at \(P < .05\).

\(^1\)GV = Germinal vesicle; MI = metaphase I; MII = metaphase II.

\(^2\)Follicle diameter (millimeters): small, 2 to 4; medium, 5 to 9; and large, 10 to 20.
**TABLE 4. Effect of bovine follicular fluid (FF) collected from small, medium, and large follicles on inhibiting germinal vesicle (GV) breakdown of bovine oocytes.**

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Estrus</th>
<th>Late metestrus</th>
<th>Early diestrus</th>
<th>Mid-diestrus</th>
<th>Early proestrus</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF From small follicles</td>
<td>87</td>
<td>54.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71</td>
<td>47.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>74</td>
</tr>
<tr>
<td>From medium follicles</td>
<td>71</td>
<td>50.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66</td>
<td>44.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>70</td>
</tr>
<tr>
<td>From large follicles</td>
<td>60</td>
<td>34.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72</td>
<td>52.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75</td>
</tr>
</tbody>
</table>

<sup>a</sup><sup>b</sup>Superscripts denote differences within rows at \( P < .05 \).
<sup>c</sup><sup>b</sup>Superscripts denote differences within columns at \( P < .05 \).

1Follicle diameter (millimeters): small, 2 to 4; medium, 5 to 9; and large, 10 to 20.

However, we found that the most maturation inhibition activity in FF from the large follicles occurred at stages other than estrus. These follicles, if not atretic, will probably become atretic. This bovine inhibition activity might be different from that described as OMI in pigs.

**CONCLUSIONS**

Bovine FF inhibited the resumption of meiosis. The GVBD inhibition activity of bovine FF declined during the course of follicular development in small and medium follicles, although it was high in large, probably atretic, follicles. The GVBD inhibition activity was least in large follicles at estrus and early proestrus.

**REFERENCES**

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