Effect of Different Charged Groups of Cow Follicular Fluid Proteins on
*In Vitro* Oocyte Maturation

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ABSTRACT

Follicular fluid (FF) provides a very important microenvironment for the development of oocyte maturation, ovulation and fertilization. The objective of this study was to determine the effect of different charged groups of cow follicular fluid proteins on *in vitro* oocyte maturation (IVM) when added to cultured maturation medium. The follicular fluid were obtained from dominant follicle and precipitated by ammonium sulphate (30-50%). The precipitated protein was fractionated into acidic and basic fractions using Macro-Prep High S and Macro-Prep High Q columns, respectively. The fractionated proteins were used as media supplement in IVM medium which replace hormones. The *in vitro* maturation rates were comparable to those obtained with a positive control medium (TCM 199+ 10% Fetal Bovine Serum+ 10 mg/ml porcine Follicle Stimulating Hormone+ 5 mg/ml Luteinizing Hormone and 1 ng/ml Estradiol). The results showed that the proteins from basic fraction stimulated *in vitro* maturation rate of oocytes (79.3%) when compare to other groups (78.8%: crude FF and 66.3%: acidic fraction) but did not different with positive control (79.8%). Following cleavage rate, the protein from basic fraction was more efficient (67.1%) than crude FF (65.0%) and acidic fraction (62.5%) but less than control (71.4%). In conclusion, we were able to generate preliminary information on the effect of charged groups of follicular fluid proteins on oocytes maturation. However, the roles of individual proteins from the basic fraction will need more investigation for its future application.

Key words: charge, protein, cow, follicular fluid, oocyte maturation

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INTRODUCTION

In vitro production of cow embryos provides an excellent opportunity for cheap and abundant embryos for carrying out basic research and for the application of emerging biotechnologies like cloning and transgenesis. In vitro production of cow embryos comprises harvesting of good quality immature oocytes, maturing them in vitro in the laboratory, in vitro fertilization of matured oocytes and cultures of the embryos up to the transferable stages. The most important step in the laboratory embryo production is maturation of the follicle with enclosed immature oocytes which is arrested at the diplotene stage of prophase of the first meiotic division (Nandi et al., 2002).

Mammalian oocytes mature in follicular fluid (FF), surrounded by follicular cells. FF contains several proteins that derive from blood plasma or are secreted both by granulosa and theca cells (Nandedkar et al., 1992). Thus, the chemical composition of the FF might reflect the stage of oocyte development and the degree of follicular maturation. Active proteins are not only transcribed and translated in eukaryotic cells but also undergo posttranslational modification, including the addition of chemical function groups and/or proteolytic cleavage (Ducolomb et al., 2013). Therefore, the biochemical of FF surrounding the oocyte might be play critical role in the differentiation of follicle cells, rupture of follicle wall, determining oocyte quality for archiving fertilization, and embryo development. However, IVF and embryo production require culture media that mimic natural physiological condition.

As most of the molecular secreted into the follicle lumen are protein in nature, they can fractionated based on their binding property with different ion exchangers. In this study we report isolation and purification of cow follicular fluid protein and their biological effect on in vitro maturation and fertilization.

MATERIALS AND METHODS

1. Follicular fluid collection and isolation of FF proteins

Bovine FF was obtained by aspiration from large healthy follicles 15–17 mm in diameter. The follicles were preliminarily evaluated under a stereomicroscope as described above. Fluid samples from two or three follicles were pooled to prepare one batch of FF. Phenyl methyl sulfonyl fluoride (PMSF) was added (20 µg/ml) to FF. Then, FF was made cell-free by centrifugation at 4°C at 1,500×g for 30 minutes, the supernatant was filtered through a 0.45-µm membrane filter. The supernatant containing proteins was precipitated by ammonium sulfate (30%, 40%, 50% and 60%). After precipitation the protein samples were dialyzed in 50 mM Tris–HCl buffer (pH 7.0) to remove ammonium sulfate.
2. Ion exchange chromatography of FF proteins

To fractionate the basic proteins, dialyzed protein samples were loaded into a Macro-Prep High S support in a 5 ml column (Bio-Rad, USA) equilibrated in 10 mM Tris buffer (pH 7.0). The bound proteins were extensively washed using the equilibration buffer till the optical density of the washed buffer became less than 0.05 at 280 nm. The bound proteins were eluted from the Macro-Prep High S column in 10 mM Tris–HCl (pH 7.0) containing 350 mM NaCl. To purify the acidic FF proteins, unbound proteins from the Macro-Prep High S column were loaded into Macro-Prep High Q support in a 5 ml column (Bio-Rad, USA) column equilibrated in 10 mM Tris buffer pH 7.0. Bound proteins were also extensively washed in the same buffer till the O.D. of the buffer at 280 nm was less than 0.05. The bound proteins were eluted from the column in 10 mM Tris–HCl pH 7.0 containing 350 mM NaCl. The bound and unbound proteins were collected separately and dialyzed in phosphate buffer saline, quantified, and used to see the effect on in vitro maturation.

3. SDS-PAGE

Samples were analyzed according to Laemmli in reductive conditions (Laemmli, 1970). From each fraction 20 mg of protein was loaded into each lane. Electrophoresis was performed on a 12.5% acrylamide separation gel and a 4% stacking gel (Acrylamide/Bis 30%; 37.5:1; Bio-Rad) in a Mini-Protean Cell (Bio-Rad) at 200 V for 45 minutes; gels were stained with Coomassie blue. The protein bands were analyzed based on the standard proteins (Bio-Rad).

4. Oocyte collection, IVM, and IVF

Cattle ovaries were collected from the local abattoir and transported to the laboratory within 3–4 h. After dissection of extra adjacent tissues and thorough washing with pre warmed saline the visible surface follicles (3–5 mm diameter) were aspirated with a sterile 18 gauge hypodermic needle in a TCM-HEPES. The oocytes were washed 3 times with the TCM-HEPES medium. Cumulus oocyte complexes (COCs) having more than 5 layers of cumulus cells with homogeneous ooplasm were taken for in vitro maturation (IVM). The IVM medium was TCM-199 supplemented with 10% Fetal Bovine Serum + 10 mg/ml porcine Follicle Stimulating Hormone + 5 mg/ml Luteinizing Hormone and 1 ng/ml Estradiol. To evaluate the effect on oocyte maturation; the oocytes were sorted into the following 5 groups:

4.1 Group 1 (negative control), matured in IVM medium without hormones
4.2 Group 2 (positive control), matured in IVM medium with hormones
4.3 Group 3 (crude FF; CFF), matured in IVM medium without hormones supplemented with 10% crude FF
4.4 **Group 4** (basic fraction; BFF), matured in IVM medium without hormones supplemented with 10% basic fraction

4.5 **Group 5** (acidic fraction; AFF), matured in IVM medium without hormones supplemented with 10% acidic fraction

Oocytes matured in above experiments were fertilized *in vitro*. Frozen semen was thawed in a water bath at 37 °C for 30 s. The semen mixture was transferred to tubes containing 1 ml of sperm-TALP. After 1-h of a “swim-up” procedure at 38.5 °C under 5% CO₂ in air, approximately 700 ml of the upper layer of the medium was removed, pooled and centrifuged at 250 × g for 10 min. The semen pellet (approximately 100 ml) was diluted with an equal volume of the TALP medium (containing 10 µg/ml heparin; Sigma) and incubated for 15 min at 38.5 °C under 5% CO₂ in air. The suspension was then diluted with an equal volume of TALP medium. The sperm concentration was determined with a hemocytometer. After IVM, the COC were washed in fert-TALP, 1 mM sodium pyruvate, 10 mM hypotaurine, and 1 mM epinephrine. The sperm suspension was added to fertilization drops overlaid with mineral oil (final concentration, 1 × 10⁶ cells/ml). After co-incubation with sperm for 18–20 h at 38.5 °C in 5% CO₂ and high humidity, the oocytes were freed from cumulus cells by gentle pipetting, washed and placed in TCM 199 containing 20% FCS. At 48 h post insemination (hpi; d 0 = insemination; zygote stage), oocytes were evaluated for evidence of cleavage.

5. Assessment of IVM and IVF

At the end of the culture period, the maturation rate was processed according to the procedure of Nandi et al., 2002. Oocytes were examined for first polar body by identifying them in the perivitelline space after denuding the oocytes by repeated pipetting the COCs after culturing in different media. Cleavage was evaluated on day 2 after insemination (at 48 hpi).

6. Statistical analysis

All treatments were repeated 4–8 times. Results are expressed as mean (S.E.M.). One-way ANOVA followed by LSD test (SPSS Inc., Chicago, IL, USA) was used to analyze differences among groups. Differences were assumed to be significant at p < 0.05.

**RESULTS AND DISCUSSION**

1. Fractionated and SDS-PAGE of cow follicular fluid

The follicular fluid protein extracts were subjected to ammonium sulphate precipitation at following concentration: 0-30%, 30-40%, 40-50% and 60-70%. Each of precipitated protein fractions after dialysis was subjected to SDS-PAGE. From the SDS-PAGE profile of ammonium sulfate
precipitated proteins it was observed that most of the proteins were precipitated at 30–50% ammonium sulfate concentration (Figure 1).

**Figure 1** SDS-PAGE profile of native CFF protein precipitated by ammonium sulfate. (cFF: Cow follicle fluid, F1: 0–30% cut off protein pellet, F2: 30–40% cut off protein pellet, F3: 40–50% cut off protein pellet, F4: 50–60% cut off protein pellet, F5: 60–70% cut off protein pellet.

The eluted proteins from Macro-Prep High S support column were cationic (basic: BFF) in nature, those proteins which eluted from Macro-Prep High Q support column were anionic (acidic: AFF) in nature. The purified protein extracts were separated by SDS-PAGE by using 12% polyacrylamide gels and stained with Coomassie Brilliant BlueR-250 (Figure 2).

**Figure 2** SDS-PAGE profile of acidic (AFF) and basic protein (BFF) isolated by ion exchange chromatography.
2. Effect of different fraction of CFF on maturation and cleavage rate

The effect of CFF fractions on IVM and IVF is shown in Table 1. There were significant increases noticed in all groups compared with negative control (P<0.05). Supplementation of CFF and BFF increased the rate of in vitro maturation than AFF. There was no significant different in vitro maturation rate of CFF and BFF with the positive control. Regarding the functional effect on oocyte maturation, in the present study, the CFF and BFF groups had a similar response as positive control group. Cleavage rates for cow oocytes matured in the CFF, AFF and BFF groups also increased than negative control.

Table 1 Mean (± S.E.M.) effect of different fraction of cFF on maturation and cleavage rate.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>No. oocytes</th>
<th>Maturation rate (%)</th>
<th>Cleavage rate (%)</th>
<th>Cleavage rate/ maturation oocyte (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>86</td>
<td>53.5±2.0</td>
<td>34.9±1.2</td>
<td>65.2±1.2</td>
</tr>
<tr>
<td>Positive control</td>
<td>84</td>
<td>79.8±2.1</td>
<td>71.4±2.4</td>
<td>89.6±2.4*</td>
</tr>
<tr>
<td>CFF</td>
<td>80</td>
<td>78.8±3.2</td>
<td>65.0±2.9</td>
<td>82.5±3.0**</td>
</tr>
<tr>
<td>AFF</td>
<td>80</td>
<td>66.3±2.2</td>
<td>62.5±3.2</td>
<td>79.3±2.2**</td>
</tr>
<tr>
<td>BFF</td>
<td>82</td>
<td>79.3±3.0</td>
<td>67.1±3.7</td>
<td>84.6±3.7**</td>
</tr>
</tbody>
</table>

*Each treatment was replicate five times.

a Increase (P<0.05) with respect to negative control group

b Decrease (P<0.05) with respect to negative control group

In the past few years, the impact of diluted and pure FF on in vitro oocyte maturation, fertilization and embryonic development has been extensively studied in cattle (Ali et al., 2004), buffalo (Nandi et al., 2004), swine (Yoon et al., 2000), and horses (Boigh et al., 2002). Previous studies have demonstrated that both porcine and bovine FF inhibits oocyte nuclear maturation. This inhibitory effect is dependent on the size of the follicle from which the FF proceeds (Driancourt et al., 1998). FF from large follicles had a lesser inhibitory effect on oocyte maturation than fluid from small and medium follicles (Dostal et al., 1996). Thus, cow oocytes were matured in CFF from similar sized dominant follicles obtained from abattoir-derived ovaries in this study. Yoshida et al. (1992) reported that one or more acidic factors (in porcine FF) with a molecular weight between 10 and 200 kDa were responsible for oocyte maturation and developmental capacity. However, there are limited data on fractionated FF proteins and their effects on in vitro maturation in cow oocytes. To assess its effect, the cFF was fractionated in two charged groups by ion-exchange column (basic: BFF and acidic: AFF). Present results showed that BFF enhanced maturation and cleavage rate.
CONCLUSION

The results of present study demonstrated the presence of an oocytes stimulatory protein in follicular fluid of cow, which can be isolated (in partially purified form) by ion-chromatography of the protein fraction obtained by saturation (30-50%) with ammonium sulphate. The present study also able to generate preliminary information on the effect of charged groups of follicular fluid proteins on oocytes maturation. Further characterizations of basic protein fraction are essential to find molecules with positive effect on oocyte maturation that could be used as supplement in culture media.

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REFERENCES


