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Effect of Proline on First Polar Body Formation in Porcine Primary Oocyte

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ABSTRACT

Follicular fluid (FF) contains an oocyte maturation inhibitor with unknown chemical properties. This study was carried out to chemically define the factor(s) inhibiting cumulus cell denudation (CD) and first polar body formation (PBF). Porcine FF (PFF) was extracted with methanol and the extract was serially separated using gel filtration on Superose 12 and Superdex columns. A Superdex fraction was derived with PITC and analyzed with an amino acid analysis column. The results obtained are as follows; PFF had an activity inhibiting both CD and PBF of porcine primary oocytes. Superdex fractions RV2.11 prepared from PFF exhibited an activity inhibiting CD and PBF. By amino acid analysis, the fraction RV2.11 appeared to be proline having the same activity inhibiting CD and PBF. In conclusion, PFF had oocyte maturation inhibitors, of which proline should inhibit CD and PBF.

(Key words: Oocyte maturation inhibitor, Follicular fluid, Proline)

Ⅰ. INTRODUCTION

Growing mammalian oocytes explanted from their follicles undergo meiotic maturation spontaneously. However, when oocytes isolated from their follicles are co-cultured with follicular granulosa cells, granulosa cell extract or follicular fluid, their spontaneous maturation is inhibited (Tsafriri and Channing, 1975). That is, follicular fluid contains an oocyte maturation inhibitor (OMI). OMI concentration in FF decreased with an increasing follicle diameter (van de Wiel et al., 1983) and with progressing oocyte maturation and fertilization competence (Channing et al., 1983). The putative OMI activity declines with follicular maturation (Hillensjo et al. 1985). Winer-Sorgen et al. (1986) supported these results by suggesting that human oocytes recovered from follicular fluid containing high OMI activity tended to have lower fertilization rates *in vitro*.

The chemical property of OMI has been controversial. Some studies suggested that an OMI was a protein of approximately 60 kDa (Dostzbrl and Pavlok. 1996) or a peptide of about 8 kDa (Kadam and Koide, 1991). In particular, Kadam and Koide (1990) suggested that hypoxanthine is the predo-

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minant low Mr component of FF that inhibits mouse oocyte maturation. However, Kadam and Koide (1991) suggested that an OMI, which was isolated from bovine follicular fluid and purified by ultra-filtration through a PM-10 membrane, gel filtration on Sephadex G-25 column, gel permeation chromatography on Superose 12 column and reversed -phase HPLC on Partisil ODS-3 column, is a peptide with an estimated Mr of 8 kDa. However, Jagiello et al. (1977) and Daen et al. (1994) suggested that the estimated Mr of OMI is below 1 kDa. Downs and Eppig (1984) also insisted that the Mr of the porcine FF inhibitor is less than 1 kDa. Considering the chemical property of OMI, Downs and Eppig (1984) suggested that the OMI is a hydrophobic molecule but not a peptide or nonpolar lipid. In addition, Chari et al. (1983) suggested that the inhibition effect of OMI was not abolished by ether extraction, trypsin treatment, heating to 56℃ for one hour or boiling for five minutes, whereas heating to 105℃ for 18 hours decreased the effect.

Although many studies suggest that OMI had to be in follicular fluid, its chemical composition has not been clearly identifiable. Thus, this study was carried out to elucidate the factor(s) inhibiting cumulus cell denudation and first polar body formation of porcine primary oocyte using medium-sized follicles that is assumed to contain high OMI.

Ⅱ. MATERIALS AND METHODS

1. Follicular Fluid Preparation, Methanol Extraction and Ultra-filtration

Follicular fluid (FF) was aspirated from medium follicles (2～5mm in diameter) of porcine ovaries obtained at a local abattoir and centrifuged at 1000 × g for 30 minutes to recover a supernatant for OMI extraction. Five ml of FF prepared was slowly diluted with 25ml of methanol at 5℃, then added with 50ml of isooctane (Fetterolf et al. 1994). The mixed solution was stood for 30 minutes at 5℃ to be separated as two layers. A methanol layer was recovered and concentrated under N_2. The concentrate was filtrated with a filter membrane of 0.22 μm pore size and centrifuged at 10,000 × g for 30 minutes at 4℃ with an Ultrafree-DEAE$_{MC}$ Filter Unit (Sigma Chemical Co., St. Louis, Mo. USA) to recover the ultra-filtrate containing components less than 30KD.

2. Cumulus Oocyte Complexes (COCs) Preparation

COCs were collected from medium follicles and washed thrice with M199 (Gibco, St. Louis, Mo. USA) using the pipettes, of which the inner diameter was 200 ± 5 μm. COCs containing 4～5 cumulus layers (200±5 μm in diameter) were selected for the experiments.

3. Experimental Design

1) Experiment 1

COCs were incubated at 39℃ for 48 hours in M199 containing of 0, 10, 20 and 30% follicular fluid. Cumulus detachment (CD) and polar body formation (PBF) of oocytes were examined at the end of incubation.

2) Experiment 2

Ultra-filtrate was loaded onto a Superose 12 PC 3.2/30 (Pharmacia Biotech AB., Uppsala, Sweden) equipped to Smart system (Pharmacia Biotech AB., Uppsala, Sweden). Components of the ultra-filtrate were eluted with buffer S (150mM NaCl, 20mM HEPES, pH 7.0) at a flow rate of 40 μl/min and detected by UV monitor at OD_{280nm}. The collected fractions were concentrated with N_2 before further re-separation with Superdex Peptide PC 3.2/30 (Pharmacia Biotech AB., Uppsala, Sweden). The Superose fractions were loaded onto a Superdex Peptide PC

3.2/30 connected to the Smart system. The components were eluted with buffer S at a flow rate of 100 μl/min and detected by UV monitor at $OD_{280\ nm}$. The fractions were auto-collected and concentrated with N_2 before investigating the CD and PBF of the COCs, which were incubated at 39℃ for 48 hours (5% CO_2 in air) in M199 containing the Superdex fractions obtained from 20% follicular fluid.

3) Experiment 3

Superdex fraction was hydrolyzed with boiling HCl at 110℃ for 24 hours and labeled with the derivation solution, ethanol/DW/triethylamine/phenylisolthiocyanate (7/1/1/1, v/v) (Tarr, 1971). Using the Waters HPLC system equipped with a Pico-Tag Free Amino Acid Analysis column (3.9 × 300mm) that was previously equilibrated with buffer A (140 mM sodium acetate and 6% acetonitrile), a PITC -derived component was eluted with buffer B (60% acetonitrile) at 40℃ at a flow rate of 1000 μl/min and detected by UV monitor at OD_{254nm}.

In order to investigate the effects of the Superdex fraction component, proline on CD and PBF, the COCs were incubated at 39℃ for 48 hours (5% CO_2 in air) with 0 and 0.35mM of proline contained in modified Whitten's medium (Whitten, 1971; 87.67 mM NaCl, 4.83mM KCl, 1.72mM Ca-lactate $5H_2O$, 1.17mM $MgSO_47H_2O$, 1.18mM KH_2PO_4, 22.62mM $NaHCO_3$, 5.56mM glucose, 0.31mM Na-pyruvate, 22.20mM Na-lactate, 1mg/ml BSA).

4. Statistics

The data to be analyzed as mean ± SEM were obtained by four repetitions. The differences in CD and PBF among the various groups of incubated oocytes were statistically analyzed using the Duncan multiple test. A probability of $p<0.05$ was considered statistically significant.

Ⅲ. RESULTS

1. Effect of Follicular Fluid on Oocyte Maturation Inhibition

When COCs were cultured in a medium containing follicular fluid as much as 0 (Control), 10, 20, and 30%, CD rates were 90.1, 83.5, 63.6 and 61.7 %, respectively, and PBF rates were 57.9, 48.8, 29.7 and 29.3%, respectively. That is, the more follicular fluid added, the less the CD and PBF rates were. CD and PBF rates were significantly decreased in COCs cultured in a medium consisting of more than 20% follicular fluid (Table 1).

Table 1. Effect of follicle fluid (FF)[A] on cumulus cell denudation (CD) and first polar body formation (PBF) in porcine primary oocytes

FF volume (%)	No. of COCs [B] examined	% (Mean±SE) of oocytes with CD	% (Mean±SE) of oocytes with PBF
Control [C]	89	90.1 ± 4.3^{a}	57.9 ± 4.1^{a}
10 [D]	88	83.5 ± 8.4^{ab}	48.8 ± 1.3^{a}
20 [E]	87	63.6 ± 4.0^{bc}	29.7 ± 0.9^{b}
30 [F]	87	61.7 ± 2.6^{c}	29.3 ± 1.1^{b}

[A] Obtained from medium follicles of 2~5 mm in diameter.

[B] Recovered from medium follicles and were 195~205 μm in size with 4~5 cumulus cell layers.

[C,D,E,F] COCs were incubated in M199 (Control), M199 with 10, 20 or 30% follicular fluid, respectively.

Values bearing superscripts a, b, and c in the same column are significantly different ($p<0.05$).

2. Separation of Follicular Fluid Components

When follicular fluid extracted with methanol was separated with Superose 12 column, it showed six major peaks at retention volumes (RVs) of 1.86, 2.15, 2.35, 2.57, 2.79 and 3.35ml (Fig. 1). Of these Superose fractions, RV1.86 and RV2.15 fractions were re-separated using Superdex column. The Superose fraction RV1.86 contained some components related to four major peaks shown at RVs of 1.67, 1.75, 2.01 and 2.11ml (Fig. 2). However, the Superose fraction RV2.15 contained some components related to two major peaks shown at RVs of 2.01 and

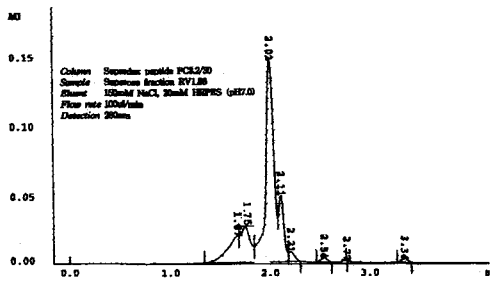

Fig. 2. Superdex fractions of Superose fraction RV1.86. The fraction RV1.86 had some components related to four major peaks shown at RVs of 1.67, 1.75, 2.01 and 2.11 ml.

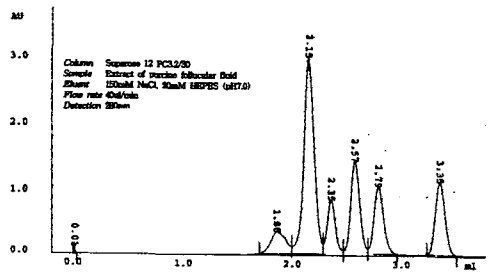

Fig. 1. Superose fractions of micro-filtered porcine follicular fluid (PFF). PFF had some components related to six major peaks shown at retention volumes (RVs) of 1.86, 2.15, 2.35, 2.57 2.79 and 3.35 ml.

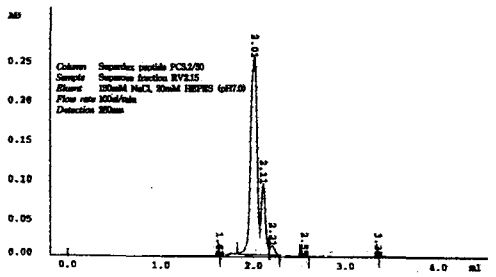

Fig. 3. Superdex fractions of Superose fraction RV2.15. The fraction RV2.15 had some components related to two major peaks shown at RVs of 2.01 and 2.11 ml.

Table 2. Effect of Superdex fractions on cumulus cell denudation (CD) and first polar body formation (PBF) in porcine primary oocytes

Superdex fractions	No. of COCs [A] examined	% (Mean±SE) of oocytes with CD	% (Mean±SE) of oocytes with PBF
Control [B]	75	91.6±1.8 [a]	51.3±1.9 [a]
RV1.67 [C]	75	89.8±2.2 [ab]	45.5±1.7 [ab]
RV1.75 [D]	73	82.2±2.8 [bc]	43.7±1.2 [b]
RV2.01 [E]	74	68.6±5.8 [c]	25.8±2.8 [c]
RV2.11 [F]	75	64.0±6.4 [d]	19.3±1.5 [d]

[A] Recovered from medium follicles and were 195~205 μm in size with 4~5 cumulus cell layers.

[B,C,D,E,F] COCs were incubated in M199 (Control), M199 with fractions RV1.67, 1.75, 2.01 and 2.11, respectively. Values bearing superscripts a, b, c and d in the same column are significantly different ($p<0.05$).

2.11ml (Fig. 3).

When COCs were incubated in M199 (Control), M199 containing Superdex fractions RV1.67, RV 1.75, RV2.01 and RV2.11, CD rates were 91.6, 89.8, 82.2, 68.6 and 64.0%, respectively and PBF rates were 51.3, 45.5, 43.7, 25.8 and 19.3%, respectively. In particular, Superdex fraction RV2.11 significantly decreased the CD and PBF rates of the COCs (Table 2).

3. Identification of CD and PBF Inhibitor in Follicular Fluid

An active fraction for oocyte maturation inhibition, Superdex fraction RV2.11 was fractionated with Pico-Tag Free Amino Acid Analysis column, and turned out to be proline (Fig. 4).

When COCs were incubated in modified Whitten's medium supplemented with 0 (Control) and 0.35 mM of proline, CD rates were 88.7 and 44.0%, and PBF rates were 54.3 and 18.6%, respectively. That is, proline significantly decreased the CD and PBF rates (Table 3).

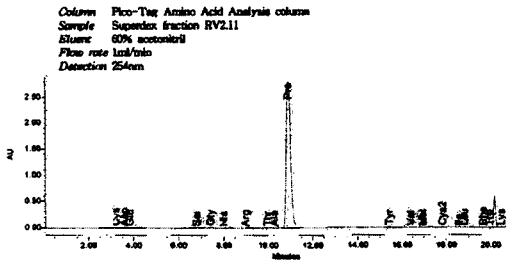

Fig. 4. Amino acid analysis of Superdex fraction RV2.11. The fraction RV2.11 had a major peak that is proline.

Ⅳ. DISCUSSION

Follicular fluid and inhibition of CD and PBF. CD and PBF rates were significantly decreased in COCs cultured in a medium consisting of more than 20% follicular fluid (Table 1). This result confirms our previous findings that porcine follicular fluid contains a component inhibiting oocyte maturation (Oh et al., 2000). Many studies report that follicular fluid inhibits oocyte meiosis and decreases oocyte maturation *in vitro* (Romero-Arredondo and Seidel, 1994; Vatzias and Hagen, 1999). Tsafriri and Channing (1975) suggested that granulosa cells are responsible for the maintenance of the oocytes in the dictyate stage by secretion of a chemical message into the follicular fluid. These data suggest that PFF should contain an oocyte maturation inhibitor (OMI).

Follicular fluid components and CD and PBF inhibitor. Superose fraction RV2.15 of methanol extract of follicular fluid significantly decreased CD and PBF. Of fractions of Superose fraction RV2.15, Superdex fraction RV2.11 also significantly decreased the CD and PBF rates of the COCs (Table 2).

Table 3. Effect of proline on cumulus cell denudation (CD) and first polar body formation (PBF) in porcine primary oocytes

Treatment	No. of COCs [A] examined	% (Mean±SE) of oocytes with CD	% (Mean±SE) of oocytes with PBF
Control[B]	131	88.7±1.6[a]	54.3±2.3[a]
Proline[C]	132	44.0±1.1[b]	18.6±2.7[b]

[A] recovered from medium follicles and were 195~205 μm in size with 4~5 cumulus cell layers.

[B,C] : COCs were incubated in modified Whitten's medium (Control) and modified Whitten's medium containing 0.35 mM proline, respectively.

Values bearing superscripts a and b in the same column are significantly different ($p<0.05$).

This result confirms the result of our previous study (Oh et al. 2000). Superdex fraction RV2.11 was turned out to be proline by fractionating with Pico-Tag Free Amino Acid Analysis column and by incubating with COCs to investigate CD and PBF rates. This result suggests that proline of follicular fluid may be a CD and PBF inhibitor.

Proline and inhibition of oocyte maturation. The proline activity may be explained by two assumptions. Firstly, *in vivo* development of antral oocytes requires amino acid uptake depending on cumulus cells (Colonna and Mangia, 1983). But, proline inhibits glycine uptake by follicular cells in a moderate manner (Haghighat and Van Winkle, 1990). Dotzbrl and Pavlok (1996) reported that, without compact cumulus cells, the inhibiting fraction of follicular fluid has no meiosis retarding effect on the oocytes. Considering these results, proline is assumed to inhibit oocyte maturation by inhibiting the transport of an amino acid required to oocyte maturation. Secondly, Mos degradation required for meiosis I of oocyte maturation is mediated primarily by the N-terminal Pro2-dependent ubiquitin pathway (Nishizawa et al. 1993). But, proline in follicular fluid (or *in vitro*) is assumed to inhibit the degradation of Mos by hindering the bonding between ubiquitin and proline of the N-end rule substrate (Mos)

In conclusion, porcine follicular fluid contains an inhibitor of cumulus denudation and first polar body formation, which is proline that may be a type of oocyte maturation inhibitors.

Ⅴ. 요 약

난포액에는 난자의 성숙을 억제하는 인자를 함유하고 있으나 화학적 성질이 정확히 알려져 있지 않다. 본 연구에서는 1차 난모세포에서 난구세포의 해리와 제1극체의 형성을 억제하는 난포액 성분을 화학적으로 동정하기 위하여 수행되었다. 이를 위하여 돼지 난포액을 methanol로 추출한 다음 이 추출물을 Superose 12 및 Superdex column을 이용 연속적으로 분리하였으며, Superdex 분절은 PITC로 처리한 다음 아미노산 분석용 column을 이용 분석하였다. 얻어진 결과는 다음과 같다.

난포액은 1차 난모세포에서 난구세포의 해리와 제1극체의 형성을 억제하였다. 난포액에서 추출 분리한 Superdex 분절 RV2.11 역시 난구세포의 해리와 제1극체의 형성을 억제하였다. 아미노산 분석 결과, 분절 RV2.11은 proline으로 추정되며, proline은 난구세포의 해리와 제1극체의 형성을 억제하였다.

결론적으로 난포액에는 난구세포의 해리와 제1극체의 형성을 억제하는 성분인 proline을 함유하고 있으며, 이는 난자의 성숙을 억제하는 성분일 것으로 추정된다.

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