Effect of Follicle-stimulating Hormone and Luteinizing Hormone on Zona Pellucida Gene Expression during Bovine Oocyte Maturation In Vitro

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Abstract: *Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) play important roles in bovine oocyte maturation. This study tested the effect of FSH and LH on bovine ZP gene expression during oocyte maturation in vitro with RNase protection assay. These gonadotropins had no effect on ZP gene expression in bovine oocytes cultured for 5 or 15 hours, but significant suppression of germinal vesicle breakdown (GVB) and metaphase II (MII) generation were observed with FSH. These data suggest that neither FSH nor LH has a significant effect on ZP gene expression in bovine oocytes. And the ZP gene expression could be independent of the mechanism of oocyte nuclear maturation in cattle.*

Key words: *Zona pellucida, In vitro maturation, Gonadotrophins, Gene expression, Cattle*

The zona pellucida, which plays an important role in fertilization, is a glycoprotein originates from oocytes [1, 2]. ZP gene expression in ovarian oocytes has been observed in the mouse [3, 4]. Its expression in bovine oocytes during maturation *in vitro* was recently reported [5]. In the process of bovine oocyte maturation, FSH and LH are important factors that affect its progress such as in GVB [6], polar body formation [7] and oocyte metabolism [8, 9]. The objective of this study was to find out the effect of LH and FSH on ZP gene expression during bovine oocyte maturation *in vitro*.

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Materials and Methods

Collection and culture of bovine cumulus-oocytes complexes

Cumulus-oocytes complexes (COCs) were collected by aspirating small follicles (−5 mm) in bovine ovaries obtained at a slaughterhouse. These complexes were cultured in 750 μl M199 (Gibco-BRL, Grand Island, NY, USA) with 5% calf serum and gonadotropin (0.1 IU/ml FSH (Denka Seiyaku, Kawasaki, Japan) or 0.01 U/ml LH (Denka Seiyaku, Kawasaki, Japan)) at 38.5°C in an atmosphere of 5% CO_2 and 95% air. After the culture, cumulus cells were removed in 1 mg/ml hyaluronidase (Sigma Chemical Co., St. Louis, MO, USA) in PBS(−). The denuded oocytes were washed 3 times to remove somatic cells completely by using PBS(−) with 1 mg/ml bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) and 10 oocytes were taken in a 500 μl microtube with minimum amount of medium. They were stored at −80°C until use.

Preparation of probe for quantification of gene expression

The intron region of ZPC [10] was amplified with an RNA-PCR kit (Perkin-Elmer, Foster City, CA, USA). The sequences of primers were as follows: 5'-CGTGCTCTAGGCTGTTCGTC-3' and 5'-CCCGACCGCAAACCTAACCA-3'. These generated 275 bp sequence with polymerase chain reaction (PCR). The size of the PCR product was confirmed with 5% polyacrylamide gel electrophoresis. The PCR product was cloned into pCR-SCRIPT with pCR-SCRIPT TM SK (+) (Stratagene, La Jolla, CA, USA). White colonies were selected and insert orientation and identity of plas-

mid were analyzed by ABI 373 DNA sequencer (Perkin-Elmer, Foster City, CA, USA). Plasmid with the correct insert was linealized with KpnI or NotI (TOYOBO, Osaka, Japan). Antisense RNA probe labeled with [α-^{32}P] CTP (Amersham Pharmacia Biotech UK Ltd, Bucks, UK), was generated from the linearlized plasmids with a MAXIscript kit (Ambion, Austin, TX, USA).

Quantification of gene expression with RNase protection assay

Gene expression in bovine oocytes was quantified with RNase protection Assay [11] with a Direct protect lysate ribonuclese protection kit (Ambion, Austin TX, USA). Namely, 20 μl lysis buffer was put into a microtube containing 10 bovine oocytes, and mixed quickly to make a cell lysate without RNA degradation. 1–5 $\times$ 10^4 cpm antisense RNA probe was added and hybridized overnight in a chamber at 50°C. After the hybridization, single strand RNAs were digested with an RNase cocktail. At this time, hybridized RNAs were protected from RNase digestion. The RNase digested sample was precipitated with isopropanol for electrophoresis with 6% urea-polyacrylamide gel. After running, the gels were dried and analyzed with a Bio-imaging analyzer (Fuji film, Tokyo, Japan). The gene expression was estimated as a percentage when the amount of gene expression in oocytes cultured without gonadotoropins was shown to be 100%.

Examination of oocyte maturation

Oocyte nuclear maturation was examined with a whole mount preparation fixed with Carnoy's fixative and stained with 1% orcein in 45% acetic acid [12].

Statistical analysis

Statistical analyses for gene expression and oocyte maturation were carried out by ANOV and χ^2 test, respectively.

Results and Discussion

The relative amounts of ZP gene expression in bovine oocytes cultured for 5 hours with FSH and LH were 88.0 ± 4.7 (mean ± SEM, n=4) and 105.5 ± 3.5% (mean ± SEM, n=4), respectively. These gonadotropins showed no effect on this gene expression (Fig. 1 (A)), but FSH suppressed the GVB significantly ($p<0.01$; Fig. 1 (B)). In 15 hour cultured bovine oocytes, these gonadotropins also showed no significant effect on ZP gene expression (Fig. 2 (A)); that is, the relative gene expressions for FSH and LH ware 105.3 ± 5.6 (mean ± SEM, n=4) and 89.8 ± 14.1% (mean ± SEM, n=4), respectively. Significant suppression of MII generation was observed in oocytes cultured for 15 hours with FSH ($p<0.01$; Fig. 2 (B)).

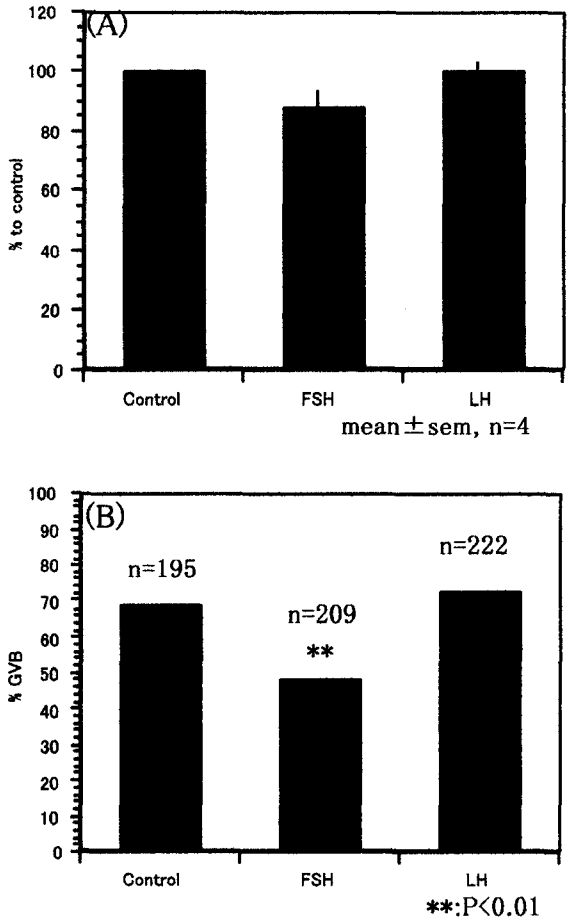

Fig. 1. Effect of FSH and LH on ZP gene expression (A) and GVB (B) in bovine oocytes at 5 hrs. of culture *in vitro*.

The relationships between gonadotropins and nuclear maturation have been studied in cattle oocytes. FSH triggers GVB of cumulus oocyte complexes connected to a piece of membrane granulosa (COCGs) [6]. LH enhances the speed of polar body extrusion in COCs when compared to FSH [7]. In this experiment, FSH suppressed GVB at 5 hours and generation of MII at 15 hours in COCs when compared to the control and LH, but no effect of gonadotropins on ZP gene expression was observed in cattle COCs. ZP protein synthesis and secretion is considered one of cytoplasmic maturation [13], which is defined as the ability to fertilize and develop to the blastocyst stage. This could be one of the reasons why gonadotropins have a different effect on

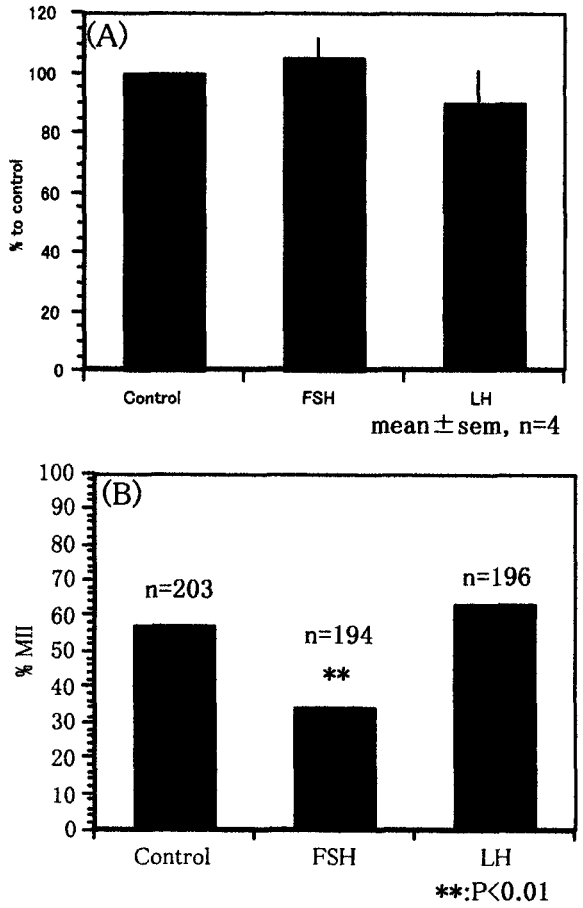

Fig. 2. Effect of FSH and LH on ZP gene expression (A) and MII generation (B) in bovine oocytes at 15 hrs. of culture *in vitro*.

ZP gene expression when compared to nuclear maturation in bovine oocytes.

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