Effects of MMP-2 activation and FSH or LH Hormone Supplementation on Embryo Development in *In Vitro* Fertilization of Porcine

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Abstract

The purpose of this study was to analyze whether FSH and LH hormone treatment directly or indirectly affect embryo development in embryonic development. To determine this, we compared the development of embryonic cells through the expression pattern of MMPs. As a result, 33.8% of blastocysts were formed in FSH added group, 20.8% in LH added group and 10% in FSH + LH added group. In addition, the activity of MMP-9 was highly detected in the FSH-added group, and the expression of Casp-3 was much lower than that of the other groups. These results suggest that the addition of FSH seems to increase the activity of MMP-9 in embryonic cells, and that LH, on the contrary, may activate MMP-2 activity. In addition, the expression level of MMP-2 in the FSH-added group was high in the Trophoblast cell group and in the LH-added group, the hormone ideal secretion might affect the development of the embryonic cell.

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INTRODUCTION

In vitro fertilization of pigs and development of embryos is a very important study in reproduction and production of transgenic animals (Abeydeera 2001). The study development of in vitro fertilization system of pigs has resulted in the production of many embryos (Yoshioka et al., 2001), but the production of in vitro fertilized eggs is still very lowly compared with the production of in vitro fertilized eggs (Kikuchi et al., 2008). Especially in the production of successful in vitro fertilized eggs, the action of FSH and LH hormone expressed in early oocyte maturation is very important. The role of FSH hormone is to regulate the activity of cumulus cells in the maturation of the oocyte, to affect the cytoplasm and nuclear maturation of the oocyte and to make a functional change in the hormone to form estrogen biosynthesis, it is important to play a role in the final differentiation of the oocyte (Hsueh et al., 2010). In particular, several studies have demonstrated that FSH plays a role in the maturation of early oocytes (development of cumulus cell and nuclear maturation) and in the development of cumulus cells (Roy and Greenwald 1989; Cain et al., 1995), and is known to act on the formation of primary follicles and preantral follicles, other studies did not improve the growth of preantral follicles (Li et al., 1995; Boland et al., 1993). The action of LH hormones is accommodated in granulosa cells and cavernosal cells from large antral follicles (Bao et al., 1997) and can play many roles in the development of follicles, but most studies focus on the physiological function of follicles. Regarding the maturation of oocytes, the important function of FSH and LH is that FSH affects the development of oocytes, but does not directly participate in nuclear maturation (Suss et al., 1988). In the end, hormones involved in the nuclear maturity of the oocytes can be seen to be formed by the action of the LH (Dominko et al., 1992). Studies by Keefer et al., 1993 have shown that the action of LH in the maturation of porcine oocytes will play an important role in the final blastocyst production. Previous studies have focused on the effects of FSH and LH on the maturation of oocytes, but there is little information on the effects of FSH and LH on development of embryos. Therefore, this study was conducted to investigate the effects of apoptosis and MMPs (Matrix metalloproteinases) and TIMPs (Tissue inhibitor of metalloproteinase) on embryo development in zygotes cultured on in vitro culture media supplemented with FSH or LH hormone.

MATERIALS AND METHODS

1. Oocytes maturation

Pig ovaries were collected from gilts at a local slaughterhouse and transported within 2 h to the laboratory in preincubated saline solution (with 100 IU/mL penicillin G and 100 µg/mL streptomycin) at 30 - 35 °C. Follicular fluid from follicles 3 to 6 mm in diameter was aspirated using an 18-gauge needle attached to a 10 ml disposable syringe. With the aid of a dissecting microscope, all naked oocytes, cumulus-oocyte complexes (COCs), and debris were removed from the aspirate and washed twice in phosphate buffered saline (PBS). Grade 1(oocytes with cumulus cells and good cytoplasm) oocytes in the cumulus - oocyte complexes were collected, and placed under a microscope after they were washed and prepared in Hepes-buffered tissue culture medium-199 (TCM-199; Gibco, MD, USA : 50 µg/mL gentamycin (SK chemical, Geyonggi, Korea), 0.3% (w/v) fatty acid free bovine serum albumin (BSA; Sigma, MO, USA)). After preparation of the oocytes in TCM-199, they were washed once with preincubated IVM medium (2.5 µg/mL FSH (Sigma, MO, USA), 1 µg/mL estradiol-17β (Sigma, MO, USA), 20ng/mL epidermal growth factor (Sigma, MO, USA) and 50 µg/mL gentamycin (SK chemical, Geyonggi, Korea) with 10%fetal bovine serum (FBS, Gibco, MD, USA)) and checked for the affinity of final oocytes and binding ability of cumulus cells. They were transferred into about 500 uL of IVM medium in a 4-well dish (20 to 25 oocytes in each well) and matured in vitro at 39°C in a 5% carbon dioxide incubator at 40 h.

2. In-vitro fertilization

After maturation for 24 h in vitro, small COCs were removed from mature medium (IVM-M199) and washed three times in TL-Hepes (Bio-Whittaker, Walkersville, MD, USA) and divided into 20-25 groups. Then wash three times in the in-vitro fertilization medium (BO : Brackett and Zuelke 1993) and transfer the mineral oil (Becton Dickinson, Franklin Lake, New Jersey, USA) to 50 µL of fertilized medium in a Petri dish. The dishes were kept at 5% CO₂ in air at 39 ° C. Sperm used for fertilization were washed twice by centrifugation at 453 x g for 8 minutes. Subsequently, sperm pellets were re-suspended in the appropriate sperm wash media to a volume of 250 µL. After final washing, sperm motility, and concentration was determined. Thirty microliters (30 µL) of the re-suspended sperm was added to each fertilization drop, giving a total concentration of 1×10^7 sperm/mL in each of the

IVF media. Oocytes were then incubated with the washed sperm for 6 h in 5% CO₂ in air at 39 °C.

3. In-vitro culture

At the completion of IVF, presumptive zygotes were denuded of remaining cumulus cells and loosely bound sperm, washed in NCSU-32, placed in 50 µl droplets of NCSU-32 (Four hormone treatment groups : 1) Hormones non-treatment group, 2) FSH (2.5IU/ml), 3) LH (2.5IU/ml), 4) FSH and LH (each : 2.5IU/ml)), and incubated in 6% CO₂, 5% O₂ and 89% N₂ at 38.5°C. On Day 4 of IVC, cleavage was assessed and the NCSU-32 was supplemented with 10% fetal bovine serum (FBS; heat inactivated, Australian origin; Gibco). Blastocyst formation was assessed on Day 7 of IVC.

4. Gelatin Zymography

Gelatinase activity was localized by in situ zymography following a previously described method (Khandoker et al., 2001; Nemori and Tachikawa 1999). Used GN film (Fuji Photo Film Co., Ltd, Tokyo, Japan) coated with a gelatin base emulsion was employed to detect and localize the gelatinase activity of the underlying tissue. Seven-micron-thick, unfixed ovarian cryosections were mounted onto the coated film, followed by incubation for 24 h at 37°C and staining with bearish scarlet (BS; Chroma-Gesellschaft, mbH & Co., Munster, Germany) and hematoxylin. Gelatinase activity was detected and localized when a specific pattern of gelatin digestion was indicated by a white color caused by the weaker staining of BS.

5. ELISA

For western blots and ELISA, total protein was extracted from ovarian tissues using Pro-Prep solution (Intron) according to the manufacturer's instructions. Total protein was quantified using a Bradford protein assay kit (Bio-Rad). For quantification of specific protein from the culture medium and cell proteins, samples diluted in assay buffer were used to coat a 96-well ELISA plate overnight at 4°C. The plate was then washed twice using washing buffer (1× PBS with 2.5% Triton X-100), and blocked using 1% BSA blocking solution at RT for 3 h. Primary antibodies (MMP-2 (ab78796, Abcam, Cambridge, UK), MMP-9(Santa Cruz Biotechnology Inc., Texas USA), TIMP-2 (sc-9905, Santa Cruz Biotechnology Inc., Texas USA), TIMP-3 (sc-6836, Santa Cruz Biotechnology Inc., Texas USA), Casp-3, PCNA, FSH-r and LH-r) were detected overnight at 4°C. After

washing, immune reactions were detected using secondary antibodies (anti-rabbit, (sc-2054, Santa Cruz Biotechnology Inc., Texas USA) and anti-mouse (sc-2054 and sc-2031, Santa Cruz Biotechnology Inc., Texas USA)) for 2 h, and substrate solution (BD) was added to initiate the reaction. The reaction was stopped with 1 M NH₂SO₄, and absorbency was measured at 450 nm.

6. Western Blot

Samples containing 30 µg of protein were separated by SDS-PAGE (in duplicate) on a 12% SDS-polyacrylamide gel and transferred to an Immuno-Blot PVDF membrane (Bio-Rad, CA, USA). The membrane was blocked using blocking buffer consisting of 3% BSA in TBST (0.1% Tween 20, 50 mM Tris-HCl (pH 7.6), 200 mM NaCl) for 3 h at RT. Following this, the membrane was washed 3 times for 15 min with washing buffer (TBST), followed by overnight incubation at 4°C with primary antibodies (TIMP-2, TIMP-3 and β-actin(Santa Cruz Biotechnology Inc., Texas, USA)) (diluted 1:1000 in blocking buffer). The membrane was then washed thrice with TBST buffer for 15 min each, and incubated for 2 h with HRP-conjugated secondary antibodies (diluted 1:5000 in blocking buffer). Detection was carried out using an ECL detection kit with 5 min incubation in a dark room. The detection reagent was drained, and the membrane was exposed to a sheet of diagnostic film in a film cassette for 1 to 30 min.

7. Immunofluorescence

We cultured samples (Blastocyst) on sterilized glass coverslips and fixed them with 4 % paraformaldehyde, followed by blocking with 0.1 % BSA in PBS. Dehydration and permeabilization were performed by freezing the slides at -20 °C in 5 mM 0.1 % Triton X-100 in PBS. After blocking with 3 % BSA in PBS, slides were incubated with an antibody against the active form of MMP-2, MMP-9, TIMP-2, and TIMP-3 at 1:150 dilutions. The slides were washed and incubated with anti-rabbit and anti-mouse IgG conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes). Nuclei were counterstained with 1 µg/mL Hoechst 33258, and slides were mounted using fluorescent mounting medium (Dako, Carpinteria, CA). Images were acquired using Olympus AX70 fluorescence microscope fitted with a CCD color camera.

8. Statistical analysis

Data were subjected to t-test and general linear model analysis

using the Statistical Analysis System (SAS Institute, version 9.4, Cary, NC, USA). Differences among treatments were determined by using Duncan's multiple range tests. The statistical significance was established at $p < 0.05$.

RESULTS

1. Development of blastocysts and expression of MMPs by hormone addition during in vitro culture

The results of analysis of embryonic development and MMPs expression in FSH, LH and FSH + LH supplemented groups after in vitro fertilization are the same as those of Table 1. In our results, the development of more than 4 cells of total embryos was $17.1 \pm 2.1\%$ in FSH treated group, $12.8 \pm 1.8\%$ in LH and $7 \pm 1.1\%$ in FSH + LH. The development of blastocysts according to the above results was $33.8 \pm 2.1\%$ in FSH group, which was the highest ($p < 0.05$). The concentration of MMPs was significantly

higher in FSH + LH ($53.43 \pm 10.1 \mu\text{l/ml}$) in MMP-2 and $38.37 \pm 3.3 \mu\text{l/ml}$ in LH, the lowest in hormone group. MMP-9 was higher in the LH group ($75.24 \pm 7.3 \mu\text{l/ml}$) than the other groups, but not significantly different from the FSH group.

2. Expression of MMPs and TIMPs in embryos

The results of analysis of MMPs and TIMPs in mature embryos in each hormone treated group are shown in Figure 1. Expression of MMPs was similar to that of Table 1. However, the activity of MMPs was slightly different, and the activity of MMP-9 was confirmed in all treatment groups except FSH + LH group. However, the activity of MMP-2 was the lowest in the control group and very high in the LH-treated group. In the case of TIMPs which are inhibitors of MMPs, TIMP-3, which is an inhibitor of MMP-9, was significantly higher in FSH + LH group and TIMP-2 was relatively higher in control group and very low in LH group.

Table 1. Mean (%) fertilization rates and embryo development on the IVC media

Treatment	No. of oocyte	No. of fertilized Ova.	No. of embryos (%)		MMPs of medium (ug/ml)	
			Cleavage(4cell<)	Blastocyst	MMP2	MMP9
NCSU-32	500	340	140($41.2 \pm 1.2\%$) ^a	85($60.7 \pm 1.2\%$) ^a	28.24 ± 11.2^d	63.78 ± 9.5^c
FSH(2.5IU/ml)	500	380	65($17.1 \pm 2.1\%$) ^b	22($33.8 \pm 2.1\%$) ^b	42.21 ± 9.2^b	73.68 ± 7.3^b
NCSU-32 LH(2.5IU/ml)	500	375	48($12.8 \pm 1.8\%$) ^c	10($20.8 \pm 1.8\%$) ^c	38.37 ± 3.3^c	75.24 ± 7.3^a
FSH+LH(5IU/ml)	500	300	20($7 \pm 1.1\%$) ^d	2($10 \pm 1.1\%$) ^d	53.43 ± 10.1^a	32.58 ± 1.4^d

*FSH : Follicle stimulation hormone, LH : Luteinizing Hormone, MMPs : Matrix metalloproteinases.

Figure 1. Expression of activation MMPs and TIMPs. A : Zymography, B : Western blot, C : ELISA analysis, 1) Hormone non-treat group, 2) FSH group, 3) LH group, 4) FSH+LH group. ^{a,b,c,d} Different letters within the same column represent a significant difference ($p < 0.05$).

3. Expression of Casp-3, PCNA and hormone-receptor in embryos

In the present study, expression of Casp-3 was significantly higher in the LH group than in the other treatment groups, and PCNA was highly expressed in the FSH group. FSH-receptor was significantly higher in FSH group, but it gradually decreased to LH group and FSH + LH group, but it was found that FSH + LH group was gradually expressed in LH-receptor group (Figure 2).

4. Expression of MMP-2 and Casp-3 in blastocysts developed in each hormone-treated group

The expression of MMP-2 and Casp-3 in blastocysts developed in each hormone-treated group is shown in Figure 3. As a result, the expression of MMP-2 was found to be expressed around the trophoblast cell of the blastocyst. In the control group not added to the hormone, the expression of MMP-2 was very low, but the expression of MMP-2 was high in the hormone-added control group. In the FSH group, expression was higher in the periphery

of the blastocyst but in the LH group and FSH + LH group, the expression was higher in the inner cell mess section. Expression patterns of Casp-3 were also similar to those of MMP-2 expression. In the inner cell mess of LH and FSH + LH, expression was higher than that of the other groups.

DISCUSSION

The purpose of this study was to analyze the effects of hormone addition on the blastocyst development during in vitro maturation of pig embryos. In the development of porcine oocytes, according to Anderiesz et al., 2000, the combination of LH and FSH suggests that LH and FSH-induced effects on the oocyte nutrition and appropriate embryonic developmental environment control the intercellular signaling pathways. It can improve embryo development. Cortvrindt et al., 1998 also reported that FSH and LH hormones affect the ability of the oocyte to synthesize proteins and affect cell differentiation (Moor et al., 1985). However, the mechanism by which the addition of FSH

Figure 2. ELISA assay for Casp-3, PCNA, FSH-r and LH-r protein expression in porcine embryo. ^{a,b,c,d}Different letters within the same column represent a significant difference ($p < 0.05$).

Figure 3. Immunofluorescence analysis of MMPs and TIMPs proteins in porcine embryo. A-1) Hormone non-treat group, A-2) FSH group, A-3) LH group, A-4) FSH+LH group.

and LH plays a direct role in embryonic development is unknown. In our study, we observed that the incidence of blastocysts was significantly different in the in vitro culture of FSH, LH, FSH and LH in NCSU-32 culture medium. This suggests that the addition of hormones does not play a positive role in embryo development and is different from the results presented by Anderiesz et al., 2000. In particular, the effects of MMPs on the development of embryonic cells were analyzed, suggesting that LH stimulation expresses both increased MMPs and increased Casp-3 in embryonic cells. However, the results of FSH were different. The expression of MMP-2 was found in the trophoblast cell and the expression of Casp-3 was significantly lower than that of the other treatment groups. These results are similar to the results of Wen-Jui et al., 2015, which shows that embryonic development affects embryonic development depending on the presence or absence of MMPs. In addition, as in the study of Imai et al., 2002, as the expression of MMPs in the cytoplasm has a positive effect on cell division, the differences in the expression and activity of MMPs in this study may clearly affect embryo development. However, it is not known exactly how FSH and LH hormone affect the expression mechanism of MMP-2 and MMP-9, and it cannot be concluded that it directly affects embryo development. However, this study suggests that the

treatment of FSH and LH may have some effect on embryonic development. Therefore, this study will be an important basic data on the effect of inappropriate secretion of hormone on embryo development.

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