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Treatment of Exogenous GDF9 and BMP15 during *In Vitro* Maturation of Oocytes increases the Cell Number of Blastocysts in Pigs

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ABSTRACT

Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are oocyte-specific growth factors that regulate many critical processes involved in early folliculogenesis and oocyte maturation. In this study, effects of GDF9 and BMP15 treatment during *in vitro* maturation of porcine oocytes upon development after parthenogenetic activation were investigated. Neither GDF, BMP15 alone nor in combination affects the number and viability of cumulus cells or the rates of oocyte maturation and blastocyst development. However, the treatment of GDF9 on porcine oocytes increased the number of trophectodermal (TE) cells of blastocysts derived from activated oocytes ($P<0.05$). The treatment of BMP15 increased the cell numbers of both inner cell mass (ICM) and TE cells ($P<0.05$). The treatment with the combination of GDF9 and BMP15 further increased the numbers of ICM and TE cells, compared with GDF9 or BMP15 treatment alone ($P<0.05$). In conclusion, the treatment of GDF9 or BMP15 (or both) enhanced the quality of blastocysts via the increased number of ICM and/or TE cells.

(Key words: GDF9, BMP15, oocyte, blastocyst, pig)

INTRODUCTION

Oocytes are matured through mutual interaction of oocytes and their surrounding follicular cells which include granulosa and cumulus cells. When ovarian follicles grow and mature, paracrine growth factors secreted from oocytes play a crucial role. These oocyte-secreted factors (OSF) regulate differentiation of granulosa cells, separating them into two distinct sub-types, cumulus and mural granulosa cells, which become phenotypically and functionally distinct from each other (Eppig *et al.*, 1997; Li *et al.*, 2000). Such regulation eventually affects maturation of oocytes and later development of preimplantation embryos. Among these factors, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) regulate many critical processes involved in early folliculogenesis and oocyte maturation. Both have important roles in maturation of oocytes by the functions that GDF9 reduces apoptosis and BMP15 promotes proliferation of granulosa cells (Eppig, 2001; Hussein *et al.,* 2005).

Culture medium to support *in vitro* maturation (IVM) of porcine oocytes often includes supplements such as epidermal growth factor, L-cysteine, and porcine follicular fluid that are known to be beneficial for meiotic maturation of oocytes (Abeydeera *et al.*, 1998). However, the developmental ability of oocytes matured in such media is still lower than that of oocytes matured *in vivo* and needs to be improved (Moor *et al.*, 2001). Therefore, it would be important to study on underlying mechanism of IVM as well as improvement of techniques to produce developmentally competent oocytes. In particular, the beneficial effects of GDF9 and BMP15 on increasing the rates of cleavage and blastocyst development were observed when they were treated in combination during *in vitro* maturation of porcine oocytes (Lin *et al.*, 2014). However, whether the treatment improves the quality of blastocyst has not been assessed.

In the present study, the effects of recombinant GDF9 and BMP15 treatment during IVM of porcine oocytes upon development both in quantity and quality were investigated.

MATERIALS AND METHODS

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1. Animal Ethics

All procedures in this study were carried out in accordance with the Code of Practice for the Care and Use of Animals for Scientific Purposes and approved by the Institutional Animal Care and Use Committee, Dankook University.

2. Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise.

3. *In Vitro* Maturation of Oocytes

Porcine ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in a warm box (25～30℃) within 2 h. Follicular fluid and cumulus-oocyte complexes (COC) from follicles (5～6 mm in diameter) were aspirated using an 18-gauge needle attached to a 5-ml disposable syringe. Compact COC were selected and washed five times in HEPES-buffered tissue culture medium (TCM)-199 (Invitrogen, Carlsbad, CA, USA). *In vitro* maturation medium was TCM-199 supplemented with 10 ng/ml of epidermal growth factor, 10 IU/ml equine chorionic gonadotropin (eCG; Intervet, Boxmeer, The Netherlands), 10 IU/ml human chorionic gonadotropin (hCG; Intervet), and 10% (v/v) porcine follicular fluid. A group of 50 COC was cultured in 500 μL of IVM medium at 39℃ in a humidified atmosphere of 5% CO_2 in air. After culturing for 20 h, the COC were transferred to eCG- and hCG-free IVM medium and cultured for another 20 h. During 40 h of IVM, 200 ng/ml recombinant human GDF9 or 200 ng/ml recombinant human BMP15 (or both) were added to the IVM medium.

4. Viability Test of Cumulus cells

After 40 h of IVM, cumulus cells were removed from oocytes by repeated pipetting in the IVM medium containing 0.5 mg/ml hyaluronidase for 1 min. Cumulus cells collected from individual oocytes were counted and stained with 0.4% trypan blue to test the viability.

5. Parthenogenetic Activation of Oocytes

The denuded oocytes with clearly extruded polar bodies were considered to be reached to the matured second metaphase (MII) stage and used for subsequent activation and culture. Matured oocytes were transferred to 0.3 M mannitol solution containing 0.5 mM HEPES, 0.05 mM $CaCl_2$, and 0.1 mM $MgCl_2$ in a chamber containing two electrodes. Using BTX Electro-Cell Manipulator 2001 (Harvard Apparatus, Holliston, MA, USA), oocytes were activated with a double DC pulse of 1.5 kV/cm for 45 μsec. Activated oocytes were cultured for 4 days in porcine zygote medium-3 (PZM-3) containing 4 mg/ml fatty acid-free BSA and transferred to PZM-3 containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and cultured for another 4 days. All embryos were cultured at 39℃ in humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Cleavage and blastocyst formation were evaluated on days 2 and 8 after the activation, respectively.

6. Differential Staining

In blastocysts, the numbers of inner cell mass (ICM) and trophectoderm (TE) cells were determined using differential nuclei staining as described previously (Papaioannou *et al.*, 1988). Briefly, blastocysts on day 8 after activation were transferred to 100 μg/ml propidium iodide (PI) with 0.5% Triton X-100 in phosphate-buffered saline (PBS). Blastocysts were then stained overnight with Hoechst 33342 in ethanol at 4℃. Embryos were then washed in 100% ethanol and mounted in a glycerol drop on a glass slide. Observation was performed under Nikon TE-300 inverted microscope (Nikon Corporation, Tokyo, Japan) equipped with epifluorescence based on ICM and TE cells appeared to be blue and pink, respectively.

7. Statistical Analysis

Statistical analyses were carried out using SPSS version 11.0 for Windows. At least three replicates were conducted for each experiment. Percentage data were subjected to arcsine transformation prior to statistical analysis. All data were analyzed by one-way ANOVA. A value of $P<0.05$ was considered significant.

RESULTS

None of the treatments including GDF9 or BMP15 (or both) affects the number and viability of cumulus cells (Table 1). Similarly, none of the treatments promote the rates of oocyte maturation, cleavage and blastocyst formation from activated oocytes (Table 2). As shown in Table 3, however, GDF9 treatment significantly increased ($P<0.05$) the number of total cells in blastocysts due to an increased number of TE cells. The numbers of both ICM and TE cells were significantly increased ($P<0.05$) by the treatment of BMP15. Combined treatment of

Table 1. Effect of GDF9 and BMP15 on the number and viability of cumulus cells

Treatment	Average no. of cells	Viability (%)
Control	7.31×10^4	66.7±2.5
GDF9	6.82×10^4	68.8±3.1
BMP15	6.45×10^4	68.7±2.5
GDF9 + BMP15	6.02×10^4	69.8±2.9

Table 2. Effect of GDF9 and BMP15 on maturation and development of oocytes

Treatment	No. of oocytes	% maturation*	% cleavage**	% blastocysts**
Control	461	73.3±1.7	83.1±6.6	43.8±5.5
GDF9	462	72.1±1.0	87.4±5.1	44.0±4.4
BMP15	462	74.6±4.3	85.5±7.4	45.8±4.8
GDF9 + BMP15	461	74.4±4.7	87.5±6.0	47.3±4.7

* Calculated from the number of oocytes.
** Calculated from the number of matured oocytes.

Table 3. Effect of GDF9 and BMP15 on the number of cells in blastocysts

Treatment	No. of ICM cells	No. of TE cells	No. of total cells
Control	8.4±3.2[a]	68.1±17.1[a]	76.9±21.2[a]
GDF9	9.1±2.7[a]	73.4±19.7[b]	82.5±23.4[b]
BMP15	9.4±3.2[b]	74.6±17.9[b]	84.1±22.1[b]
GDF9 + BMP15	10.2±3.0[c]	77.5±18.0[c]	87.7±22.5[c]

[a,b] Values with different superscripts within the same column differ significantly ($P<0.05$).

GDF9 and BMP15 further increased ($P<0.05$) the number of cells in blastocysts by increasing both ICM and TE cells.

DISCUSSION

Oocyte-secreted factors GDF9 and BMP15 regulate many critical processes involved in early folliculogenesis (Hussein *et al.*, 2006). They promote follicle growth beyond the primary stage to reach MII stage by regulating proliferation and apoptosis of granulosa cells (Huang and Dagan, 2010). In the present study, effects of recombinant human GDF9 and BMP15 treatment during IVM of porcine oocytes upon development after parthenogenetic activation were investigated.

As shown in Table 1, neither GDF9 nor BMP15 affected the number and viability of cumulus cells. Combined treatment of GDF9 and BMP15 did not promote survival and proliferation of cumulus cells. Both GDF9 and BMP15 have been known to enhance the function of cumulus cells by simultaneously increasing cell proliferation and reducing apoptosis (Eppig, 2001; Hussein et al, 2005). However, as shown in the present study, addition of exogenous GDF9 and BMP15 either individually or in combination did not increase proliferation and survival of cumulus cells. Rather, these factors may promote expansion of cumulus cells by weakening extracellular matrix (ECM)-mediated cell attachment as a previous report demonstrating scant ECM in co-culture with denuded oocytes that may secrete OSF (Gomez *et al.*, 2012).

Addition of GDF9 and BMP15 either individually or in combination did not enhance meiotic maturation and subsequent development of parthenogenetically activated oocytes into the cleavage and blastocyst stage (Table 2). Hussein *et al.* (2006) reported that these two factors could enhance developmental competence of bovine oocytes and substantially increase blastocyst formation. However, a wide range of species variability in GDF9 and BMP15 mRNA levels in oocytes was reported (Crawford and McNatty, 2012). Hence, the species-specific ratio of GDF9:BMP15 mRNA between pigs and cattle might cause variation in developmental competence of oocytes. Recently, Lin *et al.* (2014) reported slight increases in cleavage and blastocyst rate of parthenogeneticcally activated oocytes after combined treatment of GDF9 and BMP14 during IVM. However, such increases have not been observed in the present study.

As shown in Table 3, GDF9 treatment significantly increased ($P<0.05$) the number of total cells in blastocysts due to the increased number of TE cells, while the numbers of both ICM and TE cells were significantly increased ($P<0.05$) by the treatment of BMP15. Combined treatment of GDF9 and BMP15 further increased the number of cells in blastocysts by increasing both ICM and TE cells. Previous studies using denuded oocytes as a source of OSF reported increases in the numbers of ICM cells in mice (Sudiman *et al.*, 2014) and TE cells in cattle (Hussein *et al.*, 2006). In the present study, we demonstrated

that exogenous GDF9 more specifically targets TE cells, whereas BMP15 does both ICM and TE cells. Moreover, we showed a synergistic action of two factors to further increase proliferation of both ICM and TE cells. Peng *et al.* (2013) reported that GDF9 and BMP15 synergistically activate Smad2/3 with 30- to 1,000-fold when compared with either GDF9 or BMP15 alone. This synergistic activation is mediated by binding of GDF9-BMP15 complex with receptors that include ALK4/5/7 and BMPR2.

Overall, the treatment of GDF9 or BMP15 (or both) during IVM enhanced the quality of blastocysts via increased number of ICM and/or TE cells in pigs. Results obtained from the present study may be beneficial not only to study the mechanism involved in maturation of porcine oocytes but also to practically improve efficiency of *in vitro* fertilization and somatic cell nuclear transfer in pigs which the quality of *in vitro* matured oocytes are critical.

REFERENCES

Abeydeera LR, Wang WH, Cantley TC, Rieke A and Day BN. 1998. Co-culture with follicular shell pieces can enhance the developmental competence of pig oocytes after *in vitro* fertilization: Relevance to intracellular glutathione. Biol. Reprod. 58:213-218.

Crawford JL and McNatty KP. 2012. The ratio of growth differentiation factor 9:bone morphogenetic protein 15 mRNA expression is tightly co-regulated and differs between species over a wide range of ovulation rates. Mol. Cell Endocrinol. 348:339-343.

Eppig JJ. 2001. Oocyte control of ovarian follicular development and function in mammals. Reproduction 122:829-838.

Eppig JJ, Wigglesworth K, Pendola F and Hirao Y. 1997. Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. Biol. Reprod. 56:976-984.

Gomez MN, Kang JT, Koo OJ, Kim SJ, Kwon DK, Park SJ, Atikuzzaman M, Hong SG, Jang G and Lee BC. 2012. Effect of oocyte-secreted factors on porcine *in vitro* maturation, cumulus expansion and developmental competence of parthenotes. Zygote 20:135-145.

Huang ZW and Dagan W. 2010. The human oocyte and cumulus cells relationship: New insights from the cumulus cell transcriptome. Mol. Hum. Reprod. 16:715-725.

Hussein TS, Froiland DT, Amato F, Thompson JG and Gilchrist RB. 2005. Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins. J. Cell Sci. 118:5257-5268.

Hussein TS, Thompson JG and Gilchrist RB. 2006. Oocyte-secreted factors enhance oocyte developmental competence. Dev. Biol. 296:514-521.

Li R, Norman RJ, Armstrong DT and Gilchrist RB. 2000. Oocyte-secreted factor(s) determine functional differences between bovine mural granulosa cells and cumulus cells. Biol. Reprod. 63:839-845.

Lin ZL, Li YH, Xu YN, Wang QL, Namgoong S, Cui XS and Kim NH. 2014. Effects of growth differentiation factor 9 and bone morphogenetic protein 15 on the *in vitro* maturation of porcine oocytes. Reprod. Domest. Anim. 49:219-227.

Moor R and Dai Y. 2001. Maturation of pig oocytes *in vivo* and *in vitro*. Reprod. Suppl. 58:91-104.

Papaioannou VE and Ebert KM. 1988. The preimplantation pig embryo: cell number and allocation to trophectoderm and inner cell mass of the blastocyst *in vivo* and *in vitro*. Development 102:793-803.

Peng J, Li Q, Wigglesworth K, Rangarajan A, Kattamuri C, Peterson RT, Eppig JJ, Thompson TB and Matzuk MM. 2013. Growth differentiation factor 9:bone morphogenetic protein 15 heterodimers are potent regulators of ovarian functions. Proc. Natl. Acad. Sci. U. S. A. 110:E776-785.

Sudiman JI, Ritter LJ, Feil DK, Wang X, Chan K, Mottershead DG, Robertson DM, Thompson JG and Gilchrist RB. 2014. Effects of differing oocyte-secreted factors during mouse *in vitro* maturation on subsequent embryo and fetal development. J. Assist. Reprod. Genet. 31:295-306.

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