

## Original Article

# Effects of Hyaluronidase during *In Vitro* Maturation on Maturation and Developmental Competence in Porcine Oocytes

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**ABSTRACT** The aim of this study was to investigate effects of hyaluronidase during IVM on oocyte maturation, oxidative stress status, expression of cumulus expansion-related (*PTX*, pentraxin; *GJA1*, gap junction protein alpha 1; *PTGS2*, prostaglandin-endoperoxide synthase 2) and fatty acid metabolism-related (*FADS1*, delta-6 desaturase; *FADS2*, delta-5 desaturase; *PPAR $\alpha$* , peroxisome proliferator-activated receptor-alpha) mRNA, and embryonic development of porcine oocytes. The cumulus-oocyte complexes (COCs) were incubated with 0.1 mg/mL hyaluronidase for 44 h. Cumulus expansion was measured at 22 h after maturation. At 44 h after maturation, nuclear maturation, intracellular glutathione (GSH) and reactive oxygen species (ROS) levels were measured. Gene expression in cumulus cells was analyzed using real time PCR. The cleavage rate and blastocyst formation were evaluated at Day 2 and 7 after insemination. In results, expansion of cumulus cells was suppressed by treatment of hyaluronidase at 22 h after maturation. Intracellular GSH level was reduced by hyaluronidase treatment ( $p < 0.05$ ). On the other hand, hyaluronidase increased ROS levels in oocytes ( $p < 0.05$ ). Only *PTGS2* mRNA was enhanced in COCs by hyaluronidase ( $p < 0.05$ ). Population of oocytes reached at metaphase II stage was higher in control group than hyaluronidase treated group ( $p < 0.05$ ). Both of cleavage rate and blastocyst formation were higher in control group than hyaluronidase group ( $p < 0.05$ ). Our present results showed that developmental competence of porcine oocytes could be reduce by hyaluronidase via inducing oxidative stress during maturation process and it might be associated with prostaglandin synthesis. Therefore, we suggest that suppression of cumulus expansion of COCs could induce oxidative stress and decrease nuclear maturation via reduction of GSH synthesis and it caused to decrease developmental competence of mammalian oocytes.

**Keywords:** cumulus expansion, developmental competence, hyaluronidase, *in vitro* maturation, porcine oocytes

## INTRODUCTION

During the maturation process of mammalian oocytes,

cumulus cells surrounding oocytes undergo morphological and physiological changes and these changes of cumulus cells directly influence to maturation and develop-

mental competence of oocytes. These changes in cumulus cells are known as cumulus expansion. In pigs and cows, removal of cumulus layer of cumulus-oocyte complexes (COCs) decreased nuclear maturation, fertilization and further embryo development *in vitro* (Zhang et al., 1995; Maedomari et al., 2007). On the other hand, enhanced cumulus expansion by epidermal growth factor (EGF) and insulin-like growth factor (IGF-1) increased population of mature oocytes (Lorenzo et al., 1994). These researches demonstrated that function of cumulus during oocyte maturation is important for successful maturation and developmental competence and it was used as indicator of oocyte quality.

Cumulus cells synthesize and secrete extracellular matrix (ECM) for cumulus expansion, and cross-talk between cumulus cells and oocytes is mediated by secreted ECM components. Hyaluronic acid (HA) produced by cumulus cells is most abundant ECM molecules and plays a crucial role in meiotic oocyte maturation and developmental competence. It is also associated with embryonic morphogenesis and the migration of embryonic cells because embryonic tissues contain HA (Vabres, 2010).

Gutnisky et al. (2007) reported that treatment of 6-diazo-5-oxo-L-norleucine (DON) during maturation of bovine oocytes suppressed cumulus expansion and embryonic development, and reduced embryo development was recovered by HA treatment. In pigs, expression of CD44, which is one of hyaluronic acid-binding proteins, was corresponded with cumulus expansion of COCs during maturation (Yokoo et al., 2002). On the other hand, treatment of HA did not influence to cumulus expansion (Marei et al., 2012). These findings indicated that exogenous HA could not affect to physiological processes of oocytes, and synthesis of HA by cumulus is essential for cumulus expansion and enhancement of oocyte quality. Therefore, study regarding with role of secreted HA from cumulus cells is needed.

In our previous study, cumulus expansion of porcine oocytes was enhanced by 50  $\mu$ M alpha-linolenic acid (ALA) during *in vitro* maturation, and it was corresponded with monospermic fertilization and improvement of early embryonic development (Lee et al., 2018). Auclair et al. (2013) reported that amount of total lipid in bovine oocytes was lower in denuded oocytes than cumulus-enclosed oocytes and it suggested that role of cumulus layer is closely associated with lipid store and metabolism in oocytes. Based

on these results, we hypothesized that HA from cumulus cells is necessary for successful oocyte maturation and further embryo development, and it is related with lipid metabolism. To confirm role of HA from cumulus cells, hyaluronidase was used for degradation of secreted HA by cumulus cells during maturation of porcine oocytes, and oocyte quality was evaluated using nuclear maturation, oxidative stress status, cumulus expansion-related and fatty acid-related genes, and embryo development.

## MATERIALS AND METHODS

### Oocytes collection and *in vitro* maturation (IVM)

All procedures that involved the use of animals were approved by the Kangwon National University Institutional Animal Care and Use Committee (KIACUC-09-0139). The ovaries were collected from local slaughter house and were transferred to laboratory in 0.9% (w/v) saline within 2 h. The COCs were aspirated from antral follicles 3–6 mm in diameter using 10 cc syringe connected with 18-gauge needle. After aspiration, Only COCs with compact cumulus up to 3 layers and homogeneous cytoplasm were selected and were incubated in medium-199 medium (Invitrogen, MA, USA) supplemented with 10% (v/v) porcine follicular fluid (pFF), 0.5  $\mu$ g/mL follicle stimulating hormone (FSH; Sigma-Aldrich, St. Louis, MO, USA), 10  $\mu$ g/mL luteinizing hormone (LH; Sigma-aldrich), 10 IU/mL human chorionic gonadotropin (hCG; Intervet) and 10 ng/mL epidermal growth factor (EGF; Sigma-aldrich) with 0.1 mg/mL hyaluronidase at 38.5°C in 5% CO<sub>2</sub> for 22 h. Then, they were subsequently incubated with hormone-free maturation medium with hyaluronidase for 22 h.

### Assessment of cumulus cell expansion

To evaluate effect of hyaluronidase treatment on cumulus expansion, COCs were matured with hyaluronidase for 22 h and cumulus area of COCs was observed using inverted microscope at 0 or 22 h after maturation. Then, area from 65 COCs in each groups was measured using Image J software (Version 1.46; National Institutes of Health, Bethesda, MD, USA) and was normalized to control group (without hyaluronidase at 0 h).

### Measurement of intracellular GSH and ROS levels

Intracellular GSH and ROS levels were measured for evaluation of oxidative stress status in oocytes. At 44 h

after maturation, the cumulus cell surrounding oocytes were removed and denuded oocytes were fixed by 4% (v/v) paraformaldehyde at room temperature for 7 min. GSH and ROS in 15-20 oocytes from each treatment group were incubated with 5  $\mu$ M CellTracker™Red CMTPX and 20  $\mu$ M carboxy-DCFDA in at 38.5°C for 30 min in dark room, respectively. Then, oocytes were three washed with PBS-PVA and observed under fluorescent microscope with optical filter (GSH, 580 nm; ROS, 510 nm). The fluorescence intensity were analyzed using Image J software.

### Evaluation of nuclear maturation

The nuclear maturation rate was measured by aceto-orcein stain method. The cumulus cells were removed by gently pipetting after maturation and denuded oocytes were fixed in fixation solution (acetic acid : ethanol = 3 : 1; v/v) for 48 h. Fixed oocytes were stained with 1% (w/v) aceto-orcein for 7 min. The morphology of nuclear was observed under light microscope and metaphase II (MII) stage reached oocytes were classified as mature oocyte.

### Quantitative real time PCR

After maturation, total RNA in cumulus cells were extracted using Trizol reagent (Takara, Japan) and cDNA was synthesized using PrimeScript 1<sup>st</sup> standard cDNA Synthesis Kit (Takara) according to manufacturer's protocol. In brief, 1  $\mu$ g of total RNA were incubated with 5  $\mu$ M Oligo dT primer and 1 mM dNTP mixture at 65°C for 5 min,

and it was immediately cooled on ice after incubation. Then, RNA was mixed with 1 units/ $\mu$ L RNase inhibitor and 10 unit/ $\mu$ L RTase and mixture was reacted to synthesize cDNA at 42°C for 1 h. Synthesized cDNA was quantified using NanoDrop 2000 (Thermo Scientific Nanodrop, Wilmington, DE, USA) and was diluted concentration at 50 ng/ $\mu$ L for real time PCR. All of primers for fatty metabolism (delta-6 desaturase, *FADS1*; delta-5 desaturase, *FADS2*, peroxisome proliferator-activated receptor-alpha, *PPAR $\alpha$* ) and cumulus expansion-related genes (pentraxin, *PTX3*; gap junction protein 1, *GJA1*; prostaglandin-endoperoxide synthase 2, *PTGS2*) were designed using Primer-BLAST in NCBI (Table 1). Thunderbird SYBR qPCR mix (Toyobo, Japan) was used for real-time PCR and real-time PCR was carried out using AB7500 real-time PCR system (Applied Biosystem®, Foster City, CA, USA). cDNA amplification was performed following conditions: denaturation; 95°C for 15 sec, annealing; 60°C for 30 sec, extension; 72°C for 30 sec. Relative expression level of genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and analyze by 2<sup>- $\Delta\Delta$ Ct</sup> method.

### In vitro fertilization and culture

Fifty mature oocytes were placed in 250  $\mu$ L drop of modified tris-buffered medium (mTBM) containing 0.2% (w/v) BSA (Sigma-aldrich) in 4-well culture plate. Boar spermatozoa were washed twice with modena B and diluted with mTBM supplemented with 0.4% (w/v) BSA and

Table 1. Primer sequences used for real-time PCR

| Gene                           | Primer sequence (5' → 3')                           | Product size (bp) | Annealing temp. | Accession Number |
|--------------------------------|-----------------------------------------------------|-------------------|-----------------|------------------|
| <i>FADS1</i>                   | F: CGTGATTGACCGGAAGGTGT<br>R: CCACAAAGGGATCCGTAGCA  | 110               | 60°C            | NM_001113041.1   |
| <i>FADS2</i>                   | F: CCGGTTGAGTACGGCAAGAA<br>R: CCAGGACACCGTAGAAAGGG  | 217               | 60°C            | NM_001171750.1   |
| <i>PPAR<math>\alpha</math></i> | F: GTTGAAGGGCTTCTTCGG<br>R: CCGAGAGGCACTTGCGAAA     | 129               | 60°C            | NM_001044526     |
| <i>PTX3</i>                    | F: GCCAGCAGGTTGTGAAACAG<br>R: GGCTTTGACCCAAATGCAGG  | 121               | 60°C            | NM_001244783     |
| <i>GJA1</i>                    | F: CTCTGAGGCGTGCCTACTTC<br>R: CTAAGGCACTCCAGTCACCC  | 77                | 60°C            | NM_001244212.1   |
| <i>PTGS2</i>                   | F: ATCCTCCGACAGCCAAAGAC<br>R: AGCCGTTTCATCGTCCCATTC | 199               | 60°C            | NM_214321        |
| <i>GAPDH</i>                   | F: CATGGTTCAGCCATCACA<br>R: TCCCGCCAACATCAAATGGG    | 170               | 60°C            | NM_001206359     |

*FADS1*, delta-6 desaturase; *FADS2*, delta-5desaturase; *PPAR $\alpha$* , peroxisome proliferator-activated receptor-alpha; *PTX3*, pentraxin; *GJA1*, gap junction protein alpha 1; *PTGS2*, prostaglandin-endoperoxide synthase 2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

2 mM caffeine (Sigma-aldrich) at a final concentration of  $6 \times 10^5$  spermatozoa/mL. Then, 250  $\mu$ L aliquots of sperm were injected into oocyte drop and were co-incubated with oocytes for 6 h. After fertilization, cumulus cells and sperm surrounding zygotes were removed and twenty fertilized zygotes were transferred to 100  $\mu$ L drop of porcine zygote medium-3 (PZM-3) containing 0.3% (w/v) BSA. The cleavage rate and blastocyst formation were evaluated at 2 and 7 days after insemination, respectively.

### Statistical analysis

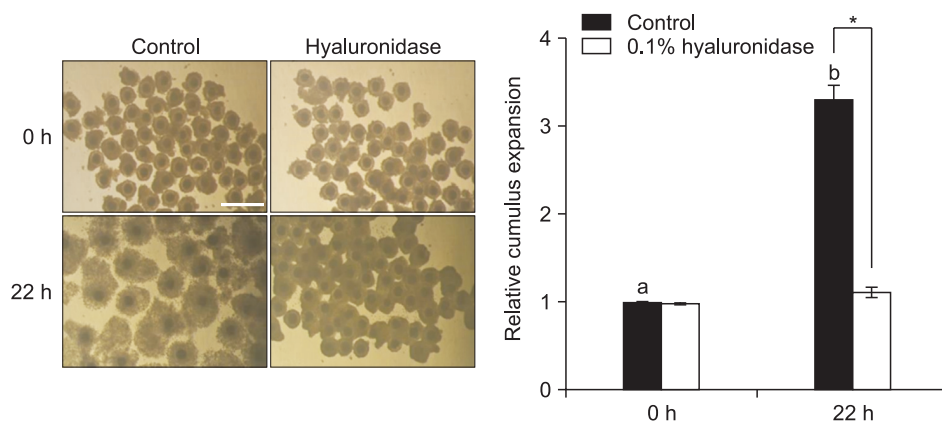
All numerical data representing each parameter were analyzed using the Statistical Analysis System Software

(SAS version 9.4). Data are presented means  $\pm$  SEM and comparisons among treatment groups were conducted by t-test using a generalized linear model (GLM) in the SAS package. A value of  $p < 0.05$  was considered to indicate a statistically significant difference.

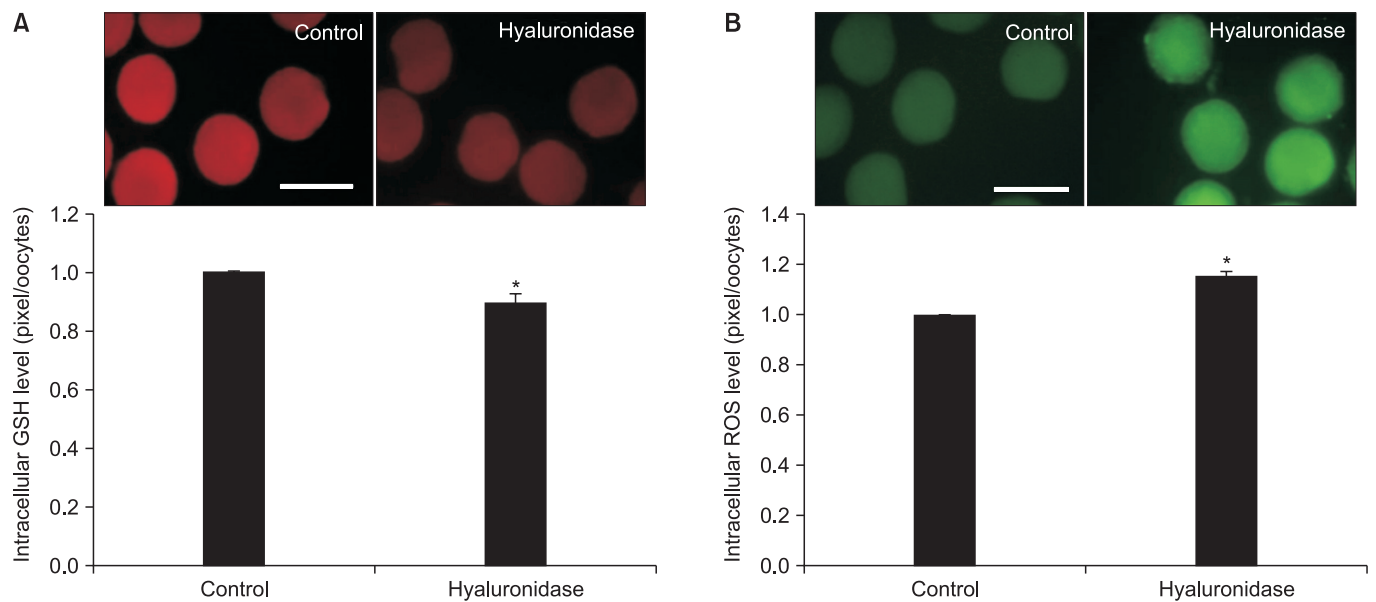
## RESULTS

### Effects of hyaluronidase on cumulus expansion and oxidative stress status

In control group, cumulus expansion was increased after 22 h maturation (Fig. 1;  $p < 0.05$ ), whereas it was not differ in hyaluronidase group. And it was higher in control



**Fig. 1.** Suppression of cumulus expansion by hyaluronidase (0.1 mg/mL) in porcine oocytes at 22 h after *in vitro* maturation. Scale bar: 200  $\mu$ m. <sup>a,b</sup>Different superscripts indicate significant difference between 0 h and 22 h in control group after *in vitro* maturation ( $p < 0.05$ ). Asterisk indicates difference between control group and hyaluronidase group at 22 h after *in vitro* maturation ( $p < 0.05$ ).



**Fig. 2.** Effect of hyaluronidase (0.1 mg/mL) on intracellular glutathione (GSH; A) and reactive oxygen species (ROS; B) level during *in vitro* maturation in porcine oocytes. Scale bar: 200  $\mu$ m. Asterisks indicate difference between control and hyaluronidase group *in vitro* maturation ( $p < 0.05$ ).

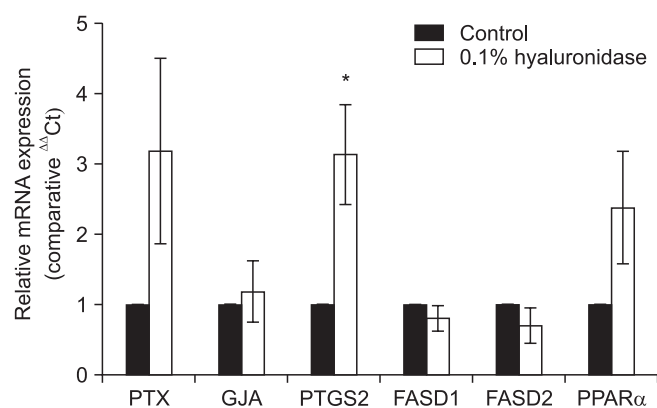
group than treatment group ( $p < 0.05$ ). Treatment of hyaluronidase reduced intracellular GSH levels and increased ROS levels in oocytes (Fig. 2;  $p < 0.05$ ).

### Changes of mRNA expression by hyaluronidase

The result of effect of hyaluronidase on fatty acid metabolism and cumulus expansion related genes was showed in Fig. 3. The expression of *PTX* and *PPAR $\alpha$*  mRNA in cumulus cells was enhanced by hyaluronidase after maturation, however, there was no significant differences. Only *PTGS2* mRNA was increased in treatment group compared to control group ( $p < 0.05$ ).

### Nuclear maturation and embryonic development

Table 2 showed nuclear maturation and developmental competence of porcine oocytes. The population of oocytes reached MII stage was decreased by treatment of hyaluronidase at 44 h after maturation ( $p < 0.05$ ). Furthermore, hyaluronidase treatment during IVM reduced



**Fig. 3.** Change of cumulus expansion-related (*PTX*, pentraxin; *GJA1*, gap junction protein alpha 1; *PTGS2*, prostaglandin-endoperoxide synthase 2) and fatty metabolism-related (*FASD1*, delta-6 desaturase; *FASD2*, delta-5desaturase; *PPAR $\alpha$* , peroxisome proliferator-activated receptor-alpha) genes in cumulus cells by hyaluronidase (0.1 mg/mL) during *in vitro* maturation. Asterisk indicates a significant difference ( $p < 0.05$ ).

both of cleavage rates and blastocyst formation of porcine oocytes ( $p < 0.05$ ).

## DISCUSSION

During the oocyte maturation, cumulus cells surrounding oocytes synthesize and secrete HA that is abundant in ECM of COCs. The area of cumulus oophorus is increased by this phenomenon and it is known as cumulus expansion. The cumulus expansion is induced by cross-talk between cumulus cells and oocytes and these cross-talk were mediated by various factors including gonadotropins, prostaglandin E2 (PGE2), and growth factors. The changes of morphology and metabolic activity of cumulus cells during cumulus expansion influence oocyte maturation and developmental competence (Auclair et al., 2013).

This study was conducted to investigate effect of secreted HA by cumulus cells on oocyte maturation and further embryonic development of porcine oocyte. Thus, hyaluronidase was treated for suppression of cumulus expansion by degradation of secreted HA from COCs during IVM. Our findings showed that cumulus expansion, nuclear maturation and developmental competence were suppressed by hyaluronidase treatment, and oxidative stress was induced.

Cell communication between cell to cell and cell to oocyte during meiotic oocyte maturation plays a crucial roles in both of nucleic and cytoplasmic maturation of oocyte and subsequent embryonic development. This communication is mediated ECM between cumulus cells and gap junction. These two types of interaction are closely associated with synthesis and secretion of HA that lead to cumulus expansion, and a variety of substances including low size molecules and metabolites were transported to other cells or oocytes via gap junction. In present study, treatment of hyaluronidase suppressed cumulus expansion at 22 h and GSH level in oocytes at 44 h after

**Table 2.** Effects of hyaluronidase during *in vitro* maturation on nuclear maturation and developmental competence of porcine oocytes

| Treatment     | No. of examined oocytes | Oocytes reached to MII* (%) | No. of oocytes used for IVF | Embryo development to (%) |                         |
|---------------|-------------------------|-----------------------------|-----------------------------|---------------------------|-------------------------|
|               |                         |                             |                             | Cleavage                  | Blastocyst              |
| Control       | 207                     | 94.34 ± 3.9 <sup>a</sup>    | 149                         | 55.6 ± 2.5 <sup>a</sup>   | 18.8 ± 0.5 <sup>a</sup> |
| Hyaluronidase | 225                     | 72.25 ± 3.8 <sup>b</sup>    | 165                         | 37.5 ± 1.1 <sup>b</sup>   | 3.8 ± 2.1 <sup>b</sup>  |

Data from 3 replication are presented as mean ± SEM.

<sup>a,b</sup>Different superscripts indicate a significant difference within a same column ( $p < 0.05$ ).

\*MII: metaphase II.

maturation. On the other hand, intracellular ROS level was increased. Activity of cell communication including gap junction could be suppressed by cumulus expansion and HA (Chen et al., 1990; Yokoo et al., 2010). In particular, cysteine and glutamine that are sources for GSH synthesis were transported from cumulus cells to oocytes through gap junction (Mori et al., 2000). As one of antioxidants in oocytes, GSH plays role in reduction of ROS and preventing ROS-induced damages. Thus, we expected that suppression of cumulus expansion by degradation of HA decreased GSH synthesis via reduction of cysteine and glutamine transport into oocytes by inactivation of gap junction, and it was caused to increase ROS level in oocytes.

Oocyte maturation and cumulus expansion are closely involved in fatty acids (FAs) metabolism, prostaglandin (PG) synthesis and energy production. During cumulus expansion, energy production by FAs metabolism and interaction between cumulus cells and oocytes are mediated by a various genes such as *PTX*, *PTGS2*, *FASDs* and *PPAR $\alpha$*  (Calder et al., 2001; Salustri et al., 2004). Our previous study had shown that treatment of ALA during IVM of porcine oocytes enhanced cumulus expansion and reduced several gap junction and FAs metabolism genes (Lee et al., 2018). We expected that suppression of cumulus expansion could influenced to gene expression that are related with FAs metabolism and cumulus expansion. In present study, expression of *PTX*, *PTGS2* and *PPAR $\alpha$*  mRNA in cumulus cells were increased by hyaluronidase treatment during maturation, however, only *PTGS2* mRNA was significantly enhanced (Fig. 3). PGs are known to play an important role in cumulus expansion and are converted from omega-6 FAs by *PTGS2*. Culture medium, which is used for oocyte maturation, contain several FAs including linoleic acid (one of omega-6 FAs). Therefore, these findings lead to predict that cumulus cells treated hyaluronidase tried to use linoleic acid in culture medium for induction of cumulus expansion via PG synthesis by expression of *PTGS2* mRNA.

Embryonic development was one of parameters for evaluation of developmental ability of *in vitro* mature oocytes (Fayezi et al., 2018). Because incomplete cytoplasmic maturation lead to reduce low development of embryos, synthesis of GSH in oocyte during maturation is used for indicator of developmental competence as cytoplasmic maturation of mammalian oocytes as well as role

of antioxidant (Wang and Tsujii, 1997; De Matos and Furnus, 2000). In numerous studies, enhanced intracellular GSH level after maturation correspond with developmental competence (You et al., 2012; Wang et al., 2014; Lee et al., 2016). Our present showed that treatment of hyaluronidase decreased both of GSH contents in oocytes and subsequent embryonic development after IVM. As mentioned above, transport of various substrates including cysteine and glutamine is contributed to GSH synthesis in oocytes. Therefore, these results demonstrated that suppression of cumulus expansion inhibited GSH synthesis via decreasing transport of source of GSH and it reduced further embryonic development.

In conclusion, our findings showed that treatment of hyaluronidase during IVM of oocytes suppressed cumulus expansion, intracellular GSH level, expression of *PTGS2* mRNA and embryonic development, whereas ROS level was increased. These results suggest that enhanced cumulus expansion is important for developmental ability of *in vitro* mature oocyte because it could regulate transport of substrate for synthesis of GSH. Therefore, induction of cumulus expansion during oocyte maturation is necessary to improve quality of *in vitro* produced oocytes and embryos.

## CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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