



Effect of hyaluronan to inhibit caspase activation in porcine granulosa cells

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ABSTRACT

We studied the ability of hyaluronan (HA) to inhibit apoptosis in porcine granulosa cells. The granulosa layer with cumulus–oocyte complex is cultured in media supplemented with follicle stimulating hormone (FSH) and 4-MU an inhibitor of hyaluronan synthases. The concentration of HA significantly increased after supplemented with FSH, but significantly decreased with 4-MU. CD44, receptor of HA, expressed after cultured with FSH, decreased in addition low concentration of 4-MU, whereas not detected in high concentration of 4-MU, indicating parallel relation between the amount of HA and CD44 expression. The 4-MU treatment also decreased the expression of procaspase-3, -8, -9 suggesting that inhibition of HA synthesis leads to activation of these caspases. Moreover, addition of anti-CD44 antibody decreased the expression of procaspases suggesting that perturbation of HA–CD44 binding leads activation of caspases. Hence, HA has ability to inhibit apoptosis and HA–CD44 binding is important on apoptosis inhibitory mechanism in porcine granulosa cells.

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Introduction

In the mammalian ovary most of follicle (>99%) die rather than ovulate because of atresia. The formation of an atretic follicle is believed to be initiated by apoptosis of granulosa cells followed by oocyte degeneration [1]. Moreover, atresia is primarily observed in follicles at preantral stage in which granulosa cells start expressing follicle stimulating hormone (FSH) receptors. Hence, FSH is believed to be an important factor in controlling atresia [2]. Recently, FSH is known to function as an apoptosis suppressor in granulosa cells under *in vitro* condition in many species including pig [3], cow [4], and rat [5].

Our previous study [6] revealed that FSH stimulates the mRNA expression of *hyaluronan synthase* (*has*), the enzyme for hyaluronan (HA) synthesis, in porcine cumulus cells. Similar results have also been reported by other researchers [7]. Studies in mouse revealed that FSH is involved in HA synthesis under *in vitro* condition [8]. This result evidenced the close correlation between FSH and HA synthesis.

HA is a glycosaminoglycan that is widely distributed in most mammalian tissues. We have previously reported that HA produced by cumulus cells prevents the fragmentation of oocytes in culture [9]. Recently, we have reported that HA can be detected in expanded cumulus cells during cumulus expansion and oocyte maturation [10], indicating that HA is an important factor for oo-

cyte survival and maturation. Moreover, HA inhibits apoptosis in human cumulus and mural granulosa cells [11]. On the basis of these findings, HA could be a potential candidate for inhibiting apoptosis in porcine granulosa cells.

Since HA is distributed in the extracellular matrix, it requires hyaluronan binding protein(s) to organize the hyaluronan-rich matrix, and CD44 as its principal cell surface receptor [12,13]. Our previous study confirmed that FSH stimulated the expression of both *has2* and *cd44* mRNA in porcine cumulus cells [6,10]. Meanwhile HA–CD44 interaction is involved in the phosphorylation of MAPK in oocytes and in the interruption of Cx43-derived gap junctional channels [14]. Also sufficient interaction between HA and CD44 during cumulus expansion plays important role in oocyte maturation [13]. However HA–CD44 interaction in porcine granulosa cells is unclarified.

A recent study reported that caspases are actively involved in apoptosis of the antral follicles [15]. There are two major upstream of apoptosis-signaling pathways in cells: extrinsic and intrinsic pathway. The extrinsic pathway involves caspase-8 as an initiator. Procaspase-8 (i.e. precursor of caspase-8) binds to FADD via the death effector domain and forms a homodimer of procaspase-8. This activates the procaspase-8. The intrinsic pathway involves caspase-9 as an initiator. The activation of procaspase-9 occurs in the presence of cytochrome *c*, which is released into the cytosol following an interaction between caspase-9 and Apaf-1. The activated caspase-8 and -9 then cleave caspase-3, the downstream effector/executor, this activates the nucleases and apoptosis is induced [16]. Interestingly, it has been observed that increased HA

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production stimulates several antiapoptotic signaling pathways in human carcinoma cell [17]. These reports suggested that HA probably regulates caspase activation cascade in porcine granulosa cells.

Therefore, the objective of this study was to elucidate the ability and the possible mechanisms by which HA inhibit apoptosis in porcine granulosa cells. We evaluated: (1) HA synthesis in granulosa cells, (2) the ability of HA to inhibit the activation of caspase-3, -8, -9 in porcine granulosa cells, and (3) the binding of HA to CD44 and its relation with inhibition of the activation of caspase-3, -8, -9.

Materials and methods

Isolation and culture of porcine granulosa cells. Porcine ovaries were obtained from prepubertal gilts from a local slaughterhouse. Follicles having a diameter of 3–5 mm were isolated from the ovaries using a scissor. The follicles were classified as healthy by using morphological determination as previously described [18]. Cumulus-oocyte complex (COC) with granulosa layer (COCG) was mechanically dissected from the follicles using fine forceps. It was placed in a 30 mm dish with basic medium: DMEM/F12 (Gibco BRL Life Technologies, Grand Island, NY) supplemented with 1% BSA (Sigma, St. Louis, MO) and streptomycin and penicillin (Meiji Seika, Tokyo, Japan). It was then cultured for 48 h in a CO₂ incubator (37 °C; 5% CO₂; 95% air) and was supplemented with either 50 mU/ml FSH (porcine pituitary FSH, Sigma); 50 mU/ml FSH and 0.1, 0.5, and 1 mM 4-methylumbelliferone sodium salt (4-MU; Sigma, a HAS inhibitor [19]); or simultaneously 50 mU/ml FSH and 0.1, 0.5, 1 mM 4-MU and 200 µg/ml HA from porcine skin (molecular weight: 100,000–150,000, Seikagaku Corporation, Tokyo, Japan). Control had only medium. Moreover, the COCG cells also cultured in basic medium which was supplemented with either 50 mU/ml FSH; 50 mU/ml FSH and 20 µg/ml rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) or 50 mU/ml FSH and 20 µg/ml IM7 (anti-mouse CD44 antibody, BD Bioscience, San Jose, CA).

HA synthesis. The COCG cells were cultured with 50 mU/ml FSH and 0, 0.1, 0.5, and 1 mM of 4-MU dissolved in DMSO. The concentration of DMSO did not exceed 0.1%. A single COCG was cultured in 1 ml of DMEM/F12 and placed in a 1.5 ml tube. After incubation for 48 h, the cells were removed and the medium was recovered. HA released into the culture medium was quantified by an HA measuring kit according to the manufacturer's instruction (Seikagaku). The analysis method of this kit is based on a competitive inhibition method using hyaluronic acid binding protein (HABP) specific to HA.

The sample and standard solutions were evaluated in duplicate and the average absorbance values of each duplicate set were calculated. HA concentration in the sample was determined against a reference curve of HA standard solution, provided with the kit. The concentration of HA in the culture medium were analyzed using a one-way ANOVA followed by a Bonferroni/Dunn test. *P* values less than 0.05 were considered significant.

Immunoblotting. Protein fraction was prepared from COCG cells cultured with FSH, 4-MU, HA and IM7 treatments. Extraction was then performed using RIPA buffer (50 mM Hepes, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% deoxycholate, 10% nonidet P40, 1% SDS, and 100 mM DTT) and the proteins were separated by 10% or 15% SDS-PAGE (50 µg/lane) and then transferred onto membranes for 1.5 h. The membranes were immersed in a blocking solution (5% w/v skim milk, 3% w/v BSA in TBS-T) for 1 h and incubated overnight at 4 °C with IgG diluted in 5% (w/v) BSA in TBS-T. The antibodies used for detection were as follows: anti-CD44 antibody (1:1000 dilution; PORC24A; VMRD Inc., Pullman, WA), rabbit polyclonal anti-procaspase-9 (1:1500 dilution; BD Bioscience),

anti-caspase-8 (Ab-3) human/mouse (1:1000 dilution; Calbiochem, Merck chemicals Ltd., Darmstadt, Germany), and anti-caspase-3 (1:1000 dilution, Cell Signaling Technology, Inc., Beverly, MA). The membranes were washed and then incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:10,000 dilution; Sigma), goat anti-rabbit IgG (1:7500 dilution; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), anti-mouse IgG (1:10,000 dilution; Sigma) and anti-rabbit IgG (1:2000 dilution; Cell Signaling), respectively. These conjugated antibodies were diluted with 5% (w/v) skim milk in TBS-T for 1 h at room temperature. The chemiluminescence was visualized using an ECL system (Amersham Bioscience, GE Healthcare, UK).

Results and discussion

Effect of FSH on HA synthesis

We established an *in vitro* culture system using granulosa layer with oocyte cumulus cells complex anchored on it. The maintenance of metabolic couplings between oocyte and granulosa cells is essential for oocyte growth [20]. On the other hand, oocyte produces regulatory factors that control specific granulosa cell functions, suggesting that bi-directional interaction between oocytes and their neighboring somatic cells is crucial right from follicle formation to ovulation [21]. Meanwhile, the difficulty of culturing porcine granulosa cell *in vitro* is granulosa cell easily luteinized (data not shown). Previous study showed that oocyte act to inhibit follicular luteinization [22]. Hence, it is thought that *in vitro* COCG culture system could be a valid model to study apoptosis of porcine granulosa cell *in vivo*.

Since HA is produced at the inner side of the plasma membrane and be extruded into the extra cellular space, we hypothesized that the synthesized HA might not be retained in granulosa cells but will be secreted into the culture medium. Hence, we analyzed the amount of HA secreted in culture medium. Fig. 1 shows a significant difference between the HA concentration in the granulosa cells treated with FSH (125.1 ± 22.5 ng/ml) and those without FSH (11.1 ± 0.9 ng/ml) indicate that HA synthesis can be stimulated by FSH treatment. Furthermore, the concentrations of HA in the media in the presence of 0.1, 0.5, and 1 mM of 4-MU were as follows: 89.2 ± 16.0, 19.8 ± 0.7, and 15.0 ± 0.9 ng/ml, respectively (*P* < 0.05) indicate when HAS function is inhibited by 4-MU, the HA synthesis by granulosa cells is perturbed as evidenced by a significant decrease in its concentration. 4-MU was previously found to inhibit HA synthesis in culture human skin fibroblasts with no effect on the synthesis of any glycosaminoglycans [23]. It is a substrate for

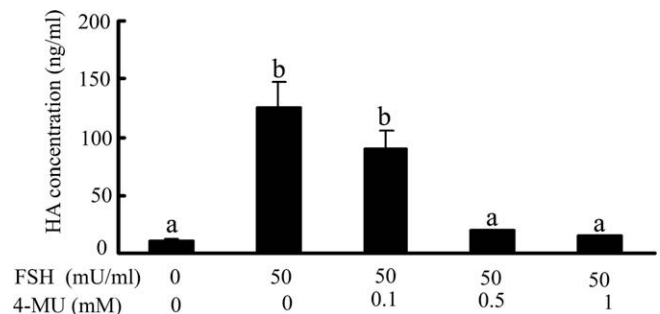


Fig. 1. Effect of FSH on HA synthesis. COCG was cultured for 48 h supplemented with FSH or FSH plus 0.1, 0.5, 1 mM 4-MU. FSH addition significantly increased HA concentration but higher concentrations of 4-MU (0.5 and 1 mM) significantly decreased HA synthesis. Values are means ± SEM of three replicates. Data were analyzed by one-way ANOVA followed by Bonferroni/Dunn test. Different letters indicate significant differences (*P* < 0.05).

glucuronosyl transferase (UGT) and the glucuronidation of 4-MU by UGT decreases the amount of UDP-glucuronic acid, the substrate for HA synthesis. Hence indirectly inhibit the synthesis of HA [24].

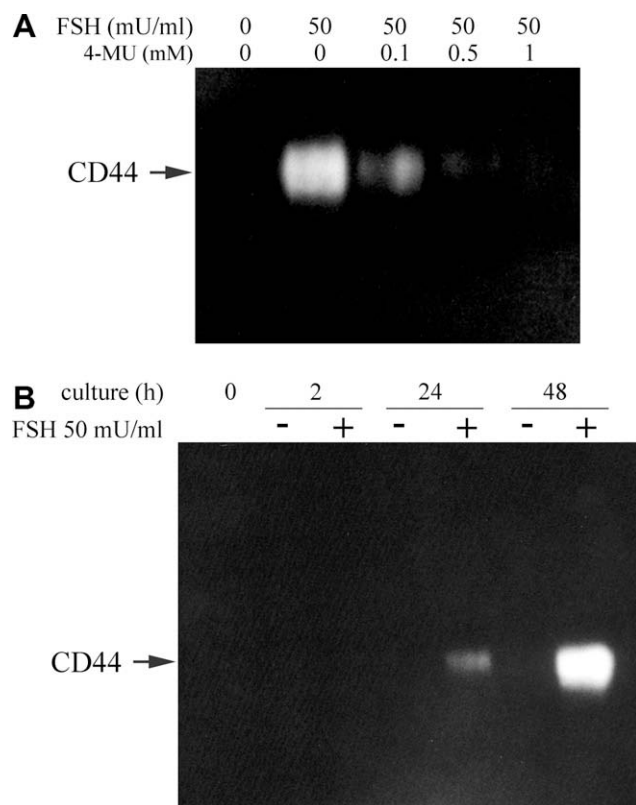


Fig. 2. Relation between HA synthesis and CD44 expression. The expression of CD44 after culturing COCG cells for 48 h with FSH or FSH plus 4-MU addition (A), and after culturing COCG cells for 0, 2, 24 and 48 h with (+) or without (–) FSH addition (B). CD44 strongly expressed after FSH addition. Furthermore higher concentration of 4-MU (0.5 and 1 mM) decreased CD44 expression. Meanwhile, CD44 expression was not detected at 0 and 2 h, slight expression after 24 h, and strong expression at 48 h.

Relation between HA synthesis and CD44 expression

HA is anchored to the surface of the cell membrane by HA-binding receptors such as CD44. Fig. 2A shows the expression of CD44 in granulosa cells after 48 h culture with 50 mU/ml FSH or 50 mU/ml FSH and 0.1, 0.5, and 1 mM 4-MU. High expression of CD44 is detected after FSH treatment, decreased expression with 0.1 mM of 4-MU, and very low to undetectable levels with 0.5 and 1 mM of 4-MU. Fig. 2B shows the expression of CD44 after FSH addition: no band is detected at 0 and 2 h, a slight band is observed after 24 h and a strong one at 48 h.

These results suggesting that when the content of secreted HA in culture medium decreased the expression of CD44 also decreased. Interestingly, the expression of CD44 gradually increased with time, indicating that when the amount of secreted HA increased, the CD44 expression also increased. Both our findings—the detection of the amount of synthesized HA by the determining the extent of CD44 expression and the lowered CD44 expression on account of low levels of HA—have not been reported earlier.

Meanwhile it can be considered that HA is secreted and it binds to the CD44 after 24 h culture and reached maximum at 48 h. Our study showed that after 2 h of culture, *has1*, 2 mRNA was expressed in granulosa layers, reached a maximum after 6 h but decreased after 24 h in porcine COCG culture system (data not shown). Probably, granulosa cells synthesize HA in the presence of *has1*, 2 mRNA and secrete it at extracellular matrix; HA then binds CD44 that transduce intracellular signals. Hence, the expression of CD44 is delayed as compared to the *has1*, 2 mRNA expression. Meanwhile, on the basis of our studies on oocyte and cumulus cells, we are the first to reveal that porcine granulosa cells synthesize HA and express CD44 under *in vitro* condition. Also, HA trigger the expression of CD44 in granulosa cells.

Effect of HA on procaspase expression

The fate of a cell, whether survive or death, depends on a balance between proapoptotic and antiapoptotic factors. Fig. 3A shows the expression of procaspase-3, -8, -9 in granulosa cells from healthy, early atretic, and progressing atretic follicle. The two major upstream apoptosis signaling pathways, extrinsic and intrinsic, were demonstrated by high expression of procaspase-8

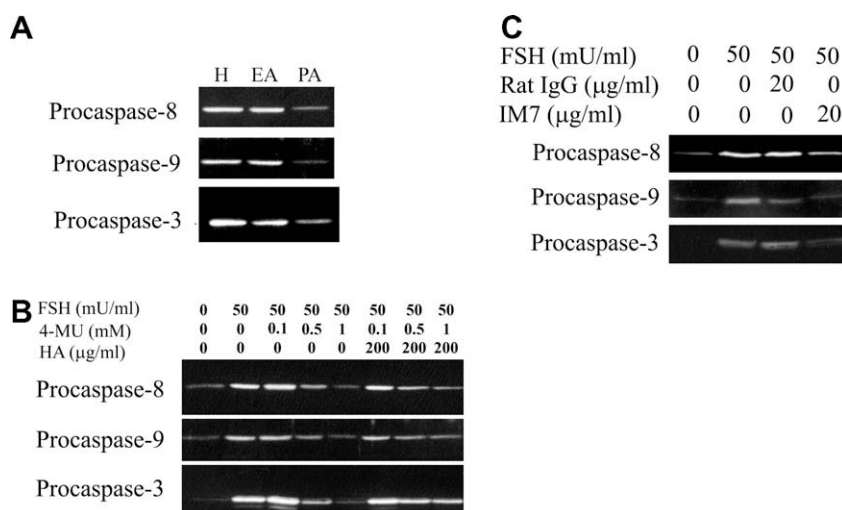


Fig. 3. Expression of procaspase-3, -8, -9 in granulosa cells. COCG 0 h (A) and after 48 h culture (B, C) H, healthy; EA, early atretic; PA, progressing atretic follicle. COCG cells after 48 h culture with the addition of FSH or FSH plus 0.1, 0.5, 1 mM 4-MU or simultaneously FSH and 0.1, 0.5, 1 mM 4-MU and 200 µg/ml HA (B). By simultaneous addition with 200 µg/ml of HA, the band of procaspase-3, -8, -9 became stronger compared to without HA addition. COCG cells after 48 h culture with addition FSH or FSH and rat IgG or FSH and IM7 (C). Addition of IM7 decreased the expression of procaspase-3, -8, -9 compared to FSH alone or FSH with rat IgG.

and -9 in granulosa cells of healthy and early atretic follicles, respectively. Accordingly, our result showed a decreased procaspase-3, -8, -9 expression was demonstrated by the progressing atretic follicle in which most cells had undergone apoptosis. This finding makes us to suppose that those procaspases are already activated. Meanwhile, both extrinsic and intrinsic pathways are observed in apoptosis of porcine granulosa cells. The expression of procaspase-3, the executor of apoptosis, decreased in progressing atretic follicles suggests that procaspase-3 was already activated into cleaved caspase-3. Our present study showed that the activation of caspase-3, -8, -9 may trigger an initiation of apoptosis. Fig. 3B shows the expression of procaspase-3, -8, -9 in COCG cells after culturing them in a medium supplemented with FSH or FSH plus various concentration of 4-MU. The expression levels of procaspase-3, -8 and -9 decreased as the concentration of 4-MU increased. Their expression was high in granulosa cells cultured in a medium supplemented with only FSH and FSH plus 0.1 mM 4-MU, whereas low expression was detected when they were cultured with FSH and 0.5 or 1 mM of 4-MU. Hence, FSH inhibits the activation of procaspase-3, -8, and -9, but they can be activated by low concentrations of HA secreted into the culture medium. In order to obtain more evidence, we supplemented the medium simultaneously with FSH, 4-MU, and HA. Simultaneous addition of 200 µg/ml of HA produced stronger bands as compared to those without HA addition, indicating that addition of exogenous HA into a medium that lacks HA derived from granulosa cells could suppress procaspase-3, -8, and -9 activation. Thus it becomes evident that HA endogenously produced by granulosa cells as well as that exogenously added control the activation of initiator and executor caspases and rescue granulosa cells from apoptosis. This finding is supported by Toole et al., 2005 who suggested that increased HA

production stimulates several antiapoptotic signaling pathways in human carcinoma cells.

Fig. 3C shows the expression of procaspase-3, -8, and -9 in COCG cells after culturing them with FSH or FSH plus rat IgG or FSH plus IM7. Addition of IM7 decreased the expression of procaspase-3, -8, -9 compared to FSH alone or FSH and rat IgG. IM7 is recognize an epitope near the proximal domain site necessary for hyaluronan binding, thus decrease HA binding ability to CD44 [26]. Although FSH stimulates granulosa cell to synthesize HA, but perturbation of the synthesized HA and CD44 bind leads to activation of caspase-3, -8, -9. These finding suggest that HA binds CD44 thus facilitate a conformational change in CD44 which then transmits a signal to the cell and function directly. Sufficient interaction between HA-CD44 was necessary for cell survival signaling. This data is supported by other researcher [27] who evidence that HA induces cell death in activated T cells through CD44.

Morphology of COCG culture

Apoptosis of the antral follicle in mouse [15] is caspase-3 dependent; this supports our results that procaspase-3 is activated in apoptotic of porcine granulosa cells. Once caspase-3 has been activated by other caspases, they cleave a variety of proteins or death substrates which contribute to the DNA condensation and fragmentation, a hallmark of apoptosis [25]. Our result indicate that the rate of apoptotic bodies in granulosa cells increased when the secretion of HA was inhibited using 4-MU, meanwhile the addition of exogenous HA suppressed it in a dose-dependent manner (data not shown). Fig. 4 shows the morphology of COCG before (Fig. 4A) and after 48 h culture with FSH (Fig. 4C); 4-MU (Fig. 4D–F); and HA (Fig. 4G–I) treatment. Granulosa cells in the COCG that

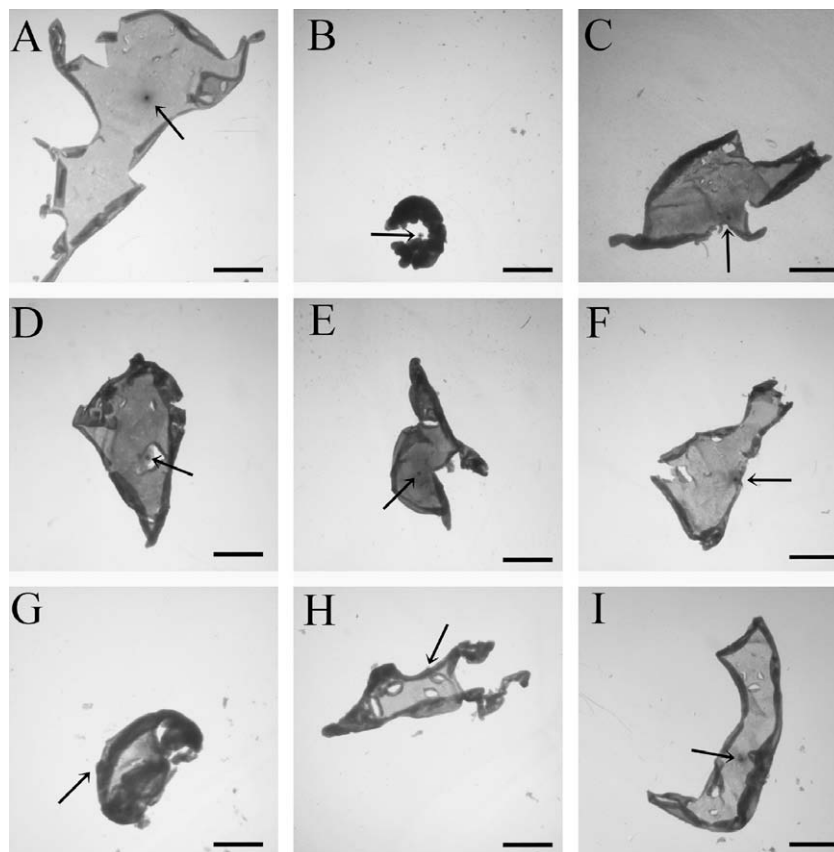


Fig. 4. Morphology of COCG. COCG 0 h (A); and after cultured for 48 h with: medium alone (B); FSH (C); FSH plus 0.1, 0.5, 1 mM 4-MU (D–F); 50, 100, 200 µg/ml HA (G–I). Granulosa cells without FSH and HA treatment (panel B) show apoptosis. The arrows indicate COCs. Bar = 1 mm.

had been cultured for 48 h without FSH and HA addition (panel B) shrunk. Further, condensation of the COCG layer with the appearance of black spots was observed. The connection between COC and granulosa cells became very weak, which is a sign of apoptosis. In the contrary, granulosa cells in the COCG that had been cultured for 48 h with the addition of 50 mU/ml FSH did not shrink, and the connection between the COC and granulosa cells was tight. The cells treated with low concentration of 4-MU and various concentration of HA treatments showed morphology similar to those treated with FSH. The differences between the morphological conditions of the COCG cells that were cultured without any addition and those cultured either with HA derived from granulosa cells or exogenous HA treatment suggest that HA have the ability to inhibit apoptosis in granulosa cells.

In summary, the present study provided evidence that HA synthesis is stimulated by FSH and this stimulation is one of the mechanism by which FSH inhibits apoptosis in porcine granulosa cells. Furthermore, HA binds CD44 and function directly from within the cell to inhibit caspase activation and finally inhibits apoptosis in porcine granulosa cells.

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