ORIGINAL ARTICLE

Regulatory effect of apoptosis on morphological changes in cell mass of porcine blastocyst through supplementation of rapamycin during *in vitro* culture

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ABSTRACT

Objective: The study of an *in vitro* embryosis is crucial in genetics for breed improvement and reproduction in livestock, identifying the causes of infertility, and stem cell application. Meanwhile, the problem of nucleic acid denaturation observed during embryo development is yet to be resolved. This study was set out to analyze the nucleic acid denaturation during the development of *in vitro* embryos.
Materials and Methods: Using an *in-vitro* fertilization-embryo in porcine, the cell development and apoptosis were evaluated by adding rapamycin by concentration to the TCM-199 containing 10% FBS or 10% porcine follicle fluid (pFF). Real-time PCR, zymography, DNA fragment, Western blot, and immunofluorescence analysis were also carried out to determine the development rate of inner cell mass in the *in-vitro* fertilization-embryo.
Results: The findings indicated that the addition of rapamycin to the 10% pFF group during *in vitro* maturation led to an increase in the rates of cleavage and blastocyst development and the expression of active matrix metallopeptidase (MMP-9), while nucleic acid denaturation was suppressed. In other words, the addition of rapamycin was found to increase the expression of MMP-2 in the inner cell mass and trophoblast, while it inhibited apoptosis.
Conclusion: The addition of rapamycin influences the regulation of apoptosis and MMPs, and based on this, it is presumed to have a positive effect on blastocyst development.

Introduction

Porcine oocytes require a more extended period for *in-vitro* maturation than other livestock animals, and the easy separation of the cumulus and oocytes, due to the small number of cells connected through microvilli during *in-vitro* maturation, leading to a fall in gap junctions that obstruct metabolic activities [1]. Besides, a disturbance is caused in glutathione synthesis in the ooplasm upon maturation [2], leading to incomplete oocyte maturation and a reduced rate of fertilization due to an increased rate of multiple sperm penetration, sperm head swelling, and incomplete pronuclear formation [3]. These are known to reduce the effect of BSA on porcine oocyte maturation and cumulus growth, compared to FBS, during the maturation and culture of oocytes [4]. As methods to overcome

such growth inhibition problems, studies have reported the following: the addition of an antioxidant to the culture to protect the *in-vitro* embryo from free radicals [5]; the addition of a thiol compound to the culture with similar effects, such as *β*-mercaptoethanol, cysteamine, and cysteine [6,7]; or the addition of growth factors [8,9], hormones [10], serum [11,12], BSA [13,14], or NO compound to the culture [15].

Furthermore, during the process of active growth of the ovarian follicle, changes occur to the follicle in the components and structures of the extracellular matrix (ECM), collagen, laminin, and fibronectin, which ultimately lead to oocyte maturation [16] and drive the primordial oocyte to grow into a mature oocyte based on the reformation of such structures. The activities of matrix metallopeptidases

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(MMPs) are known to promote the transportation of nutrients through the decomposition of the main matrix component, type IV collagen, and to exert a direct influence on oocyte maturation [17]. In other words, MMP-2 and MMP-9 are found in the ECM, where they denature the enzymes of collagen type IV, V, VII, X, casein, and gelatin, thus playing a critical role in trophoblast penetration while facilitating the activities in the metabolic process for the oocyte and the embryo [18].

Programmed cell death (PCD) is another mechanism that influences cell growth and activities. It is a process known to regulate the biological mechanisms for cell population control for embryogenesis, immune reactions, wound healing, and homeostasis of healthy tissues [19–21]. This study has determined whether the addition of rapamycin leads to the regulation of the genes engaged in apoptosis and MMPs and has analyzed the consequent influence on blastocyst development.

Materials and Methods

Ethical approval

All animal procedures followed the protocol approved by the Animal Experimentation Ethics Committee at Hankyong National University (permission number: 2018-3).

Collection of oocytes and in vitro maturation

Following the sacrifice of the animal at the slaughterhouse, the porcine ovary was placed in 35°C physiological saline (0.85%) and transported to the laboratory within 2 h. The ovary sample was washed 3–4 times with physiological saline; then, using an 18-gauge needle attached to a 10-ml syringe, the follicular fluid and oocytes were collected from 2 to 6 mm ovarian follicles through aspiration. Only those oocytes with a high density of cumulus cells adhering in three or more layers with homogenous cytosol were selected. The selected oocytes were washed 3–4 times with the basic culture media based on Hepes-buffered tissue culture medium-199 (TCM-199; Gibco, MD) containing 50 µg/ml gentamycin (SK Chemical, Geyonggi, Korea) and 0.3% (*w/v*) fatty acid-free bovine serum albumin (BSA; Sigma, MO). The samples were then divided into a 10% fetal bovine serum (FBS, Gibco, MD) group and a 10% porcine follicle fluid (pFF) group, and washed 1–2 times with TCM-199 maturation culture solution containing 2.5 µg/ml FSH (Sigma, MO), 1 µg/ml estradiol-17β (Sigma, MO), 20 ng/ml epidermal growth factor (Sigma, MO), and 50 µg/ml gentamycin (SK Chemical, Geyonggi, Korea). 1 ml containing 200 oocytes was placed into each well of a four-well dish (Nunc, Roskilde, Denmark) containing 500 µl of the same culture solution. The primary *in-vitro* maturation

happened over 22 h in a 39°C, 5% CO_2 incubator. Following that, the cells were cultured for another 22 h for the secondary *in-vitro* maturation using the same medium, but without gonadotropic hormone (GTH).

In vitro fertilization and culture

Following *in-vitro* maturation, the mature oocytes were washed once with a modified Tris buffer medium (mTBM), 500 µl of mTBM, and 200 mature oocytes were placed in each well of a four-well dish. Then, 1.5 ml of porcine sperm (Darby, Gyeonggi, Korea) was mixed with 2 mg/ml BSA (Sigma, MO) and 3 ml of mTBM containing 1 mM caffeine (Sigma, MO). The mixture was centrifuged for 3 min, and after washing, the same procedure was repeated once. The collected sperms were diluted with mTBM to a concentration of 1×10^6 sperms/ml, and after the injection of the sperms, the wells were covered with mineral oil (Sigma, MO). The fertilization proceeded for 6 h in a 39°C, 5% CO_2 incubator. Following *in-vitro* fertilization, the embryo was washed once using the North Carolina State University-23 medium (NCSU-23), then 0.1 µM, 0.3 µM, and 0.5 µM rapamycin (Sigma, MO) were added. The culture was carried out in a four-well dish (Nunc, Roskilde, Denmark), using 1 ml of NCSU-23 as the basic medium, for 72 h in a 39°C, 5% CO_2 incubator. Afterward, the medium was replaced with a fresh medium of the same type, for a further 96 h of culture, and the rate of blastocyst formation was analyzed. The *in-vitro* culture medium was retrieved for use in subsequent analyses.

Real-time PCR

The total RNA was extracted from each blastocyst and the cDNA was produced using the first strand synthesis using (Oligo dT) (Invitrogen, Carlsbad, CA). Next, real-time PCR (RT-PCR; Real Time polymerase chain reaction) was carried out according to the one-Step SYBR RT-PCR kit (TaKaRa, Kusatsu, Japan). The PCR reaction was carried out as follows: first, one cycle of 15 min at 42°C and 2 min at 95°C; then, 40 cycles of 40 sec at 95°C, 15 sec at 58°C, and 30 sec at 60°C; finally, one cycle of 5 min at 72°C. The amplified DNA was quantified according to the cycle threshold (Ct) value based on the semi-log amplification plot of the genomic regions.

Western blot analysis

Blastocyst proteins were extracted using PRO-PREP™ (Intron biotechnology, Gyeonggi, Korea). To comparatively analyze the expression pattern of a specific protein, the proteins were separated through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene difluoride membrane (0.2 µm) using a semidry electroblotter apparatus. After blotting, the blocking

of the membrane with 5% skim milk blocking reagent was carried out for 2 h; then the antigen–antibody reaction was induced overnight using primary antibody at 4°C to remove unbound antibodies, and washing was carried out three times with Tris-buffered saline Tween20 (TBS-T) (1×Tris + 1×NaCl + 0.05% Tween 20) for 15 min. For the reaction with the secondary antibody, the reaction was allowed to proceed for 2 h at room temperature, after which washing three times with TBS-T for 15 min was carried out. The membrane was then fluorescent-stained using 2 ml of Lumi-Light substrate solution and exposed on an X-ray film for 1–10 min.

Immunofluorescence

After the fixing of the blastocyst in 4% formalin and stabilization in TBS-T containing 0.2% triton X-100, washing with 1× Phosphate-buffered saline (PBS) for 3 min was carried out two times. Blocking with 5% normal horse serum was carried out for 20 min at room temperature. To induce the antigen–antibody reaction, the primary antibody was diluted with the blocking solution in a 1:250 ratio; then, the reaction was allowed to proceed for 18 h at 4°C. Next, washing with 1×PBS for 5 min was carried out two times and the reaction using the secondary antibody Alexa 594 (Red), 488 (Green) for 2 h at 37°C was carried out. Another two rounds of washing with 1×PBS for 5 min was carried out, followed by the count stain with Hoechst 33,342 and subsequent mounting using H-1000.

Zymography

For analyzing the enzymatic reaction of MMP, Fast of zymography (FOZ) loading buffer (5% bromophenol blue, 10% SDS, and 2% glycerol) was used as the zymography reaction solution. It was mixed with the *in-vitro* culture medium in a ratio of 2 μg:10 μl, and then the mixture was allowed to react for 5 min on ice. Next, electrophoresis was carried out on a gelatin SDS-PAGE gel, containing 100 mg/ml gelatin, for 1 h 30 min at 150 V. After electrophoresis, the renaturation buffer (2.5% triton X-100, 1×PBS) was applied to the gel for 20 min to induce protein renaturation

twice; then, the gel was washed with sterile water for 20 min. Following renaturation, the gel was immersed in the zymography reaction buffer (1M tris-HCl, pH 7.5, 5M NaCl, 1M CaCl2, 0.2 mM ZnCl2, 0.2% triton X-100, and 0.02% NaN3) so that the enzymatic reaction could proceed for 18 h at 37°C. For the zymography gel following the reaction, protein staining was induced for 1 h using Coomassie Brilliant Blue (Bio-rad, CA); then, after the destaining step, the resulting bands were analyzed.

DNA fragmentation

The cells and tissues were pulverized and then mixed with fragment assay buffer (20 mM EDTA, 0.5% (*v/v*) Triton X-100, and 5 mM Tris-C1, pH 8.0). After reacting for 2 h at room temperature, the solution was centrifuged for approximately 10 min at 14,000 rpm. Afterward, the DNA was extracted using the phenol extraction method. Following the dissolution in TE buffer (10 mM Tris, 1 mM EDTA, and pH 8.0), electrophoresis was carried out on a 1.2% agarose gel for 1 h.

Results

The cleavage rate and blastocyst development rate based on the addition of rapamycin during in vitro culture

The addition of rapamycin during *in-vitro* culture resulted in increased cleavage and blastocyst development rates with an increase in rapamycin. Compared to the group whose *in-vitro* maturation was induced using 10% FBS, the group whose maturation was induced using 10% pFF showed higher cleavage and blastocyst development rates (Table 1).

Regarding the blastocysts developed, the 10% FBS-induced *in-vitro* maturation group showed a fall in the trophoblast density in the blastocyst cavity, with a simultaneous fall in cellular homogeneity. On the contrary, the 10% pFF-induced group showed high homogeneity for the trophoblast and inner cell mass, while blastocoel formation was observed (Fig. 1).

Table 1. Embryo development rate of *in-vitro* culture treated with rapamycin.

| Group | Volume of rapamycin | No. oocytes used | No. zygote | Developed to (%) | |
				Cleavage(4 cell<)	Blastocyst
10% FBS	0.1 μM	211	63 (29.86%)	53 (25.12%)	10 (4.74%)
	0.3 μM	203	71 (34.98%)	58 (28.57%)	13 (6.40%)
	0.5 μM	194	77 (39.69%)	62 (31.96%)	15 (7.73%)
10% pFF	0.1 μM	228	55 (24.12%)	43 (24.12%)	12 (5.26%)
	0.3 μM	190	71 (37.37%)	57 (30.00%)	14 (7.37%)
	0.5 μM	230	124 (53.91%)	97 (42.17%)	*27 (11.74%)

Ovum were divided into groups using 10% FBS and 10% pFF during *in-vitro* maturation, and each group was divided into 0.1, 0.3, and 0.5 μM, depending on the amount of rapamycin added during the *in vitro* culture. The group with the highest incidence and blastocyst development rate was the group with 0.5 μM of rapamycin added to 10% pFF.
*Different letters within the same column represent a significant difference (*p* < 0.05).

The gene expression patterns for apoptosis and MMPs in blastocysts based on the addition of rapamycin

The results of analyzing the expression pattern of MMPs are presented in Figure 2. For MMP-2, the level of expression was generally higher in the 10% FBS group than in the pFF group, while protein activities were also higher. For MMP-9, the level was higher in the pFF group, and an identical result was shown by protein activities in the embryo. For the expression pattern of TIMPs, the MMP inhibitors and
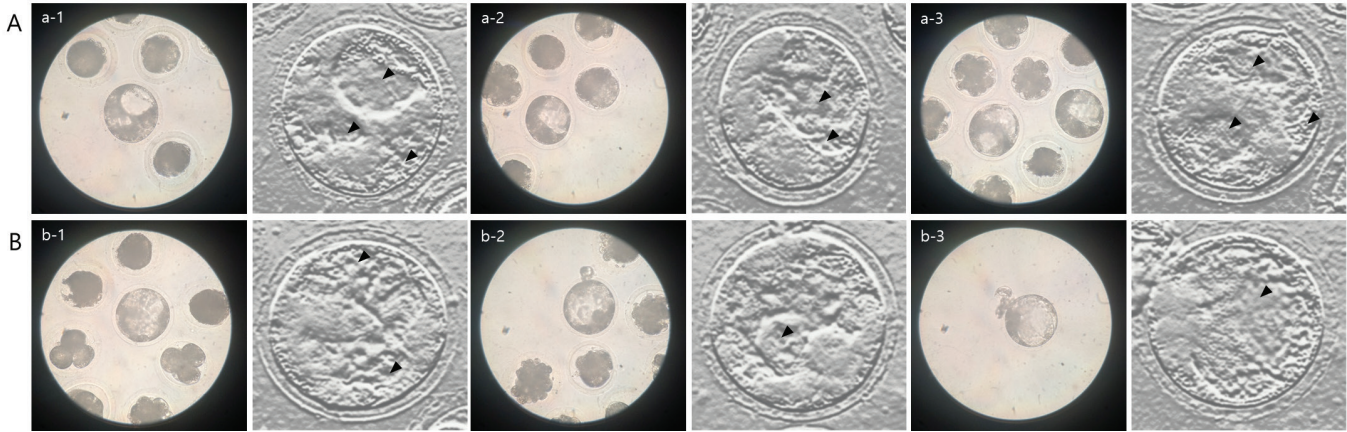

Figure 1. Development of blastocysts in each culture condition. The gray shaded photograph was taken by enlarging only one blastocyst. The black arrow points to the blastocyst cavity. (A) 10% FBS, a-1 : 0.1 µM rapamycin , a-2 : 0.3 µm rapamycin , a-3 : 0.5 µM rapamycin (B) : 10% pFF , b-1 : 0.1 µM rapamycin , b-2 : 0.3 µM rapamycin , b-3 : 0.5 µM rapamycin.
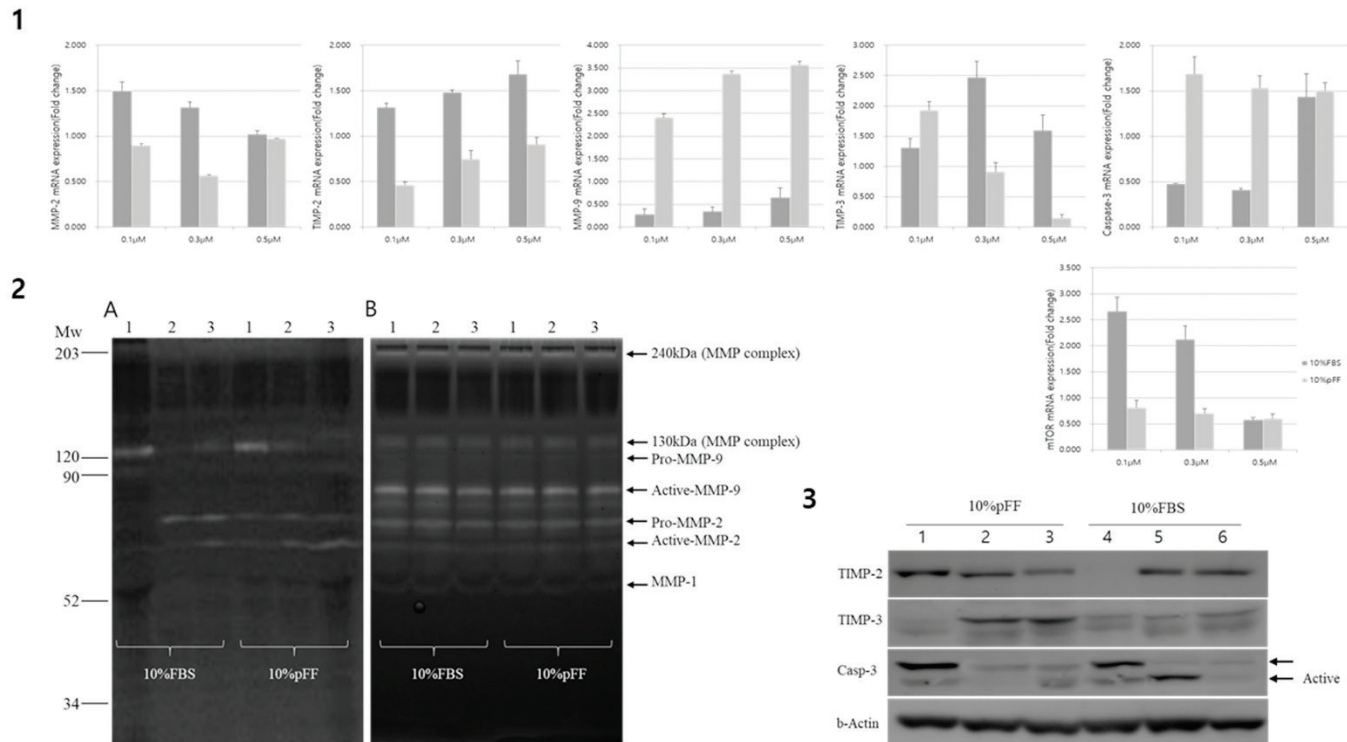

Figure 2. Expression analysis of MMP and Casp-3 gene of the FBS group and pFF group in embryos. (1) Real-time PCR analysis, (2) zymography analysis, (3) Western blot, 2-A: embryo protein, 2-B: culture medium, 3-1,4: 0.1 uM rapamycin group, 3-2,5: 0.1 uM rapamycin group, 3-3,6: 0.1 uM rapamycin group. Real-time PCR data represent mean ± SEM of five individual experiments and were normalized against β-actin (housekeeping gene) as an internal standard. *Different letters within the same column represent a significant difference ($p < 0.05$).

contrasting patterns to MMPs were found in general (Fig. 2(1) and (2)); in both FBS and pFF groups, the addition of 0.1 µM rapamycin was shown to have led to the increase in MMP-9 activity (Fig. 2(2)). On the contrary, the expression pattern of CASP-3 was such that the mRNA expression was higher in the pFF group than in the FBS group, while the expression of active-CASP-3 was higher in the FBS group than in the pFF group. In the case of the pFF group, a low-level expression of active-CASP-3 was found across all groups, with varying concentrations of rapamycin, but in the FBS group, most groups showed a relatively high level of activity, except in the group to which 0.3 µM rapamycin had been added (Fig. 2(3)).

The analyses of MMP-2 and CASP-3 expression and gDNA fragmentation in blastocysts

The level of apoptosis was shown to have increased with an increase in DNA fragmentation due to nucleic acid denaturation in the 10% FBS group. A contrasting result was found in the10% pFF group, where the level of DNA fragmentation was low, irrespective of the added concentration of rapamycin. For CASP-3, the expression pattern showed a fall dependent on the concentration of rapamycin in both 10% FBS and pFF groups. The expression favored the inner cell mass more than the trophoblast, and the level was high in the 10% FBS group, but lower in the 10% pFF group. For MMP-2, the expression was between the inner cell mass and the trophoblast, while the level was slightly higher for the trophoblast. The expression was higher in the 10% FBS group than in the 10% pFF group, and it did not significantly differ according to the added concentration of rapamycin (Fig. 3).

Discussion

The porcine *in-vitro* embryo has a substantial problem of reduced development caused by multiple sperm penetration and intracellular metabolic errors. Of note is the spread of apoptosis caused by cytoplasmic destruction that poses the most massive problems of reduced development in an *in-vitro* embryo or other types of embryos. The induction of apoptosis in an *in-vitro* embryo occurs upon embryo disintegration. As the embryo cannot derive macrophage action independently, nucleic acid denaturation may result from cytotoxicity. Thus, the present study investigated whether the addition of rapamycin, that can regulate apoptosis and promote the formation of autophagy, would control apoptosis in an embryo [21,22], or the generation of MMPs for cytoplasmic activities while analyzing the consequent influence on embryo development. Apoptosis regulation plays a critical role in embryogenesis, where it is involved in the degradation of maternal proteins and the synthesis of new proteins. The failure of these processes causes developmental arrest and low embryo development rates [23].

Especially, our results support the conclusion that rapamycin-induced apoptosis control has a positive effect on porcine blastocyst development. The findings in this study indicate that the addition of rapamycin to increase
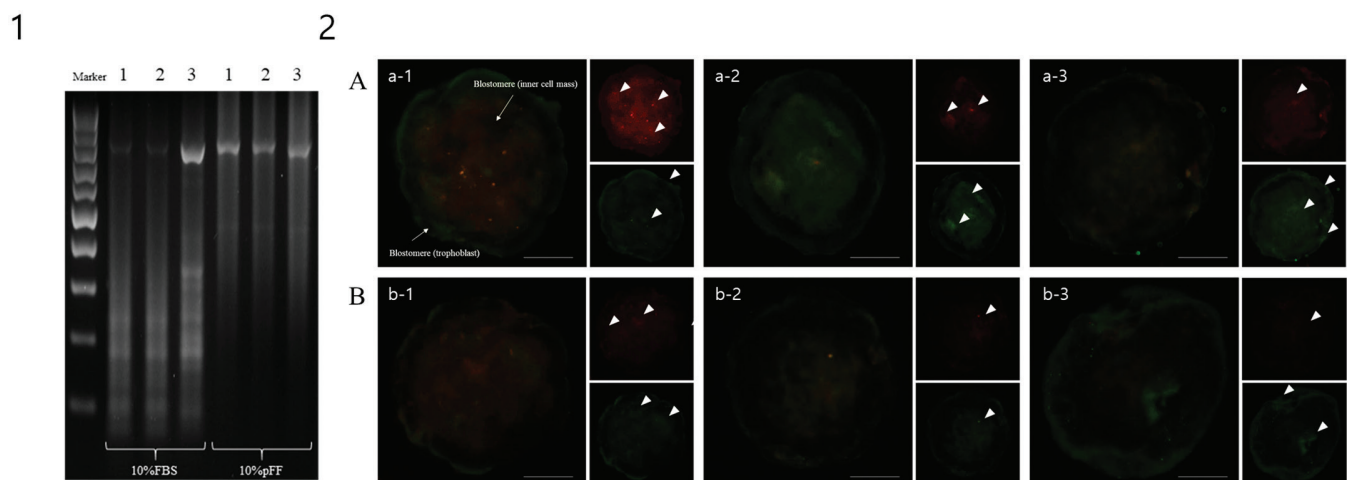

Figure 3. Analysis of cell death in blastocysts of each treatment group. (1) DNA fragmentation analysis in porcine blastocyst gDNA. 1-1: 0.1 µM rapamycin, 1-2: 0.3 µM rapamycin, 1-3: 0.5 µM rapamycin. (2) The localization analysis of Casp-3 and MMP-2 protein in blastocyst. Red fluorescence is an expression of Casp-3 and green fluorescence is an expression of MMP-2. White arrows indicate the major expression localization. The large picture is the fluorescence merging, and the small picture is the detected antibody. A: 10% FBS , a-1: 0.1 µM rapamycin , a-2: 0.3 µM rapamycin , a-3: 0.5 µM rapamycin, B: 10% pFF, b-1: 0.1 µM rapamycin, b-2: 0.3 µM rapamycin, b-3: 0.5 µM rapamycin.

autophagy, as one process of apoptosis, leads to the inhibition of CASP-3, which is involved in intracellular apoptosis. An increase in the development of embryonic cells during the regulatory process was also observed, which coincided with the report of Lee et al. [24], which states that there is a deep association between the apoptotic mechanism, oocyte maturation, and blastocyst development. At the same time, rapamycin was also shown to exert an influence on the cellular metabolism in the embryo.

In other words, rapamycin treatment can affect embryonic development through beneficial effects [22,23]. Thus, our study found that apoptosis regulation induced by rapamycin treatment may have a temporary positive effect on the incidence of *in-vitro* embryo.

Furthermore, the addition of rapamycin to FBS or pFF cultures was shown to increase embryo development to a higher degree in the pFF group than in the FBS group, while the development in the FBS group was comparatively reduced. This result is in agreement with Naito et al. [25], who reported a reduced cellular development in the FBS culture. Notably, the higher level of increase in DNA fragmentation suggested that the rapamycin effect was lowered in the FBS culture, whereas rapamycin treatment in the pFF culture was shown to prevent nucleic acid alteration or denaturation, which is thus thought to reduce the problem of apoptosis. The result, as in Lee et al. [26], indicated that the effect of rapamycin led to increased cell survival and blastocyst development. In addition to apoptosis, this study examined whether the effect of rapamycin extended to the generation of MMPs involved in cellular development and facilitated differentiation, and the result showed that the activities of MMPs differed in each culture group; in particular, the generation of MMP-9 that allows an intracellular sub-space to be acquired was found to increase in the pFF group treated with rapamycin, implying a potential role in embryo development as well [27].

The role of rapamycin was found to be critical in this study, while it showed an association with the cellular reorganization, blastocyst development, and apoptosis. This study focused on the actions of rapamycin and follicle fluid that can increase such a role, and the findings suggested that the cellular metabolic processes related to the maturation of the embryo are influenced by, first, the activities of MMPs whose expression patterns differ between trophoblast and inner cell mass; second, the expression pattern of CASP-3, a crucial apoptotic factor; and finally, the selective regulation of MMPs [28]. The findings in this study may not imply that rapamycin controls the entire process of apoptosis; nevertheless, the implications are that there is, at the very least, a substantial influence from rapamycin on cell growth. In particular, studies to increase the morphological safety and development rate of blastocysts in pigs with very low implantation efficiency of *in-vitro* fertilized embryos are very efficient. Therefore, we suggest that apoptosis regulation plays a critical and selective survival role in porcine *in-vitro* embryos, but further studies are needed to elucidate the specific molecular mechanisms underlying PCD in the development of porcine *in-vitro* embryos.

Conclusion

In this study, the formation of autophagy as a main effect of rapamycin was not discussed, while the focus was on whether the rapamycin effect in FBS or pFF culture could indeed regulate apoptosis and MMPs. As a result, the effect of rapamycin in regulating apoptosis and MMPs during cellular development was verified. These findings can serve as primary data for establishing the hypotheses regarding the cellular differentiation and facilitated development based on the concurrent actions of pFF and rapamycin. It is also anticipated that a theory may be suggested wherein the promotion or inhibition of apoptotic factors due to basic culture conditions influences the mechanism of cellular development.

Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contribution

DS Kim and SH Kim participated in developing the protocol, the sample granulosa cell, and drafting the manuscript. DS Kim and SH Kim participated in the identification of the development of the database. JT Yoon contributed to the translation of the manuscript and supervised the analysis.

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