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In vitro maturation on a soft agarose matrix enhances the developmental ability of pig oocytes derived from small antral follicles

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ABSTRACT *In vivo* oocytes grow and mature in ovarian follicles whereas oocytes are matured *in vitro* in plastic culture dishes with a hard surface. *In vivo* oocytes show a superior developmental ability to *in vitro* counterparts, indicating suboptimal environments of *in vitro* culture. This study aimed to evaluate the influence of an agarose matrix as a culture substrate during *in vitro* maturation (IVM) on the development of pig oocytes derived from small antral follicles (SAFs). Cumulus-oocyte complexes (COCs) retrieved from SAFs were grown in a plastic culture dish without an agarose matrix and then cultured for maturation in a plastic dish coated without (control) or with a 1% or 2% (w/v) agarose hydrogel. Then, the effect of the soft agarose matrix on oocyte maturation and embryonic development was assessed by analyzing intra-oocyte contents of glutathione (GSH) and reactive oxygen species (ROS), expression of *VEGFA*, *HIF1A*, and *PFKP* genes, and blastocyst formation after parthenogenesis. IVM of pig COCs on a 1% (w/v) agarose matrix showed a significantly higher blastocyst formation, intra-oocyte GSH contents, and transcript abundance of *VEGFA*. Moreover, a significantly lower intra-oocyte ROS content was detected in oocytes matured on the 1% and 2% (w/v) agarose matrices than in control. Our results demonstrated that IVM of SAFs-derived pig oocytes on a soft agarose matrix enhanced developmental ability by improving the cytoplasmic maturation of oocytes through redox balancing and regulation of gene expression.

Keywords: agarose matrix, embryonic development, *in vitro* maturation, small antral follicle

INTRODUCTION

Mammalian oocytes grow and mature in the finely controlled and specialized microenvironment of ovarian follicles. Ovarian follicles are composed of soft tissues including various types of cells such as granulosa and thecal cells. Immature oocytes are surrounded by granulosa cells (GCs). GCs are tightly attached to oocytes and differentiate into cumulus cells during follicular development to form cumulus-oocyte complexes (COCs) (Strączyńska et al., 2022). COCs communicate with the cellular and non-cellular niches of ovarian follicles (Rodgers et al.,

2003) and play a nurturing role via transferring into the oocytes of nutrients and small molecules such as glucose metabolites and RNA transcripts (Campen et al., 2017). Therefore, the functional cell-to-cell and cell-to-matrix interactions between CCs and oocytes are crucial for the development of highly fertile oocytes.

Especially, CCs perform an important role in oocyte nurturing through the transfer of glucose metabolites and RNA transcripts (Sugiura and Eppig, 2005; Campen et al., 2017). The glucose metabolic enzyme is known as phosphofructokinase platelet (PFKP) in CCs (Matsuno et al., 2016). The crucial glucose substrates produced by CCs are provided to oocytes for meiosis and ATP synthesis (Gu et al., 2015). Increasing glucose metabolism in CCs enhances oocyte developmental competence. Moreover, during *in vitro* maturation (IVM), CCs synthesize glutathione (GSH) which is a non-enzymatic antioxidant in mammalian cells. GSH is transported from CCs into oocytes through gap junctions and improves oocyte maturation and embryonic development via the protection of oocytes from the oxidative stress that is generated by reactive oxygen species (ROS) (Pu et al., 2014). Therefore, the essential role of CCs is emphasized in the energy and antioxidant generation of oocytes.

A conventional IVM system for pig COCs derived from SAFs has been developed by evaluating the effects of physiological and physico-chemical factors on oocyte development (Saraiva et al., 2012). Recently, the effectiveness of soft culture substrate using polyacrylamide gels (PAGs) on *in vitro* growth (IVG) of oocyte-granulosa cell complexes (OGCs) has been reported (Munakata et al., 2017). In a culture of various cells including oocytes and stem cells, mimicking the stiffness of soft tissues in the body induced proper cellular behaviors similar to those *in vivo* (Munakata et al., 2017; Park et al., 2017). Therefore, it has been suggested that the IVM microenvironment using a soft matrix would play a beneficial role in oocyte maturation and later development by influencing bidirectional communications between COCs and the surrounding matrix.

Agarose is a natural polysaccharide included in marine red algae and has many advantages; it is inexpensive, nontoxic, and biocompatible (Shin et al., 2016). Agarose hydrogels, due to their porosity and tissue-like soft properties, have been used for tissue engineering or three-dimensional (3D) culture of various cell types including chondrocytes (DiFederico et al., 2017) and embryonic stem cells (Baek et al., 2017). Recently, the stimulating effects of 3D agarose hydrogels on the competence of self-renewal and proliferation of spermatogonial stem cells have been reported in pigs (Park et al., 2017). Based on the previous finding, it was hypothesized in this study that an IVM microenvironment made up of a soft agarose matrix would enhance the developmental ability of pig oocytes by influencing cellular responses to the tissue stiffness adjusted similarly to that *in vivo*. To test this hypothesis, we designed an IVM system using an agarose matrix and evaluated the effect on the oocyte maturation and embryonic development of SAF-derived pig oocytes by analyzing intra-oocyte GSH and ROS contents, expression of *VEGFA*, *HIF1A*, and *PFKP*, and blastocyst formation after parthenogenesis (PA).

MATERIALS AND METHODS

Oocyte collection and *in vitro* growth (IVG)

Ovaries of pre-pubertal gilts were collected from a local slaughterhouse. Cumulus-oocyte-complexes (COCs) were aspirated from SAFs with diameters of 2 to 3 mm. The COCs were then washed in HEPES-buffered Tyrode's medium (TLH) supplemented with 0.05% (w/v) polyvinyl alcohol (PVA) (TLH-PVA). COCs were cultured for 2 days for IVG in a well of a 4-well multidish (Nalge Nunc International, Roskilde, Denmark) containing 500 μL of IVG medium consisting of minimum essential medium alpha (α-MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 1 mM dibutyryl cAMP sodium salt (dbcAMP), 0.4 mM sodium pyruvate, 75 μg/mL kanamycin, and 8 μg/mL follicle stimulating hormone (FSH) (Antrin R-10; Kyoritsu Seiyaku, Tokyo, Japan) at 39℃ with a humidified atmosphere of 95% air and 5% CO_2 (Shin et al., 2021). All reagents and chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (IACUC Approval No. KW-200302-1) and all experimental procedures were conformed to the guidelines according to the Animal Care and Use Guideline of Kangwon National University.

Preparation of an agarose matrix

The agarose matrix was prepared using a method as described previously (Park et al., 2020). Briefly, a 2% (w/v) agarose solution was prepared by dissolving agarose powder in α-MEM with heating. To make a 1% (w/v) agarose hydrogel with different stiffness, the 2% (w/v) agarose solution was diluted with α-MEM. Then, 200 μL of each 1% and 2% (w/v) agarose solution was overlaid on each well of a 4-well multidish. The coated agarose matrix was solidified by placing it for 20 min at 39℃ in humidified 95% air and 5% CO_2. Finally, the agarose matrix was equilibrated overnight under an IVM medium. The equilibrated medium was exchanged with a fresh medium before use.

IVM

IVM culture was performed as reported in our previous work (Park et al., 2021). The medium for IVM of oocytes was medium 199 supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM L-cysteine, 0.4 mM sodium pyruvate, 75 μg/mL kanamycin, 15 ng/mL mouse epidermal growth factor (EGF), 1 μg/mL insulin. Immature COCs grown for 2 days *in vitro* were cultured for 22 h in IVM medium containing 10 IU/mL human chorionic gonadotropin (hCG; Intervet International BV, Boxmeer, Holland) and 80 μg/mL FSH on a 4-well multidish coated without (control) or with a 1% or 2% (w/v) agarose at 39℃ under a humidified atmosphere of 95% air and 5% CO_2. Then, COCs were transferred to a hormone-free IVM medium and cultured for an additional 22 h. The IVM oocytes were denuded from cumulus cells by gentle pipetting in a 0.06% (w/v) hyaluronidase medium.

PA and *in vitro* culture (IVC) of PA embryos

Metaphase II (MII) oocytes were electrically activated for PA in 280 mM D-mannitol solution supplemented with 0.05 mM $MgCl_2$ (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 0.1 mM $CaCl_2$ (Wako Pure Chemical Industries) by applying two direct current pulses of 120 V/mm for 60 μs (Park et al., 2020). The activated oocytes were exposed for 4 h to 5 μg/mL cytochalasin B in an IVC medium consisting of porcine zygote medium (PZM)-3 that was supplemented with 2.77 mM myo-inositol, 10 μM β-mercaptoethanol, and 0.34 mM trisodium citrate (Wako Pure Chemical Industries) (You et al., 2012). Then, PA oocytes were cultured for 7 days in an IVC medium at 39℃ with an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Embryo cleavage and blastocyst formation were evaluated on Days 2 and 7, respectively (the day of PA was Day 0). The mean cell number of PA blastocysts was determined under an epifluorescence microscope (TE300, Nikon, Tokyo, Japan) after bisbenzimide staining.

Measurement of intra-oocyte GSH and ROS contents

After IVM, MII oocytes were examined for GSH and ROS contents by using specific blue and green fluorescence assays (Park et al., 2021). Intra-oocyte GSH and ROS contents were analyzed by staining with Cell-Tracker Blue CMF_2HC (4-chloromethyl-6,8-difluoro-7-hydroxycoumarin; Invitrogen) and H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate; Invitrogen), respectively. Oocytes were placed for 30 min in the dark in TLH-PVA supplemented with 10 μM Cell-Tracker for GSH and 20 μM H_2DCFDA for ROS detection. And then oocytes treated with Cell-Tracker were incubated in IVC medium for 30 min at 39℃ under humidified 95% air and 5% CO_2. After staining, oocytes were washed with DPBS-PVA and transferred into 2 μL washing droplets. Each fluorescence was detected under an epifluorescence microscope at 370 and 460 nm for GSH and ROS. The fluorescence intensity of GSH and ROS was quantified by the Image J software (version 1.48v).

Quantitative real-time polymerase chain reaction (RT-qPCR)

Total mRNA was extracted using a Dynabeads® mRNA Direct™ kit (Ambion, Austin, TX, USA), which was followed by cDNA synthesis using a ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan), according to the manufacturers' instructions. Subsequently, RT-qPCR was performed using Thunderbird™ SYBR® qPCR Mix (Toyobo) and a 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). PCR specificity was verified by analyzing melting curve data, and the mRNA levels of genes were normalized to that of the reference gene (*ACTB*) (Park et al., 2020). Relative mRNA levels were calculated as $2^{-\Delta\Delta Ct}$, where Ct = threshold cycle for target amplification, $\Delta Ct = Ct_{target\ gene}$ (specific gene in a sample) - $Ct_{internal\ reference}$ (reference gene in same sample), and $\Delta\Delta Ct = \Delta Ct_{sample}$ (treatment sample in each experiment) - $\Delta Ct_{calibrator}$ (control sample in each experiment). Table 1 shows the primer sequences designed using Primer3 software (Whitehead Institute/MIT Center for Genome Research), which were based on cDNA sequences derived

Table 1. Primers used for gene expression analysis

Genes	GenBank number	Primer sequence		Size (bp)
		Sense (5' > 3')	Anti-sense (5' > 3')	
ACTB	XM_003357928.1	TCCCTGGAGAAGAGCTACGA	TGTTGGCGTAGAGGTCCTTC	187
HIF1A	NM_001123124.1	AGCCAGATGATCGTGCAACT	CCATTGATTGCCCCAGGAGT	144
PFKP	XM_021065066.1	TCGAGAGCAACCTGAACACC	CACGGCGAACATCTTGTGTC	179
VEGFA	NM_214084.1	TCGGAGCGGAGAAAGCATTT	CGGCTTGTCACATCTGCAAG	129

ACTB, actin beta; BAX, Bcl-2-associated X protein; CX43, gap junction protein alpha 1 (GJA1); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFPT1, glutamine-fructose-6-phosphate aminotransferase 1; HIF1A, hypoxia inducible factor 1 subunit alpha; PFKP, phosphofructokinase platelet; VEGFA, vascular endothelial growth factor A.

Table 2. Effects of agarose matrix during *in vitro* maturation of small antral follicle-derived oocytes on embryonic development after parthenogenesis (PA)

Agarose matrix	% of oocytes reached MII	No. of PA embryos cultured[a]	% of embryos developed to		No. of cells in blastocyst
			≥ 2 cells	Blastocyst	
Non-coated	72.5 ± 5.2	174	93.8 ± 0.2	41.0 ± 2.2[b]	43.5 ± 2.5
1%	71.4 ± 3.3	169	97.5 ± 0.5	53.6 ± 2.6[c]	42.3 ± 2.0
2%	69.0 ± 1.2	166	95.6 ± 2.5	41.3 ± 1.8[b]	44.6 ± 2.2

[a]Five replicates. [b,c]Values in the same column with different superscript letters are significantly different ($p < 0.05$). MII, metaphase II.

from GenBank for pigs.

Statistical analysis

Analyses of statistical significance were performed by using the Statistical Analysis System (version 9.4; SAS Institute, Cary, NC, USA). Before analysis, percentage data were arcsine transformed to maintain the homogeneity of variance. The data were analyzed by one-way ANOVA. Post hoc analysis to identify between-group differences was performed using the least significant difference (LSD) test. Significant differences among treatments were acknowledged when a p-value was less than 0.05. All data are shown as the mean ± standard error of the mean (SEM).

RESULTS

Effects of agarose matrix on oocyte maturation and blastocyst formation

To determine the effect of soft culture matrix during IVM, pig COCs matured on non-coated (control) culture dishes or dishes coated with a 1% or 2% (w/v) agarose matrices were examined for nuclear maturation and blastocyst formation after PA. A significantly higher blastocyst formation was observed in the 1% (w/v) agarose matrix (Table 2). In our preliminary study, we identified that IVG culture on the 1% (w/v) agarose matrix improved the PA development of SAF-derived pig oocytes. Therefore, we evaluated the combined effects of IVG and/or IVM on the 1% (w/v) agarose matrix on the nuclear maturation and blastocyst formation after PA. As the results, IVG and/or IVM on the 1% (w/v) agarose matrix showed a significantly higher blastocyst formation than control (Table 3). Furthermore, both IVG and IVM culture on the 1% (w/v) agarose matrix resulted in a significantly higher blastocyst cell number than only IVG culture on the 1% (w/v) agarose matrix. However, IVG and IVM on the 1% (w/v) agarose matrix resulted in significantly lower nuclear maturation than IVG or IVM on the 1% (w/v) agarose matrix.

Effect of agarose matrix on intra-oocyte GSH and ROS contents of IVM oocytes

To determine whether the IVM on the agarose matrix affects the cytoplasmic maturation of porcine oocytes derived from SAFs, we analyzed the intra-oocyte GSH and ROS after IVM. Oocytes matured on a 1% (w/v) agarose matrix showed a significantly higher intra-oocyte GSH (Fig. 1C) and significantly lower ROS contents (Fig. 1D). Moreover, 2% (w/v) agarose matrix significantly decreased intra-oocyte ROS contents. These results show that 1% (w/v) agarose matrix improves the cytoplasmic maturation of oocytes by regulating intra-oocyte GSH and ROS contents.

Table 3. Effects of agarose matrix during *in vitro* growth (IVG) and/or *in vitro* maturation (IVM) of small antral follicle-derived oocytes on embryonic development after parthenogenesis (PA)

IVG and/or IVM on the 1% (w/v) agarose matrix		% of oocytes reached MII	No. of PA embryos cultured[a]	% of embryos developed to		No. of cells in blastocyst
IVG	IVM			≥ 2 cells	Blastocyst	
No	No	67.2 ± 3.7[bc]	136	92.7 ± 2.8	43.3 ± 1.9[b]	45.1 ± 2.7[bc]
Yes	No	72.7 ± 2.3[b]	120	96.6 ± 1.0	68.8 ± 2.5[c]	41.7 ± 1.9[b]
No	Yes	73.3 ± 2.2[b]	123	94.8 ± 2.9	58.2 ± 2.9[c]	43.1 ± 1.8[bc]
Yes	Yes	59.0 ± 2.1[c]	113	95.5 ± 2.9	61.4 ± 6.5[c]	49.0 ± 2.3[c]

[a]Three replicates. [b,c]Values in the same column with different superscript letters are significantly different ($p < 0.05$). MII, metaphase II.

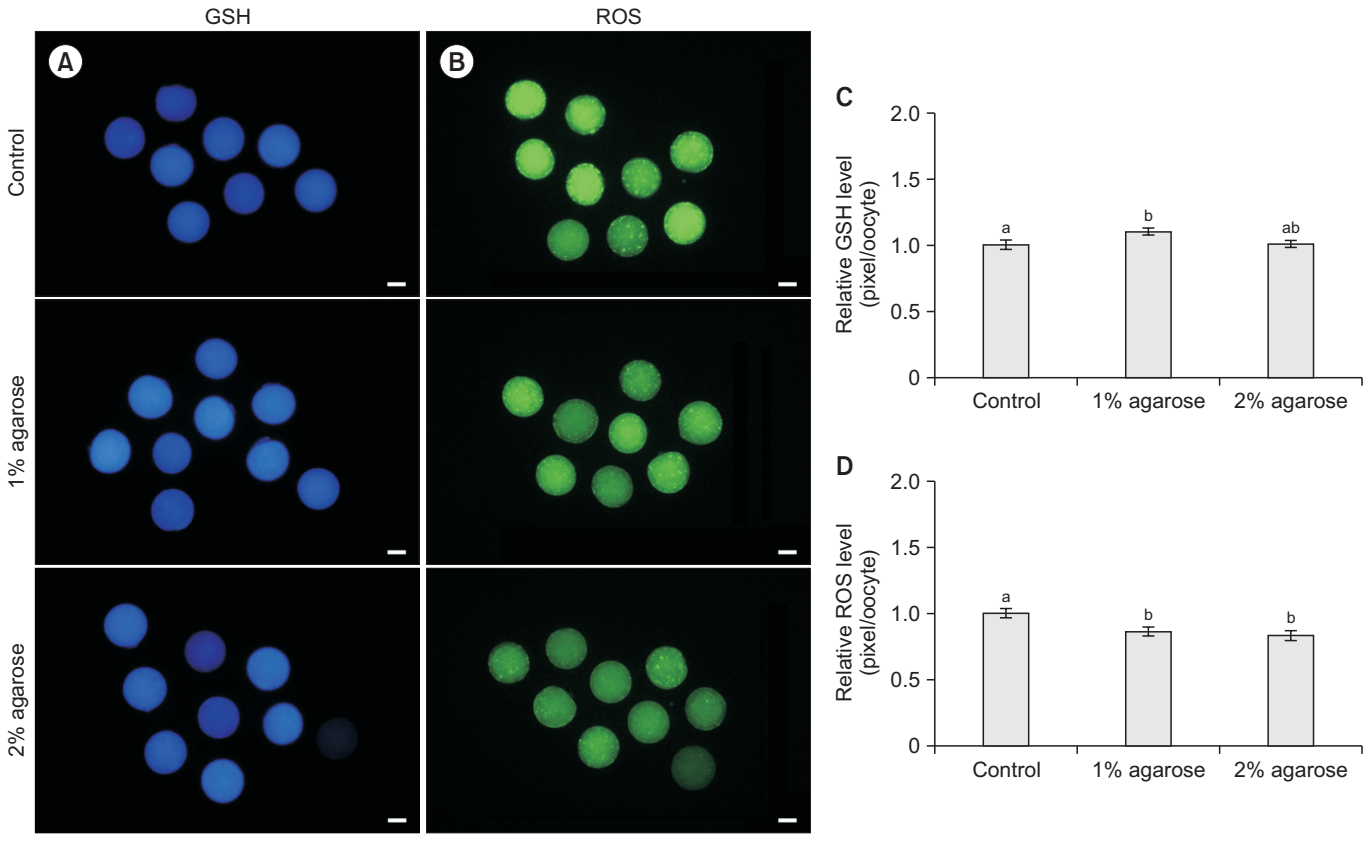

Fig. 1. Effects of an agarose matrix during *in vitro* maturation (IVM) on intra-oocyte glutathione (GSH) and reactive oxygen species (ROS) contents. Cumulus-oocyte complexes retrieved from small antral follicles were grown *in vitro* on the hard culture dish without an agarose matrix and then matured on the hard culture dish without (control) or with a 1% or 2% (w/v) agarose matrix. GSH and ROS contents in MII oocytes were analyzed by staining oocytes with Cell-Tracker Blue (A) and H2DCFDA (B), respectively, and determining fluorescence intensity. The GSH contents were significantly increased in the 1% (w/v) agarose matrix compared to control (C). A significant reduction of ROS contents was detected in the 1% and 2% (w/v) agarose matrix (D) compared to the control. All data are represented as the means ± SEM. [a,b]Bars with different letters are significantly different ($p < 0.05$). The scale bar is 50 μm.

Effects of agarose matrix on gene expression of CCs

The transcriptional regulation of *VEGFA*, *HIF1A*, and *PFKP* in CCs of IVM oocytes was determined quantitatively by real-time PCR. The transcript abundance of *VEGFA* (Fig. 2A) was significantly increased in CCs of oocytes that were matured on a 1% (w/v) agarose matrix compared to that of control. The transcript abundance of *HIF1A* was not altered by culturing oocytes on the agarose matrix (Fig. 2B). *PFKP* gene expression was significantly reduced in CCs that matured on the 2% (w/v) agarose matrix (Fig. 2C).

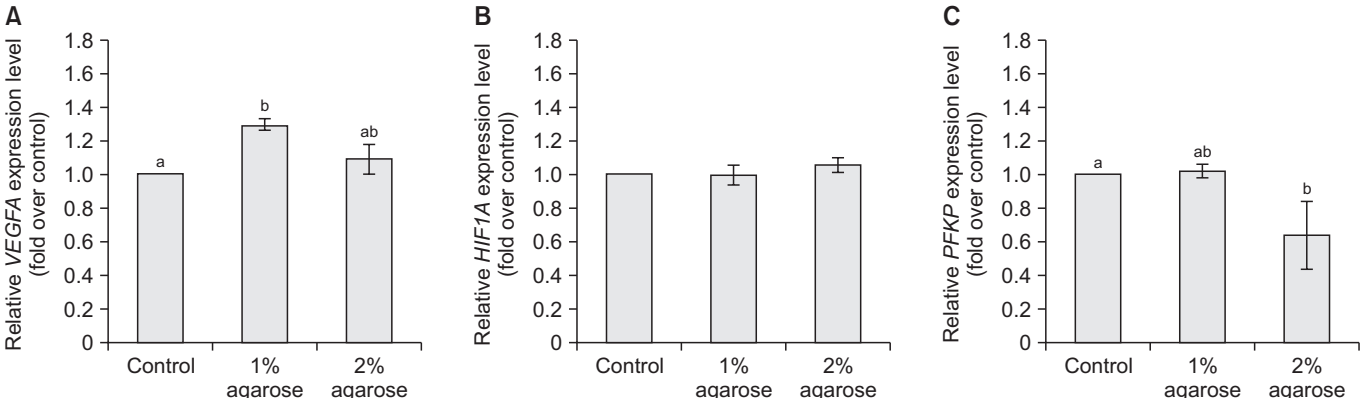

Fig. 2. Effects of an agarose matrix during *in vitro* maturation (IVM) on *VEGFA*, *HIF1A*, and *PFKP* expression in cumulus cells (CCs). Cumulus-oocyte complexes retrieved from small antral follicles were grown *in vitro* on the hard culture dish without an agarose matrix and then matured on the hard culture dish without (control) or with a 1% or 2% (w/v) agarose matrix. Gene expression levels in cumulus cells of *in vitro* matured COCs were analyzed quantitatively by a real-time PCR. The transcript abundance of *VEGFA* was significantly increased in CCs that matured on a 1% (w/v) agarose matrix compared to that of control (A). The transcript abundance of *HIF1A* in CCs of control oocytes and matured on agarose matrices were not significantly different (B). The transcript abundance of *PFKP* was significantly reduced in CCs that matured on the 2% (w/v) agarose compared to that of control (C). All data are represented as the means ± SEM. [a,b]Bars with different letters are significantly different ($p < 0.05$).

DISCUSSION

We evaluated the effects of a soft agarose matrix during IVM culture on the maturation and embryonic development of pig oocytes derived from SAFs. It was observed that oocytes matured on the 1% (w/v) agarose matrix generated more blastocysts than control oocytes that were cultured in a plastic dish without an agarose matrix. *In vivo*, cells are organized in three-dimensional structure (Levental et al., 2007), and cellular responses are controlled by cell-to-matrix and cell-to-cell communications (Yamada and Cukierman, 2007). Our results of improved embryonic development might be attributed to enhanced cytoplasmic maturation of oocytes mediated by the cellular response to the soft culture matrix of agarose.

Oocytes matured on a 1% (w/v) agarose matrix had significantly higher intra-oocyte GSH contents than control oocytes. Inversely, oocytes matured on 1% and 2% (w/v) agarose matrices expressed a significantly lower level of intra-oocyte ROS than control oocytes. Generally, GSH is synthesized in CCs during oogenesis and plays a key role in oocyte development by playing as an antioxidant because oxidative stress generated by ROS is one of the major causes for the deterioration of oocyte quality and subfertility (Pu et al., 2014; Yahfoufi et al., 2020). The GSH content was significantly increased in oocytes with a high number of CCs than in cumulus-denuded oocytes (Tatemoto et al., 2000). In this study, the 1% (w/v) agarose matrix showed up-regulation of the VEGFA gene that was known to regulate GCs proliferation (Shiratsuki et al., 2016). Therefore, it was speculated that the proliferation of CCs from COCs that were matured on a 1% (w/v) agarose matrix improved oocyte quality by increasing intra-oocyte GSH content via altered bidirectional communication between COCs and soft agarose matrix.

Generally, PFKP shows a greater expression in CCs to provide glucose as an energy source into oocytes via glycolysis (Su et al., 2009). According to Sugiura and Eppig (2005), paracrine factors secreted by oocytes influence *PFKP* expression because the mRNA level of *PFKP* was decreased in oocytectomized mouse CCs that were cultured in the absence of fully grown oocytes in comparison to those cultured with fully grown oocytes (Marchal et al., 2003). Moreover, the expression of PFKP in CCs from mature COCs was higher than those from immature COCs (Shen et al., 2020). PFKP expression in CCs is an indicator of the developmental competence of oocytes (Machado et al., 2015). Our results demonstrated that the pig COCs matured on 2% (w/v) agarose matrix expressed lower levels of gene *PFKP* in CCs than control COCs.

To understand the effects of agarose, we analyzed the expression of several genes relating to mechanosensing signals including *vascular endothelial growth factor A* (*VEGFA*) and *heterodimeric complex of α* (*HIF1A*). Mechanosensing signals are the cellular responses to mechanical cues of the microenvironment such as substrate rigidity,

topology, and adhesiveness. Specifically, HIF1A plays a key role in converting the cellular metabolism from oxidative phosphorylation (OXPHOS) to glycolysis to provide energy for oocyte development (Chen et al., 2012). VEGFA regulates folliculogenesis as a major downstream effector of HIF1A (Doyle et al., 2010). A previous study reported up-regulation of the *VEGFA* and *HIF1A* genes in GCs during *in vivo* follicular development in pigs (Munakata et al., 2016). Follicular growth by the activation of the PI3K/mTOR pathway induces up-regulation of the *VEGFA* and *HIF1A* genes in bovine (Zhang et al., 2011). Moreover, soft substrate using PAG up-regulated the *VEGFA* and *HIF1A* genes in GCs collected from early antral follicles during IVG of pig oocytes (Munakata et al., 2017). From the result of up-regulated expression of the *VEGFA* in CCs by the culture in 1% (w/v) agarose matrix, it is suggested that the regulators of folliculogenesis such as *VEGFA* and *HIF1A* are important for obtaining oocyte developmental ability through mechanosensing signals.

CONCLUSION

In summary, it has been demonstrated that an IVM system with an agarose matrix provides a soft substrate microenvironment to oocytes and resultantly improves embryonic development by influencing cytoplasmic maturation explained by the increased *VEGFA* expression and intra-oocyte GSH and decreased intra-oocyte ROS contents. In this study, we developed an improved IVM culture system for pig oocytes derived from SAFs by incorporating an agarose matrix into an IVM microenvironment. Our findings will contribute to biomedical research by increasing the production efficiency of high-quality oocytes in pigs.

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Consent to Publish: Not applicable.

Availability of Data and Materials: Not applicable.

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