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Antioxidant effect of ergothioneine on *in vitro* maturation of porcine oocytes

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ABSTRACT

Background: Ergothioneine (EGT) is a natural amino acid derivative in various animal organs and is a bioactive compound recognized as a food and medicine.
Objectives: This study examined the effects of EGT supplementation during the *in vitro* maturation (IVM) period on porcine oocyte maturation and subsequent embryonic development competence after *in vitro* fertilization (IVF).
Methods: Each EGT concentration (0, 10, 50, and 100 μM) was supplemented in the maturation medium during IVM. After IVM, nuclear maturation, intracellular glutathione (GSH), and reactive oxygen species (ROS) levels of oocytes were investigated. In addition, the genes related to cumulus function and antioxidant pathways in oocytes or cumulus cells were investigated. Finally, this study examined whether EGT could affect embryonic development after IVF.
Results: After IVM, the EGT supplementation group showed significantly higher intracellular GSH levels and significantly lower intracellular ROS levels than the control group. Moreover, the expression levels of hyaluronan synthase 2 and Connexin 43 were significantly higher in the 10 μM EGT group than in the control group. The expression levels of nuclear factor erythroid 2-related factor 2 (*Nrf2*) and NAD(P)H quinone dehydrogenase 1 (*NQO1*) were significantly higher in the oocytes of the 10 μM EGT group than in the control group. In the assessment of subsequent embryonic development after IVF, the 10 μM EGT treatment group improved the cleavage and blastocyst rate significantly than the control group.
Conclusions: Supplementation of EGT improved oocyte maturation and embryonic development by reducing oxidative stress in IVM oocytes.

Keywords: Embryonic Development; antioxidant; reactive oxygen species; ergothioneine

INTRODUCTION

Pigs are important livestock species widely used as large animal biomedical models to study human health and disease owing to their anatomical and physiological similarities to humans [1]. The *in vitro* maturation (IVM) of oocytes is an essential step in assisted reproductive

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Conflict of Interest

None of the authors have any conflict of interest to declare.

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technologies [2]. *In vitro* oocytes have reduced the developmental competence compared to *in vivo* oocytes, which is a major issue [3]. Therefore, improving the quality of oocytes *in vitro* is essential for developing oocytes. Oocyte maturation consists of nuclear and cytoplasmic maturation [4]. During nuclear maturation, oocytes progress from the germinal vesicle (GV) breakdown stage in prophase I and through anaphase I/telophase I to metaphase II (MII) arrest [5]. Cytoplasmic maturation consists of all oocyte changes, including accumulation of protein, mRNA, and substrates; restructuring of the cytoskeleton and organelles (mitochondria, ribosome, endoplasmic reticulum, and cortical granules); and changes in cellular metabolism [6]. In oocyte maturation, complete maturation, including nuclear and cytoplasmic maturation, was the primary determinant of the developmental capacity of *in vitro* mature oocytes. Nevertheless, the abnormal developmental capacity of *in vitro* mature oocytes was lower than that of *in vivo* mature oocytes because of insufficient cytoplasmic maturation [7]. The oocyte maturation process required high-energy supply of adenosine triphosphates, which upregulated reactive oxygen species (ROS) generation by mitochondria [8]. Oxidative stress, caused by the overproduction of ROS in oocytes, is one of the numerous factors inducing this outcome. ROS are highly reactive and electron-accepting molecules derived from oxygen with unpaired electrons, such as hydroxyl radical (OH), peroxyl radical ($ROO^{\cdot}$), hydrogen peroxide (H_2O_2), and superoxide ion radical [9]. Moreover, excessive ROS formation overpowered the antioxidant capacity of the system, which can lead to oxidative stress [10]. Oxidative stress damages cells and tissues by producing an imbalance between free radicals and antioxidants [11]. Thus, several studies have been actively conducted on supplementing antioxidants to improve oocyte quality [12]. Ergothioneine (EGT) is a naturally occurring amino acid and a biochemical antioxidant present in large amounts in mushrooms. It is also a powerful natural antioxidant and cytoprotective agent within the cell [13]. An evaluation of the EGT distribution in the body revealed preferential accumulation in the organs and cells and secretions that were probably exposed to high oxygen levels, such as the liver, kidneys, erythrocytes, lens, and semen [14]. Hence, EGT exerted antioxidant effects on oxidative stress [15]. A recent study showed that the presence of EGT in the follicular fluid (FF) of domestic animals indicated its antioxidant functions in the reproductive system [16]. Nevertheless, the beneficial effects of EGT on oocyte maturation and other basic mechanisms are unclear. This study examined the effects of EGT treatment during IVM on the maturation and subsequent preimplantation embryonic development after *in vitro* fertilization (IVF).

MATERIALS AND METHODS

Chemicals

Unless stated otherwise, all chemicals and reagents were obtained from Sigma–Aldrich Chemical Company (Sigma–Aldrich, USA).

Oocytes collection and IVM

Porcine ovaries were obtained from a provincial slaughterhouse, placed in saline (0.9% NaCl) at 37°C for 1 h in the laboratory, and washed with saline. Subsequently, cumulus–oocyte complexes (COCs) inside the FF of the antral follicles (3–6 mm) were collected and aspirated into a 15-mL conical tube using a 10-mL disposable syringe. The conical tubes were left to stand for 10 min at 37°C to settle the debris. The supernatant liquid was then discarded, and the sediments were washed three times with HEPES-buffered Tyrode's medium (TLH) containing 0.05% (w/v) polyvinyl alcohol (PVA). The COCs were gathered using a stereomicroscope. The COCs with the same quality of cytoplasm and more than

three layers of cumulus cells were caught from the washed FF. Approximately 50–60 selected COCs were moved to each well in a four-well dish (Nunc, Roskilde, Denmark). Medium-199 (Invitrogen, USA) was used as the basic medium for IVM of oocytes. The maturation medium supplemented with diverse components, such as 0.1% (w/v) PVA, 0.91 mM sodium pyruvate, 0.6 mM cysteine, 10 ng/mL epidermal growth factor, 75 μg/mL kanamycin, and 1 μg/mL insulin, was added to each well. These COCs were incubated with 10 IU/mL equine chorionic gonadotropin and 10 IU/mL human chorionic gonadotropin for the first 22 h of IVM at 39°C in a 100% humidity, 5% CO_2 atmosphere and then incubated in an IVM medium free of hormones for 20 h. During the whole IVM duration, EGT was added to the media at concentrations of 0 (control), 10, 50, and 100 μM for each group. EGT was dissolved using an IVM culture medium, and no replacer was added to the control group. The EGT concentrations were determined based on a previous study on EGT in the FF of several animals [16].

Evaluation of nuclear maturation

To estimate the nuclear maturation ratio, the oocytes derived from COCs were obtained by denuding the cumulus cells using 0.1% (w/v) hyaluronidase. All the denuded oocytes in each group were washed more than three times and stained for 10 min with TLH-PVA containing 10 μg/mL Hoechst-33342. The stained oocytes were mounted and examined using a fluorescence microscope with ultraviolet (UV) filters (Nikon, Japan). The oocytes were further classified according to five stages: GV, metaphase I (MI), anaphase I/telophase I (AI/TI), and metaphase II (MII) to assess the maturation stage of oocyte meiosis.

Measurement of intracellular glutathione (GSH) and ROS levels

The oocytes obtained after 42 h of IVM were denuded by pipetting using 0.1% (w/v) hyaluronidase to assess the intracellular GSH and ROS levels of oocytes. The intracellular GSH and ROS levels were measured using a previously described method [17]. Briefly, the intracellular GSH was stained with Cell Tracker Blue, and ROS was stained with 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). Ten oocytes from each group were washed three times with TLH-PVA. The washed oocytes were incubated in TLH-PVA and transferred to a 20 μL droplet, which was prepared for fluorescence measurements using an epifluorescence microscope (TE300, Nikon) with UV-2A (370 nm) and GFP-B (460 nm) filters. The oocytes from each treatment group were incubated (in the darkness) for 30 or 10 min in TLH-PVA supplemented with 10 μM H_2DCFDA or 10 μM CMF_2HC, respectively. After incubation, the oocytes were washed three times with TLH-PVA and transferred to a 10 μL droplet, which was readied for the fluorescence measurements using an epifluorescence microscope with an UV filter (ROS, 460 nm, green; GSH, 370 nm, blue). The fluorescence intensity across the cytoplasmic area of oocytes was quantified using Adobe Photoshop CS6 (Adobe, USA). These values were normalized to those of the control group. Thirty mature oocytes from each treatment were used for GSH and ROS measurements, and measurements were repeated three times.

Analysis of gene expression using real-time quantitative polymerase chain reaction (RT-qPCR)

After IVM, the cumulus cells and oocytes were severally denuded from 50–60 COCs in each group using 0.1% hyaluronidase and then sampled using a stereomicroscope to analyze the gene expression. All oocyte and cumulus cell samples were washed more than twice using Dulbecco's phosphate-buffered saline (Welgene, Korea) and stored at −80°C until further analysis. The mRNA expressions of hyaluronan synthase 2 (*HAS2*), gap junction protein Connexin 43 (*CX43*), and proliferating cell nuclear antigen (*PCNA*) were only analyzed in

cumulus cells. The levels of superoxide dismutase 1 (*SOD1*), superoxide dismutase 2 (*SOD2*), nuclear factor erythroid-2 related factor 2 (*Nrf2*), Kelch-like ECH-associated protein 1 (*KEAP1*), NAD(P)H quinone dehydrogenase 1 (*NQO1*), glutathione disulfide reductase (*GSR*), heme oxygenase 1 (*HMOX*), BCL2-like 1 (*BCL2L1*), and BCL2-associated X (*BAX*) were measured in each group of mature oocytes and cumulus cells. **Table 1** provides information on the primers used for RT-qPCR. The total RNA was isolated from the samples using TRIzol reagent (TaKaRa Bio Inc, Japan) according to the manufacturer's protocol. Moloney murine leukemia virus reverse transcriptase (Elpis-Biotech, Korea), random primers (Takara Bio Inc.), 10 mM dNTPs (BEAMS Biotechnologies, Korea), and RNase inhibitor (Intron Biotechnology, Korea) were used to synthesize the complementary DNA (cDNA) from the total RNA. RT-qPCR (Agilent Technologies, USA) analysis was performed using 1 μL of the synthesized cDNA template mixed with 2X SYBR Premix Ex Taq (Takara Bio Inc.) containing 10 pM of particular primers. Varying fluorescence intensities were assessed at the end of the extension step of each cycle. The reactions were carried out for 40 cycles using the following cycling parameters: denaturation at 95°C for 15 sec, annealing at 57°C for 15 sec, and extension at 72°C for 24 sec. The relative expression (R) was calculated using the following equation:

$$R = 2^{-[\Delta Ct\ sample - \Delta Ct\ control]}$$

All values were standardized to those of *RN18S* and *GAPDH* in oocytes and cumulus cells, respectively, to determine a regularized random value in each gene. The experiments were performed in triplicate.

Table 1. Primers for real-time quantitative polymerase chain reaction

mRNA	Primer sequences	Tm (°C)	Product size (bp)	GenBank accession number
GAPDH	F: 5′-GTCGGTTGTGGATCTGACCT-3′	59	207	NM_001206359
	R: 5′-TTGACGAAGTGGTCGTTGAG-3′	58		
RN18S	F: 5′-CGCGGTTCTATTTTGTTGGT-3′	57	219	NR_046261
	R: 5′-GGTCATTTCCGACTGAAGG-3′	58		
HAS2	F: 5′-TTACAATCCTCCTGGGTGGT-3′	57	199	NM_214053.1
	R: 5′-TCAAGCACCATGTCGTACTG-3′	57		
CX43	F: 5′-ACTGAGCCCCTCCAAAGACT-3′	60	191	NM_001244212
	R: 5′-GCTCGGCACTGTAATTAGCC-3′	59		
PCNA	F: 5′-CCTGTGCAAAAGATGGAGTG-3′	58	187	NM_001291925.1
	R: 5′-GGAGAGAGTGGAGTGGCTTT-3′	61		
SOD1	F: 5′-CTGGGATTGACGTGTGGGA-3′	60	132	NP_000116.2
	R: 5′-GTCGCAAGCCGTGTATCGTG-3′	60		
SOD2	F: 5′-GACAAATCTGAGCCCTAACG-3′	61	193	NM_214127.2
	R: 5′-GTTAGAACAAGCGGCAATCT-3′	60		
Nrf2	F: 5′-CCATTCACAAAAGACAAACATT-3′	58	75	XM_021075133.1
	R: 5′-GCTTTTGCCCTTAGCTCATCTC-3′	60		
KEAP1	F: 5′-AGCTGGGATGCTCAGTGTT-3′	60	100	NM001114671.1
	R: 5′-AGGCAAGTTCTCCCAGACA-3′	59		
NQO1	F: 5′-TATCCTCCTCTGGCCAATTC-3′	60	81	NM_001159613.1
	R: 5′-AGGCGTTTCTTCCACTCTTC-3′	60		
GSR	F: 5′-TGGGCTCTAAGACGTCACTG-3′	60	106	XM_003483635.4
	R: 5′-TCTATGCCAGCATTCTCCAG-3′	61		
HMOX	F: 5′-AAGGCTTTAAGCTGGTGATG-3′	60	104	NM_001004027.1
	R: 5′-GAAGTAGAGGGGCGTGTAGA-3′	58		
BCL2L1	F: 5′-AATGACCACCTAGAGCCTTG-3′	58	182	NM_214285.1
	R: 5′-GGTCATTTCCGACTGAAGAG-3′	58		
BAX	F: 5′-TGCCTCAGGATGCATCTACC-3′	61	199	XM_013998624.2
	R: 5′-AAGTAGAAAAGCGCGACCAC-3′	58		

F, forward; R, reverse; Tm (°C), melting temperature.

IVF and *in vitro* culture of porcine embryos

After IVM, the oocytes derived from COCs were obtained by denuding cumulus cells using 0.1% (w/v) hyaluronidase. IVF was performed according to previously described procedures [18]. Only oocytes in which extrusion of the first polar body was confirmed were selected and used for IVF. After washing the oocytes three times using a modified Tris-buffered medium (mTBM), 15 oocytes were transferred to each drop of 40 μL mTBM covered with mineral oil. The sperm motility and concentration were checked using a hemocytometer. Only sperm with a motility of 80% or more was used for IVF, and the final concentration of sperm was diluted to 1×10^6 sperm/mL. Diluted sperm and oocytes were co-cultured in mTBM for 20 min at 39°C, 5% CO_2, 5% O_2, 90% N_2, and 100% humidity. Sperm on the surface of the oocytes were detached by gentle pipetting. The oocytes were transferred to a drop of fresh mTBM and incubated for an additional six hours. Subsequently, IVF embryos were washed three times in porcine zygote medium-3 (PZM-3), transferred to fresh PZM-3 drops, and cultured for 168 h at 39°C, 5% CO_2, 5% O_2, 90% N_2, and 100% humidity.

Evaluation of developmental competence and total cell number count

To assess developmental competence in porcine embryos, the day of initiation IVF was considered as day 0. After 48 h of IVF, the cleavage rates of porcine embryos were analyzed, and the embryos from each group were assessed using a stereomicroscope. The cleaved embryos were categorized into three groups based on the number of blastomeres, wherein single-cell and fragmented embryos were not counted. The pattern of blastocyst formation was investigated through morphological characteristics observed in microscopic images with reference to the standards of Gardner [19]. On Day 7, the blastocyst formation patterns were classified into three groups in conformity with the extent of expansion and hatching shape status: early (a blastocoel equal to or less than 50% volume of the embryo), expanded (a blastocoel greater than 50% the embryo or a blastocyst with a blastocoel fully taking up the embryo), and hatched (hatching or previously hatched blastocyst). Blastocysts that were difficult to distinguish between the early and expanded visually were classified by calculating the area of the blastocoel in the total volume of the blastocyst using ImageJ 1.53e (National Institutes of Health, USA). The cell numbers of blastocysts on Day 7 were determined. The blastocysts in each group were washed twice with TLH-PVA, and the zona pellucida on unhatched blastocysts was removed using 0.5% (w/v) protease for 2 min. The blastocysts without zona pellucida were held in 0.1% (w/v) paraformaldehyde of PBS–PVA and stained with 10 μg/mL Hoechst-33342 for 5 min. Finally, the blastocysts of each group were set on glass slides in a 100% glycerol droplet, and a coverslip was placed gently on the glass slide. The total blastocysts were determined under a fluorescence microscope (Nikon) with a UV-2A filter (370 nm) at 400× magnification. The experiment was repeated three times.

Statistical analysis

All experiments were repeated a minimum of three times. The oocytes used in the experiment were selected randomly from each experimental group. The statistical data analysis was conducted using SPSS 12.0 software (SPSS Inc., USA). All data were compared using one-way ANOVA, followed by Duncan's multiple range test (post-hoc). The significant differences among the statistical data values were considered at $p < 0.05$. The data are shown as mean ± SEM.

Table 2. Effect of EGT supplementation on nuclear maturation.

EGT concentration (μM)	No. of oocytes cultured for maturation[a]	Mean ± SEM (%) of oocytes at the nuclear stage			
		Germinal vesicle	Metaphase I	Anaphase I and telophase I	Metaphase II
0	142	0.7 ± 0.7	9.9 ± 1.0[b]	2.0 ± 1.2	87.4 ± 1.0[b,c]
10	140	0.8 ± 0.8	2.8 ± 2.4[c]	4.4 ± 1.4	92.0 ± 1.6[b]
50	148	0.7 ± 0.7	3.9 ± 3.4[c]	4.1 ± 1.2	91.3 ± 2.4[b]
100	144	2.2 ± 1.3	6.4 ± 4.0[b,c]	5.9 ± 5.7	85.5 ± 0.6[c]

EGT, ergothioneine.
[a]Three-time replicates.
[b,c]Values with different superscript letters within a column differ significantly ($p < 0.05$).

RESULTS

Effect of EGT supplementation on oocyte nuclear maturation during IVM

Nuclear maturation of oocytes was assessed at four stages of GV, MI, AI/TI, and MII in each group to estimate the effect of EGT supplementation during IVM on the nuclear maturation of porcine oocytes. Oocyte maturation was assessed at the MII stage. After 42 h, the nuclear maturation rates of oocytes in the MII stage were 87.4 ± 1.0% (control), 92.0 ± 1.6% (10 μM), 91.3 ± 2.4% (50 μM), and 85.5 ± 0.6% (100 μM EGT group). Thus, there were no significant differences between the EGT-treated groups and the control group. On the other hand, among the EGT-treated groups, the nuclear maturation rates decreased with higher concentrations of treatments (**Table 2**).

Effect of EGT supplementation on intracellular GSH and ROS levels during IVM

The effects of the EGT treatment during IVM on intracellular GSH and ROS levels were determined by evaluating the MII stage oocytes from each group by staining after 42 h of maturation (**Fig. 1**). The intracellular GSH level was significantly higher ($p < 0.05$) in the oocytes from the 50 μM EGT group than those from the control group (**Fig. 1A**). The relative levels of GSH in the control, 10 μM, 50 μM, and 100 μM EGT groups were 1.00 ± 0.012, 1.01 ± 0.011, 1.05 ± 0.015, and 1.03 ± 0.011, respectively. The 10 μM and 100 μM EGT groups did not show a significant difference compared to the control group. In contrast, the ROS levels in the 50 μM and 100 μM EGT groups were significantly lower ($p < 0.05$) than those in the control group (**Fig. 1B**). The relative levels of ROS in the control, 10 μM, 50 μM, and 100 μM EGT groups were 1.00 ± 0.018, 0.98 ± 0.016, 0.95 ± 0.016, and 0.94 ± 0.078, respectively.

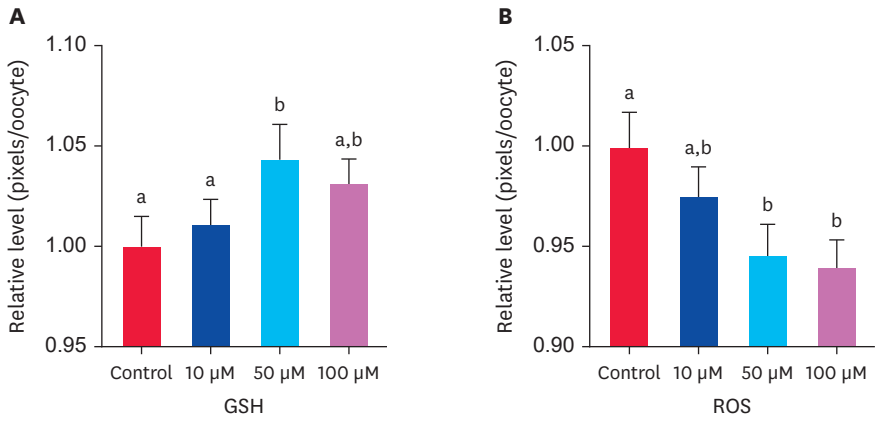

Fig. 1. Effect of ergothioneine supplementation in maturation medium on intracellular GSH (A) and ROS (B) levels in mature porcine oocytes for *in vitro* maturation. Within each endpoint of the two groups (GSH and ROS), bars with different letters (a, b) are significantly different ($p < 0.05$). For all graphs, the value was represented as mean ± SEM. GSH, glutathione; ROS, reactive oxygen species.

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These results suggest that the EGT treatment during IVM affects the intracellular GSH and intracellular ROS levels in oocytes.

Effect of EGT supplementation on gene expression on mRNA levels in cumulus cells and oocytes during IVM

The mRNA expression levels of the antioxidative pathway-related, apoptotic pathway-related, and proliferation pathway-related genes were evaluated in porcine COCs from each group to examine the effect of the EGT mechanism on IVM of porcine oocytes (**Figs. 2** and **3**). In cumulus cells, the mRNA expression levels of *HAS2* and *CX43*, a cumulus function-related gene, were significantly higher ($p < 0.05$) in the 10 μM EGT group than the control group (**Fig. 2**). On the other hand, there were no significant differences in *PCNA* expression, an embryonic developmental marker gene, in cumulus cells compared to the control group. **Fig. 3** shows the effects of antioxidative pathway-related and apoptotic pathway-related genes, including *SOD1*, *SOD2*, *Nrf2*, *KEAP1*, *NQO1*, *GSR*, *HMOX*, *BCL2L1*, and *BAX*, on all EGT-supplemented groups in cumulus cells during IVM of porcine oocytes. Regarding the antioxidative pathway-related genes, the mRNA expression level of HMOX was significantly higher ($p < 0.05$) in the 100 μM EGT group than the control group (**Fig. 3A**). On the other hand, although the EGT-supplemented cumulus cells tended to have significantly lower *Nrf2* and *KEAP1* expression ($p < 0.05$) than the control group, the apoptotic pathway-related genes showed no significant differences in all the treatment groups in cumulus cells. In the oocytes, the mRNA expression levels of the downstream (antioxidative pathway-related) antioxidant genes *Nrf2* and *NQO1* were significantly higher ($p < 0.05$) in the 10 μM EGT group than in the control group. In contrast, *KEAP1* expression, which regulates the steady-state levels of *Nrf2*, was significantly lower ($p < 0.05$) in the 10 and 100 μM EGT groups than in the control group (**Fig. 3B**). In addition, the apoptotic pathway-related gene expression of *BCL2L1* and *BAX* in oocytes were similar in the control and EGT-supplemented groups.

Effect of EGT supplementation on embryonic development after IVF during IVM

After IVF, the cleavage and blastocyst development formation rates were significantly higher ($p < 0.05$) in the 10 μM EGT group than in the control group (**Table 3**). On day 2, The one-cell and fragmentation group formation rates of the IVF embryo cleavage pattern were significantly lower ($p < 0.05$) in the 10 μM EGT groups than in the control group (**Fig. 4A**). Furthermore, the IVF embryos in the 10 μM EGT group showed significantly higher ($p < 0.05$)

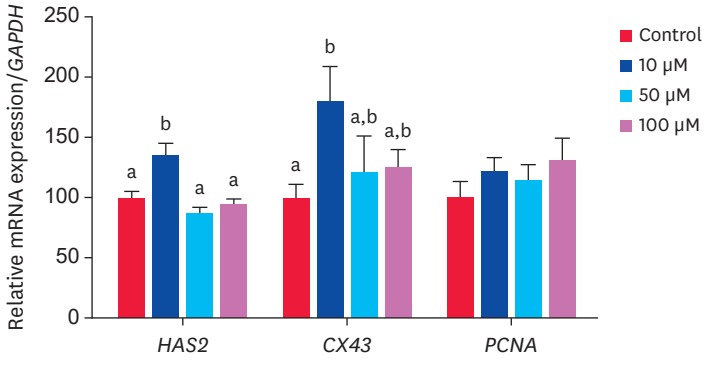

Fig. 2. Effect of mRNA expression of EGT supplementation during *in vitro* maturation on the function of porcine cumulus cells. The mRNA expression levels of the genes *HAS2*, *CX43*, and *PCNA*, related to cumulus cell function, were compared between cumulus cells from different EGT-supplemented groups. In this graph, the values represent mean ± SEM. The experiment was repeated four times. Values (a, b) with different superscript letters within the same column are significantly different ($p < 0.05$).
EGT, ergothioneine.

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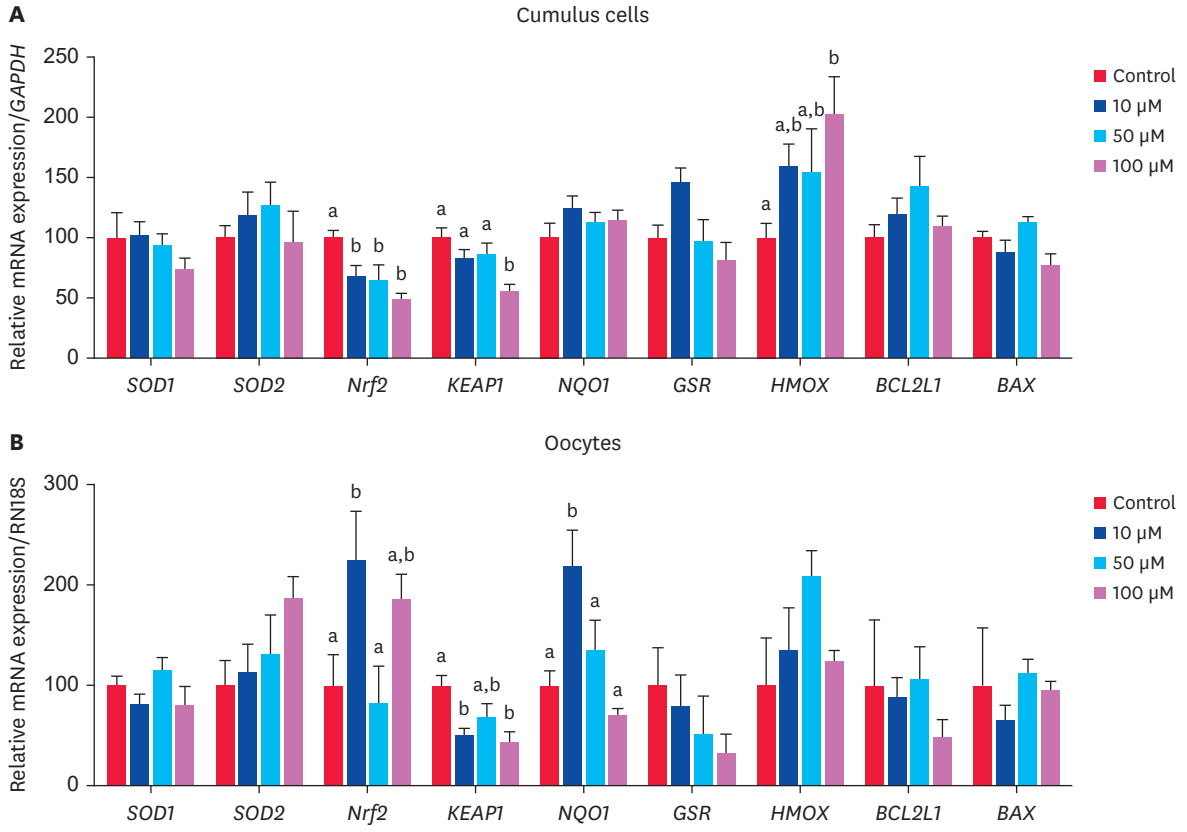

Fig. 3. Relative mRNA expression levels of antioxidative pathway- and apoptotic pathway-related genes in EGT-supplemented cumulus cells and oocytes. (A) Relative mRNA expression levels of *SOD1*, *SOD2*, *Nrf2*, *KEAP1*, *NQO1*, *GSR*, *HMOX*, *BCL2L1*, and *BAX* were assessed in cumulus cells supplemented with various concentrations of EGT. (B) The relative mRNA expression levels of *SOD1*, *SOD2*, *Nrf2*, *KEAP1*, *NQO1*, *GSR*, *HMOX*, *BCL2L1*, and *BAX* were assessed in oocytes supplemented with various concentrations of EGT. In all graphs, the values represent the mean ± SEM. The experiment was repeated three times. Values (a, b) with different superscript letters within the same column are significantly different ($p < 0.05$).
EGT, ergothioneine.

Table 3. Effect of EGT supplementation during *in vitro* maturation on embryonic development after *in vitro* fertilization

EGT concentration (μM)	No. of embryos cultured[a]	No. (%) of embryos developed to		Total cell number in blastocyst (No.[b])
		≥ 2-cell	Blastocyst	
Control	168	108 (64.6 ± 2.8)[c]	28 (22.2 ± 4.5)[c]	55.5 ± 6.9 (28)
10	179	138 (77.1 ± 3.2)[d]	49 (36.1 ± 1.7)[d]	51.5 ± 4.4 (49)
50	176	129 (73.4 ± 1.5)[c,d]	44 (33.2 ± 4.3)[c,d]	62.7 ± 4.7 (44)
100	180	129 (71.7 ± 3.2)[c,d]	33 (24.5 ± 3.3)[c,d]	58.6 ± 5.4 (33)

EGT, ergothioneine.
[a]Three-time replicates; [b]The number of blastocysts examined; [c,d]Values with different superscript letters within a column differ significantly ($p < 0.05$).

cleavage rates than those in the control group. Moreover, all the EGT-supplemented groups showed a significantly higher ratio of expanded blastocyst formation than in the control group (**Fig. 4B**). On the other hand, there was no significant difference in the cell number of IVF blastocyst formation in each group (**Table 3**).

DISCUSSION

Oocyte quality is indispensable for oocyte maturation, fertilization, embryonic development, and successful implantation [20]. Therefore, oxidative stress events associated with *in vitro*

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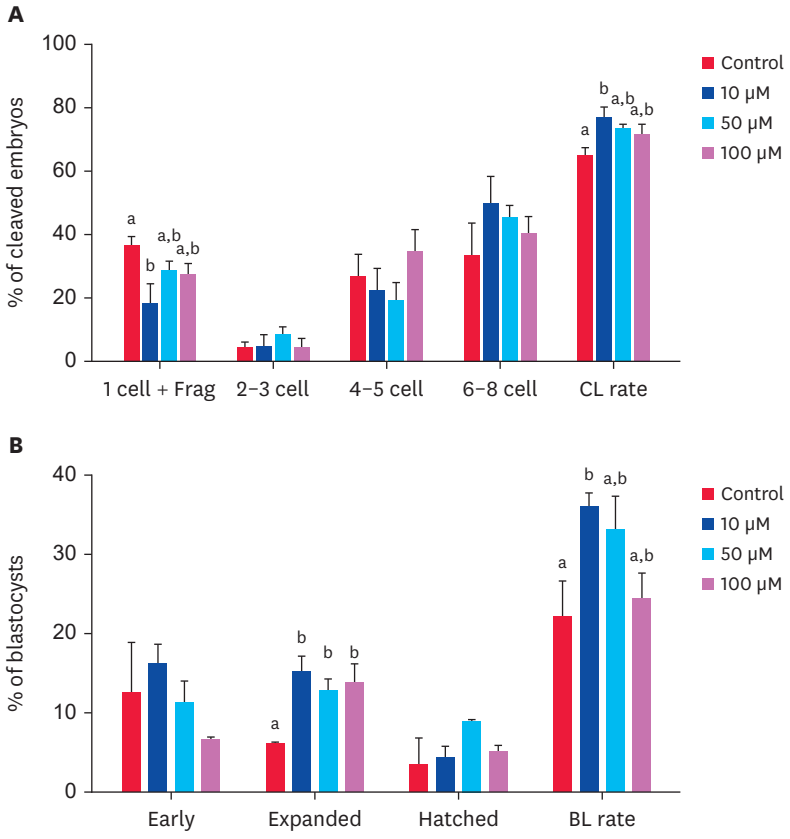

Fig. 4. Effect of EGT supplementation during *in vitro* maturation on the development of preimplantation embryos. (A) Cleavage patterns of embryos after IVF. (B) Blastocyst formation pattern of the IVF embryos. Within each endpoint, bars with different letters (a, b) are significantly different ($p < 0.05$) for various concentrations of EGT. The cleavage rate was measured on Day 2, and the blastocyst formation rate was determined on Day 7 of culture. For all graphs, the values are represented as mean ± SEM. The experiment was repeated three times.
Frag, fragmentation; CL, cleavage; BL, blastocyst; EGT, ergothioneine; IVF, *in vitro* fertilization.

environmental experiments are a major issue that might result in poor oocyte quality [21]. Oocytes can develop within the FF environment, which provides a suitable microenvironment for oocyte development and plays an important role in embryo developmental competence and the interaction of oocytes with sperms [22]. The major components of FF are metabolites, proteins, ROS, steroid hormones, and antioxidants. Sotgia et al. [16] reported that the concentration of EGT in porcine FF was 1.04 ± 0.37 μM, which was slightly higher than that of sheep and goats (0.72–0.73 μM) and lower than that of mares (3.16 ± 1.27μM). On the other hand, only a few studies examined the effects of EGT treatment during IVM on porcine oocyte maturation and the subsequent development of embryos after IVF or how EGT improves the quality of porcine oocytes. Therefore, the present study examined the effects of an EGT treatment during IVM of pig oocytes on IVM and embryonic development after IVF.

Nuclear and cytoplasmic maturation were inspected to analyze the effects of EGT during oocyte maturation. The nuclear maturation rates of oocytes at the MII stage in all treatment groups were similar to those in the control group. Although the GSH levels in the 50 μM EGT group significantly increased compared to those in the control group, the ROS levels in the 50 and 100 μM EGT groups were significantly lower than those in the control group (**Fig. 1**). Previous studies showed that the use of various antioxidants, including flavonoids, melatonin, and α-ketoglutarate, during the IVM of porcine oocytes accelerated the

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intracellular GSH levels and decreased the ROS levels in oocytes after maturation [23]. With improved cytoplasmic maturation, these diverse antioxidants benefit oocyte maturation and embryonic development. Higher levels of ROS were usually generated in oocytes under *in vitro* conditions than under *in vivo* conditions, which was one of the leading causes of reduced embryonic development [24]. Increased ROS levels can trigger mitochondrial dysfunction, cell membrane damage, cell membrane degeneration, lipid oxidation, and DNA damage [25]. EGT exerts an antioxidant effect and plays a crucial role in scavenging free radicals and ROS in the cells [26]. In this study, the reduced intracellular ROS and increased intracellular GSH levels in the EGT-supplemented oocytes suggest that EGT enhances the antioxidant capacity of oocytes under *in vitro* conditions.

RT-qPCR was accomplished using cDNA from porcine cumulus cells and oocytes that had completed maturation to determine if the effect of EGT during the maturation process was associated with changes in mRNA expression. The cumulus cells adjacent to the oocytes supported the nuclear maturation of oocytes and enhanced cytoplasmic maturation [27]. These made them capable of undergoing normal fertilization and subsequent embryo development by protecting the oocytes from oxidative and saturated fatty acid stresses [28]. Therefore, the expansion of cumulus cell layers is essential for the optimal maturation of porcine oocytes [29]. The *HAS2* enzyme was involved in regulating hyaluronan synthesis and was the main component of the cumulus expansion process. Moreover, *HAS2* expression was correlated with cumulus cell expansion [30]. Furthermore, *CX43* was the main gap junction alpha-1 protein in cumulus cells [31]. CX proteins were a primary means of conveying information from cumulus cells to the oocytes [32]. In cumulus cells supplemented with 10 μM EGT, the expression levels of *HAS2* and *CX43* genes related to cumulus function during IVM increased significantly in this study. Thus, 10 μM EGT supplementation can improve oocyte quality and maturation via cumulus cell expansion.

The *Nrf2–KEAP1* and antioxidant response element (ARE) signaling pathway is the main cellular antioxidant signaling for alleviating oxidative stress [33]. *Nrf2* is a transcription factor that leads to the expression of antioxidant proteins or genes by binding the ARE region of the promoter [34]. Within the cell, *Nrf2* remains in the cytoplasm by binding to and controlled by *KEAP1* constitutively [35]. EGT played an essential role in the mRNA expression of the antioxidant transcription factor *Nrf2* and upregulated *NQO1* and *HMOX*, which were its downstream antioxidant genes [36]. Previous studies reported that several antioxidants upregulated the expression levels of the *Nrf2* gene in porcine oocytes [37]. This study showed that the transcript levels of *Nrf2* and *NQO1* increased, but those of *KEAP1* decreased in oocytes in the 10 μM EGT group compared to the control group. Furthermore, the levels of *HMOX* expression in cumulus cells were higher in the 10 μM EGT group than in the control group. Therefore, EGT supplementation during IVM upregulates *Nrf2* expression, stimulating the activation of various antioxidant pathway-related genes to relieve oxidative stress, thus improving the oocyte quality.

Various antioxidants treated during IVM have shown that oocyte maturation increased nuclear and cytoplasmic maturation and resulted in a higher rate of IVF and subsequent embryonic development [38]. A previous study showed that an antioxidant treatment during IVM also improved embryonic development and enhanced blastocyst formation, which is a crucial indicator of the efficiency of embryonic development and conditions [39]. Furthermore, antioxidant supplementation within the oocyte medium alone for IVF showed an increase in the total cell number of blastocysts than embryos that had antioxidants only in the sperm medium for IVF [39]. The embryos with an increased level of ROS after IVF

showed low developmental competence and promoted DNA fragmentation [40]. Therefore, it is essential to protect oocytes from oxidative stress during IVM. EGT supplementation during IVM, IVF, and *in vitro* culture had a beneficial effect on the IVM of oocytes and embryo developmental competence [41]. These beneficial effects of EGT could be used in the potential applications of EGT in IVF. When analyzing embryonic development after IVF, the 10 μM EGT group showed a significantly higher cleavage and blastocyst formation rate than the control group. In this result, EGT upregulated the gene expression of *Nrf2* and activated downstream antioxidant pathway-related genes to alleviate oxidative stress. Therefore, EGT supplementation improves oocyte maturation and promotes the development of IVF embryos by activating the cellular antioxidant mechanisms. EGT supplementation during porcine IVM was identified to affect embryo developmental competence constantly.

Supplementing the maturation medium with EGT during IVM positively affects oocyte quality and embryonic development. Furthermore, 50 μM EGT had antioxidant effects by increasing the GSH content and reducing ROS accumulation. On the other hand, increased antioxidant effects did not influence embryonic developmental competence in proportion to the concentration. By contrast, 10 μM EGT treatment activated the cellular antioxidant response mechanism by activating *Nrf2*, a transcription factor, and its downstream antioxidant genes. In particular, the increased oocyte quality achieved via the upregulation of the antioxidant pathway-related signaling in porcine oocytes improved the subsequent developmental potential of porcine embryos after IVF. This study showed that the supplementation of 50 μM EGT improved the antioxidant effects of porcine oocytes, and 10 μM EGT promoted the development of IVF embryos. These findings will improve the IVM of oocytes.

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REFERENCES

1. Ito J, Shirasuna K, Kuwayama T, Iwata H. Resveratrol treatment increases mitochondrial biogenesis and improves viability of porcine germinal-vesicle stage vitrified-warmed oocytes. Cryobiology. 2020;93:37-43. PUBMED | CROSSREF
2. De Vos M, Grynberg M, Ho TM, Yuan Y, Albertini DF, Gilchrist RB. Perspectives on the development and future of oocyte IVM in clinical practice. J Assist Reprod Genet. 2021;38(6):1265-1280. PUBMED | CROSSREF
3. Tatemoto H, Ootaki K, Shigeta K, Muto N. Enhancement of developmental competence after in vitro fertilization of porcine oocytes by treatment with ascorbic acid 2-O-alpha-glucoside during in vitro maturation. Biol Reprod. 2001;65(6):1800-1806. PUBMED | CROSSREF
4. Sirard MA, Richard F, Blondin P, Robert C. Contribution of the oocyte to embryo quality. Theriogenology. 2006;65(1):126-136. PUBMED | CROSSREF
5. Eppig JJ. Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. Reprod Fertil Dev. 1996;8(4):485-489. PUBMED | CROSSREF
6. Mao L, Lou H, Lou Y, Wang N, Jin F. Behaviour of cytoplasmic organelles and cytoskeleton during oocyte maturation. Reprod Biomed Online. 2014;28(3):284-299. PUBMED | CROSSREF

7. Barnes FL, Kausche A, Tiglias J, Wood C, Wilton L, Trounson A. Production of embryos from in vitro-matured primary human oocytes. Fertil Steril. 1996;65(6):1151-1156.
PUBMED | CROSSREF
8. Chappel S. The role of mitochondria from mature oocyte to viable blastocyst. Obstet Gynecol Int. 2013;2013:183024.
PUBMED | CROSSREF
9. Halliwell B. Free radicals and antioxidants: updating a personal view. Nutr Rev. 2012;70(5):257-265.
PUBMED | CROSSREF
10. Kala M, Shaikh MV, Nivsarkar M. Equilibrium between anti-oxidants and reactive oxygen species: a requisite for oocyte development and maturation. Reprod Med Biol. 2016;16(1):28-35.
PUBMED | CROSSREF
11. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: impact on human health. Pharmacogn Rev. 2010;4(8):118-126.
PUBMED | CROSSREF
12. Kang JT, Kwon DK, Park SJ, Kim SJ, Moon JH, Koo OJ, et al. Quercetin improves the in vitro development of porcine oocytes by decreasing reactive oxygen species levels. J Vet Sci. 2013;14(1):15-20.
PUBMED | CROSSREF
13. Cheah IK, Halliwell B. Ergothioneine; antioxidant potential, physiological function and role in disease. Biochim Biophys Acta. 2012;1822(5):784-793.
PUBMED | CROSSREF
14. Borodina I, Kenny LC, McCarthy CM, Paramasivan K, Pretorius E, Roberts TJ, et al. The biology of ergothioneine, an antioxidant nutraceutical. Nutr Res Rev. 2020;33(2):190-217.
PUBMED | CROSSREF
15. Halliwell B, Cheah IK, Tang RM. Ergothioneine - a diet-derived antioxidant with therapeutic potential. FEBS Lett. 2018;592(20):3357-3366.
PUBMED | CROSSREF
16. Sotgia S, Zinellu A, Pisanu E, Vlahopoulou G, Ariu F, Ledda S, et al. Concentrations of l-ergothioneine in follicular fluids of farm animals. Comp Clin Pathol. 2015;24(5):1261-1265.
CROSSREF
17. You J, Kim J, Lim J, Lee E. Anthocyanin stimulates *in vitro* development of cloned pig embryos by increasing the intracellular glutathione level and inhibiting reactive oxygen species. Theriogenology. 2010;74(5):777-785.
PUBMED | CROSSREF
18. Hwang SU, Jeon Y, Yoon JD, Cai L, Kim E, Yoo H, et al. Effect of ganglioside GT1b on the *in vitro* maturation of porcine oocytes and embryonic development. J Reprod Dev. 2015;61(6):549-557.
PUBMED | CROSSREF
19. Gardner DK, Balaban B. Assessment of human embryo development using morphological criteria in an era of time-lapse, algorithms and 'OMICS': is looking good still important? Mol Hum Reprod. 2016;22(10):704-718.
PUBMED | CROSSREF
20. Nikbakht R, Mohammadjafari R, Rajabalipour M, Moghadam MT. Evaluation of oocyte quality in polycystic ovary syndrome patients undergoing ART cycles. Fertil Res Pract. 2021;7(1):2.
PUBMED | CROSSREF
21. Da Broi MG, Navarro PA. Oxidative stress and oocyte quality: ethiopathogenic mechanisms of minimal/mild endometriosis-related infertility. Cell Tissue Res. 2016;364(1):1-7.
PUBMED | CROSSREF
22. Pawlak P, Warzych E, Cieslak A, Malyszka N, Maciejewska E, Madeja ZE, et al. The consequences of porcine IVM medium supplementation with follicular fluid become reflected in embryo quality, yield and gene expression patterns. Sci Rep. 2018;8(1):15306.
PUBMED | CROSSREF
23. Lin T, Lee JE, Kang JW, Oqani RK, Cho ES, Kim SB, et al. Melatonin supplementation during prolonged *in vitro* maturation improves the quality and development of poor-quality porcine oocytes via anti-oxidative and anti-apoptotic effects. Mol Reprod Dev. 2018;85(8-9):665-681.
PUBMED | CROSSREF
24. Hardy ML, Day ML, Morris MB. Redox regulation and oxidative stress in mammalian oocytes and embryos developed *in vivo* and *in vitro*. Int J Environ Res Public Health. 2021;18(21):11374.
PUBMED | CROSSREF
25. Agarwal A, Said TM, Bedaiwy MA, Banerjee J, Alvarez JG. Oxidative stress in an assisted reproductive techniques setting. Fertil Steril. 2006;86(3):503-512.
PUBMED | CROSSREF

26. Fu TT, Shen L. Ergothioneine as a natural antioxidant against oxidative stress-related diseases. Front Pharmacol. 2022;13:850813.
PUBMED | CROSSREF

27. Atikuzzaman M, Koo OJ, Kang JT, Kwon DK, Park SJ, Kim SJ, et al. The 9-cis retinoic acid signaling pathway and its regulation of prostaglandin-endoperoxide synthase 2 during *in vitro* maturation of pig cumulus cell-oocyte complexes and effects on parthenogenetic embryo production. Biol Reprod. 2011;84(6):1272-1281.
PUBMED | CROSSREF

28. Fatehi AN, Roelen BA, Colenbrander B, Schoevers EJ, Gadella BM, Beverst MM, et al. Presence of cumulus cells during in vitro fertilization protects the bovine oocyte against oxidative stress and improves first cleavage but does not affect further development. Zygote. 2005;13(2):177-185.
PUBMED | CROSSREF

29. Chen L, Russell PT, Larsen WJ. Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. Mol Reprod Dev. 1993;34(1):87-93.
PUBMED | CROSSREF

30. Yung Y, Ophir L, Yerushalmi GM, Baum M, Hourvitz A, Maman E. HAS2-AS1 is a novel LH/hCG target gene regulating HAS2 expression and enhancing cumulus cells migration. J Ovarian Res. 2019;12(1):21.
PUBMED | CROSSREF

31. Kidder GM, Mhawi AA. Gap junctions and ovarian folliculogenesis. Reproduction. 2002;123(5):613-620.
PUBMED | CROSSREF

32. Gershon E, Plaks V, Aharon I, Galiani D, Reizel Y, Sela-Abramovich S, et al. Oocyte-directed depletion of connexin43 using the Cre-LoxP system leads to subfertility in female mice. Dev Biol. 2008;313(1):1-12.
PUBMED | CROSSREF

33. Baird L, Dinkova-Kostova AT. The cytoprotective role of the Keap1-Nrf2 pathway. Arch Toxicol. 2011;85(4):241-272.
PUBMED | CROSSREF

34. Kwak MK, Itoh K, Yamamoto M, Kensler TW. Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter. Mol Cell Biol. 2002;22(9):2883-2892.
PUBMED | CROSSREF

35. Itoh K, Mimura J, Yamamoto M. Discovery of the negative regulator of Nrf2, Keap1: a historical overview. Antioxid Redox Signal. 2010;13(11):1665-1678.
PUBMED | CROSSREF

36. Salama SA, Abd-Allah GM, Mohamadin AM, Elshafey MM, Gad HS. Ergothioneine mitigates cisplatin-evoked nephrotoxicity via targeting Nrf2, NF-κB, and apoptotic signaling and inhibiting γ-glutamyl transpeptidase. Life Sci. 2021;278:119572.
PUBMED | CROSSREF

37. Jiang X, Xing X, Zhang Y, Zhang C, Wu Y, Chen Y, et al. Lead exposure activates the Nrf2/Keap1 pathway, aggravates oxidative stress, and induces reproductive damage in female mice. Ecotoxicol Environ Saf. 2021;207:111231.
PUBMED | CROSSREF

38. Abeydeera LR, Wang WH, Cantley TC, Prather RS, Day BN. Presence of beta-mercaptoethanol can increase the glutathione content of pig oocytes matured *in vitro* and the rate of blastocyst development after in vitro fertilization. Theriogenology. 1998;50(5):747-756.
PUBMED | CROSSREF

39. Truong T, Gardner DK. Antioxidants improve IVF outcome and subsequent embryo development in the mouse. Hum Reprod. 2017;32(12):2404-2413.
PUBMED | CROSSREF

40. Kitagawa Y, Suzuki K, Yoneda A, Watanabe T. Effects of oxygen concentration and antioxidants on the *in vitro* developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. Theriogenology. 2004;62(7):1186-1197.
PUBMED | CROSSREF

41. Mishra A, Reddy IJ, Dhali A, Javvaji PK. l-Ergothioneine improves the developmental potential of *in vitro* sheep embryos without influencing OCTN1-mediated cross-membrane transcript expression. Zygote. 2018;26(2):149-161.
PUBMED | CROSSREF