Zinc deficiency during *in vitro* maturation of porcine oocytes causes meiotic block and developmental failure

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Abstract. The present study investigated the effects of zinc deficiency during *in vitro* maturation (IVM) of porcine oocytes. Zinc deficiency was induced by administering the membrane-permeable zinc chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylendiamine (TPEN). First, the effects of zinc deficiency during IVM on a TPEN-treated group and a TPEN+zinc-treated group compared with a control group were assessed. The oocyte maturation rates and subsequent embryonic developmental competence of the TPEN+zinc-treated oocytes were similar to those of the control oocytes (metaphase II [MII] rate, 93.0 and 92.7%, respectively, and blastocyst [BL] formation rate, 42.0 and 40.0%, respectively). These results were significantly different from those obtained for the TPEN-treated oocytes (MII rate, 0.61%; BL formation rate, 0%). Although the TPEN-treated oocytes were arrested at metaphase I (MI), the distribution of microtubules was normal. However, microfilament formation was abnormal in the TPEN-treated oocytes. Furthermore, the effect of a temporary zinc deficiency during IVM on oocyte maturation and subsequent embryonic development was assessed. TPEN (10 μM) was added to the IVM medium for 0, 7, 15 or 22 h. The 0 h-treated oocytes showed an 83.9% MII rate, while the 7 h-treated oocytes had a significantly lower MII rate (44.8%). Most of the 15- and 22 h-treated oocytes were arrested at MI (MI rate: 98.0 and 97.2%, respectively; MII rate, 0% in both groups). Reductions in the BL formation were dependent on the TPEN treatment duration (29.3, 9.2, 0, and 0% after 0, 7, 15 and 22 h, respectively). In conclusion, zinc is an essential element for successful oocyte maturation and embryonic development in pigs. Zinc deficiency caused a meiotic block and had lasting effects on early embryonic development.

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Introduction

In vitro production (IVP), including *in vitro* maturation (IVM) and somatic cell nuclear transfer, is an important technology for producing transgenic cloned pigs. Oocyte quality influences early embryonic development, fetal growth, pregnancy and the health of the offspring (1). Thus, a better understanding and improved IVM are required to generate transgenic cloned pigs efficiently.

To date, there has been extensive research regarding the optimal conditions for IVM. Addition of various hormones and growth factors, including luteinizing hormone, follicle-stimulating hormone (2), transforming growth factor, androstenedione (3), pregnant mare serum gonadotropin, human chorionic gonadotropin (hCG) (4,5), insulin-like growth factor I (6), and estradiol-17β (5,7), to IVM media exerts positive effects during meiosis (8). Cumulus cells also have an important role in oocyte maturation. Cumulus cells regulate the resumption of meiosis and protect oocytes from oxidative stress (9). Reactive oxygen species (ROS) cause oxidative damage to oocytes due to an improper *in vitro* environment (10,11), while glutathione (GSH) reduces ROS and oxidative stress (12). Various factors can increase GSH, including cysteine (13), cysteamine, glutamine (14), vascular endothelial growth factor (15) and granulocyte-macrophage colony-stimulating factor (16). However, the *in vitro* oocyte quality remains inferior to the *in vivo* oocyte quality for transgenic pig production (17). Numerous aspects of oocyte maturation have remained to be determined; for example, porcine follicular fluid (pFF) contains various unknown factors, including growth factors, cytokines and trance minerals. These factors can influence oocyte maturation and subsequent embryonal development. However, the factors or mechanisms have yet to be determined.

The mammalian body contains small amounts of trace minerals, which are necessary to maintain life and health (18). These minerals are involved in the formation of bones and teeth, acid-base balance, fluid and moisture equilibrium, and are

used as components of neurotransmitters and hormones (18). Trace minerals influence embryonic and fetal survival, as well as other aspects of reproductive performance and growth in mammals (19). Of these, zinc is an important factor during reproduction and development.

Zinc is required for normal fetal growth and development (20). Zinc deficiency causes fetal teratogenesis, prolonged gestation, difficult labor, low birth weight and weak offspring (21,22). In addition, zinc levels are increased 1.7- to 8.7-fold in porcine conceptuses compared to those in the endometrium or ovary between days 12 and 30 of gestation (23). This indicates that the developing conceptus requires increased uptake and/or utilization of zinc. Another potential pathway for zinc to influence pregnancy is by its effect on prostaglandin (PG)F2α synthesis (24-27). Zinc is involved in the formation of PGs, as the arachidonic acid cascade is regulated by zinc enzymes. Zinc also has important roles in scavenging free oxygen radicals (28), DNA synthesis and gene transcription (29). Thus, zinc is thought to influence reproduction and development, and it exists in the oocyte cytoplasm and maturational environment during oocyte maturation; however, its role and influence during porcine IVM have not been sufficiently considered by previous studies. Only a previous study by our group investigated the effects of zinc supplementation during porcine IVM, showing that supplementation of 0.8 μg/ml zinc during IVM of porcine oocytes improved embryonic development prior to implantation (30).

The aim of the present study was to clarify the role of zinc during IVM. For this, zinc deficiency was induced using the membrane-permeable zinc chelator TPEN, and the effects on cumulus cell expansion, nuclear maturation, cytoskeletal organization, GSH and ROS levels, and subsequent embryonic development were evaluated.

Materials and methods

Chemicals. Unless otherwise indicated, all chemicals and reagents used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Oocyte collection and IVM. The current study was approved by the Committee on the Ethics of Animal Experiments (permit no. CBNUA-584-13-01; Chungbuk National University, Cheongju, Republic of Korea). Porcine ovaries were obtained from 496 Landrace x Duroc crossbreed gilts (sows) from a local slaughterhouse (Dong-A, Chengju, Korea) and transported to the laboratory within 2 h in 0.9% (w/v) NaCl supplemented with penicillin-G (100 IU/ml) and streptomycin sulfate (100 mg/l) at 30-35˚C. Follicular fluid with oocytes was aspirated from antral follicles (3-6 mm) using an 18-gauge needle connected to a 10-ml disposable syringe and collected into a 15-ml centrifuge tube. Cumulus-oocyte complexes (COCs) were recovered under a stereomicroscope (SZ51; Olympus, Tokyo, Japan), and those with at least three layers of compact cumulus cells and a homogenous cytoplasm were selected for IVM. The selected COCs were washed three times in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered Tyrode's medium containing 0.05% (w/v) polyvinyl alcohol (TLH-PVA), and transferred to 500 μl tissue culture medium 199 (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 0.6 mM cysteine, 0.91 mM sodium pyruvate, 10 ng/ml epidermal growth factor, 75 μg/ml kanamycin, 1 μg/ml insulin and 10% (v/v) pFF. pFF was aspirated from follicles (3-7 mm) obtained from pre-pubertal gilt ovaries. After centrifugation at 1,600 xg for 30 min, the supernatants were collected and filtered sequentially through 1.2- and 0.45-μm syringe filters (Gelman Sciences, Ann Arbor, MI, USA). The prepared pFF was then stored at -20˚C until use. For maturation, the selected COCs were washed three times in oocyte maturation medium containing hormone supplements, and 50-60 oocytes were transferred to each well of a four-well Nunc dish (Roskilde, Denmark) containing 500 μl of culture medium and equilibrated for at least 2 h with 5% CO_2 at 39˚C in a humidified atmosphere. During the first 22 h, the COCs were matured with hormones (10 IU/ml equine chorionic gonadotropin and 10 IU/ml hCG (Intervet, Boxmeer, the Netherlands). After 22 h of maturation with hormones, the COCs were washed twice and cultured in hormone-free IVM medium for an additional 18-20 h.

Assessment of nuclear maturation. After 40-42 h of culture, the oocytes were denuded by gently pipetting them in IVM medium containing 0.1% hyaluronidase followed by washing in TLH-PVA. The denuded oocytes were fixed for 5 min in fixative solution containing 2% formaldehyde and 0.25% glutaraldehyde and then stained in TLH-PVA containing 10 μg/ml Hoechst 33342 for 10 min. The stained oocytes were examined by fluorescence microscopy (Nikon Corp., Tokyo, Japan) and classified according to their developmental stage as follows: Germinal vesicle, metaphase I (MI), anaphase I/telophase I or MII.

Experimental groups. Zinc deficiency was induced with TPEN. TPEN was prepared as a 1-mM stock solution and used at a final concentration of 10 μM. In experiment 1, nuclear maturation, cytoskeletal component organization, GSH, ROS, and subsequent embryonic development in the following three groups were evaluated to investigate the effects of zinc deficiency during IVM: i) Treatment without TPEN (control); ii) treatment with 10 μM TPEN for 22 h during IVM; and iii) treatment with 10 μM TPEN+10 μM zinc (zinc sulfide) for 22 h during IVM. In experiment 2, the effects of the zinc deficiency period during IVM on oocyte maturation and subsequent embryonic development after PA were determined. TPEN (10 μM) was added to the IVM medium for 0, 7, 15 or 22 h. After TPEN treatment, 10 μM zinc was added to the IVM medium during the second half of IVM in all but the 0-h group.

Immunofluorescence imaging. Oocytes were collected after IVM, fixed in 4% paraformaldehyde for 40 min and then permeabilized with 1% Triton X-100 for 30 min. After incubation in am Image-iTTM FX Signal Enhancer (I36933; Invitrogen Life Technologies) for 30 min, the oocytes were blocked with 1% bovine serum albumin (BSA; A9418; Sigma-Aldrich) in phosphate-buffered saline (PBS) for 1 h and then incubated with fluorescein isothiocyanate-conjugated monoclonal anti-α-tubulin antibodies (F2168, 1:100; Sigma-Aldrich) or tetramethylrhodamine-conjugated phalloidin (P1951, 1:200; Sigma-Aldrich) for 1 h at 37˚C. After

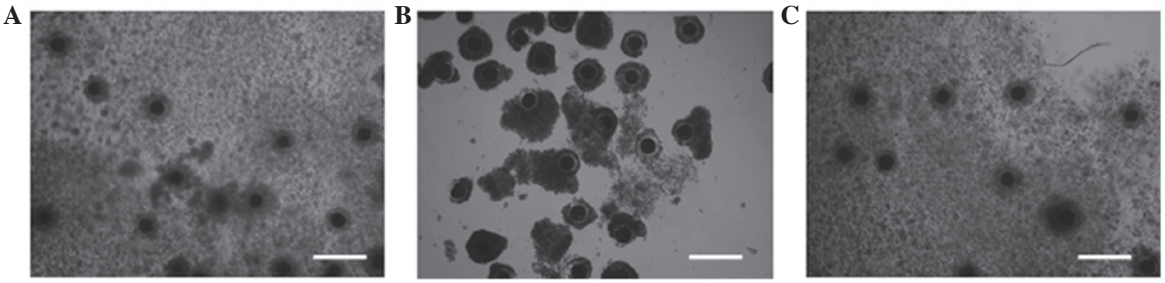

Figure 1. Effect of zinc deficiency during IVM on cumulus cell expansion at 42 h after IVM. (A) Control, (B) TPEN (10 μM), (C) TPEN+zinc. Control and TPEN+zinc treatment groups showed abundant cumulus cell expansion. However, cumulus cell expansion was inhibited in the TPEN-treated group. Scale bar, 300 μm. IVM, *in vitro* maturation; TPEN, zinc chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylendiamine.

nuclear staining with 10 μg/ml Hoechst 33342, The oocytes were mounted on slides with 1,4-diazabicyclo-(2.2.2) octane (P0126; Beyotime Institute of Biotechnology, Shanghai, China) and observed with a laser-scanning confocal microscope (TCS SP2 AOBS; Leica Microsystems, Wetzlar, Germany).

Measurement of intracellular ROS and GSH levels. The oocytes were sampled after 44 h of IVM to determine their intracellular ROS and GSH levels as described previously (31). Briefly, 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen Life Technologies) and CellTracker Blue (4-chloromethyl-6.8-difluoro-7-hydroxycoumarin; Invitrogen Life Technologies) were used to detect intracellular ROS as green fluorescence and the GSH level as blue fluorescence. Ten oocytes from each treatment group were incubated in the dark for 30 min in TLH-PVA supplemented with 10 μM H2DCFDA and 10 μM CellTracker. After incubation, the oocytes were washed with Dulbecco's PBS (Invitrogen Life Techonologies) containing 0.1% (w/v) PVA and placed in 10-μl microdrops, and fluorescence was detected under an epifluorescence microscope (TE300; Nikon) with ultraviolet filters (460 nm for ROS and 370 nm for GSH). Fluorescence images were saved as graphic files in TIFF format. The fluorescence intensities of the oocytes were analyzed with ImageJ software v. 1.41o (National Institutes of Health, Bethesda, MD, USA) and normalized to a control.

Parthenogenetic activation (PA). For PA, mature oocytes were activated with two pulses of 120 V/mm of DC for 60 μsec in 280 mM mannitol containing 0.01 mM $CaCl_2$ and 0.05 mM $MgCl_2$. Following electrical activation, the PA embryos were treated with 5 μg/ml cytochalasin B in IVC medium for 6 h at 39˚C in a humidified atmosphere of 5% CO_2 in air.

In vitro embryo culture. The PA embryos were washed three times with IVC medium (porcine zygote medium 3) and cultured in 30-μl microdrops of IVC medium. Embryos in culture medium were covered with pre-warmed mineral oil and incubated at 39˚C for 7 days under a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2.

Embryo evaluation and total cell count. The day when PA was performed was designated as day 0. The embryos were evaluated under a stereomicroscope for cleavage on day 2 (48 h). Blastocyst formation was assessed on day 7 (168 h). To determine the total cell number in the blastocysts on day 7, blastocysts were collected and the zona pellucida of (if unhatched) was dissolved with 0.5% protease. The zona-free blastocysts were washed in PBS containing 1% (w/v) BSA and stained with 10 μg/ml Hoechst 33342 for 5 min. Following a final wash in PBS-BSA, the blastocysts were fixed briefly (10 min) in 4% paraformaldehyde in PBS. The blastocysts were mounted on glass slides in a drop of 100% glycerol, gently squashed under a cover slip and observed by fluorescence microscopy (Nikon) at x400 magnification.

Statistical analysis. Each experiment consisted of at least three replicates. All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The GSH and ROS levels and embryonic development data (e.g., rate of cleavage, blastocyst formation and number of nuclei) were compared by a one-way analysis of variance, followed by Duncan's multiple range test. All values are expressed as the mean or mean ± standard error of the mean. $P<0.05$ was considered to indicate a statistically significant difference between values.

Results

Zinc deficiency inhibits oocyte maturation and subsequent embryo development. Cumulus cell expansion was observed after IVM. The control and TPEN+zinc treatment groups showed normal cumulus cell proliferation, while in the TPEN-treated group, cumulus cell proliferation did not proceed (Fig. 1).

Almost all oocytes in the TPEN-treated groups were arrested at MI. Only 0.61% of the oocytes matured to MII. By contrast, >90% of the oocytes reached MII in the control and TPEN+zinc-treated groups (Table I).

Cytoskeletal organization was investigated by immunofluorescence staining to determine an accurate meiotic rate. Almost all oocytes in the control and TPEN+zinc-treated groups displayed a metaphase spindle and polar body (Fig. 2). By contrast, almost all of the TPEN-treated oocytes displayed only a metaphase spindle (Fig. 2). Although the meiotic stages were different, the shapes of the chromatin and spindle were normal in all groups. Only a few oocytes in the TPEN-treated group did not have a spindle signal.

In the control, a typical MII-stage distribution of microfilaments was observed. Strong signals were detected at the cortex (Fig. 3). The microfilament distribution was abnormal in the

Table I. Effect of zinc deficiency during *in vitro* maturation on nuclear maturation of porcine oocytes.

Group	Oocytes cultured for maturation (n)[a]	Number of oocytes (n)			
		Germinal vesicle	Metaphase I	Anaphase and telophase I	Metaphase II
Control	150	0	3.3±0.7[b]	4.0±1.2	92.7±1.8[b]
TPEN	157	1.9±1.1	96.2±1.0[c]	1.3±0.6	0.6±0.6[c]
TPEN+Zn	156	0	5.1±1.2[b]	1.9±0.0	93.0±1.2[b]

[a]Three replicates. [b,c]P<0.05 between values with different footnotes within a column. Values are expressed as the mean ± standard error of the mean. TPEN, zinc chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylendiamine.

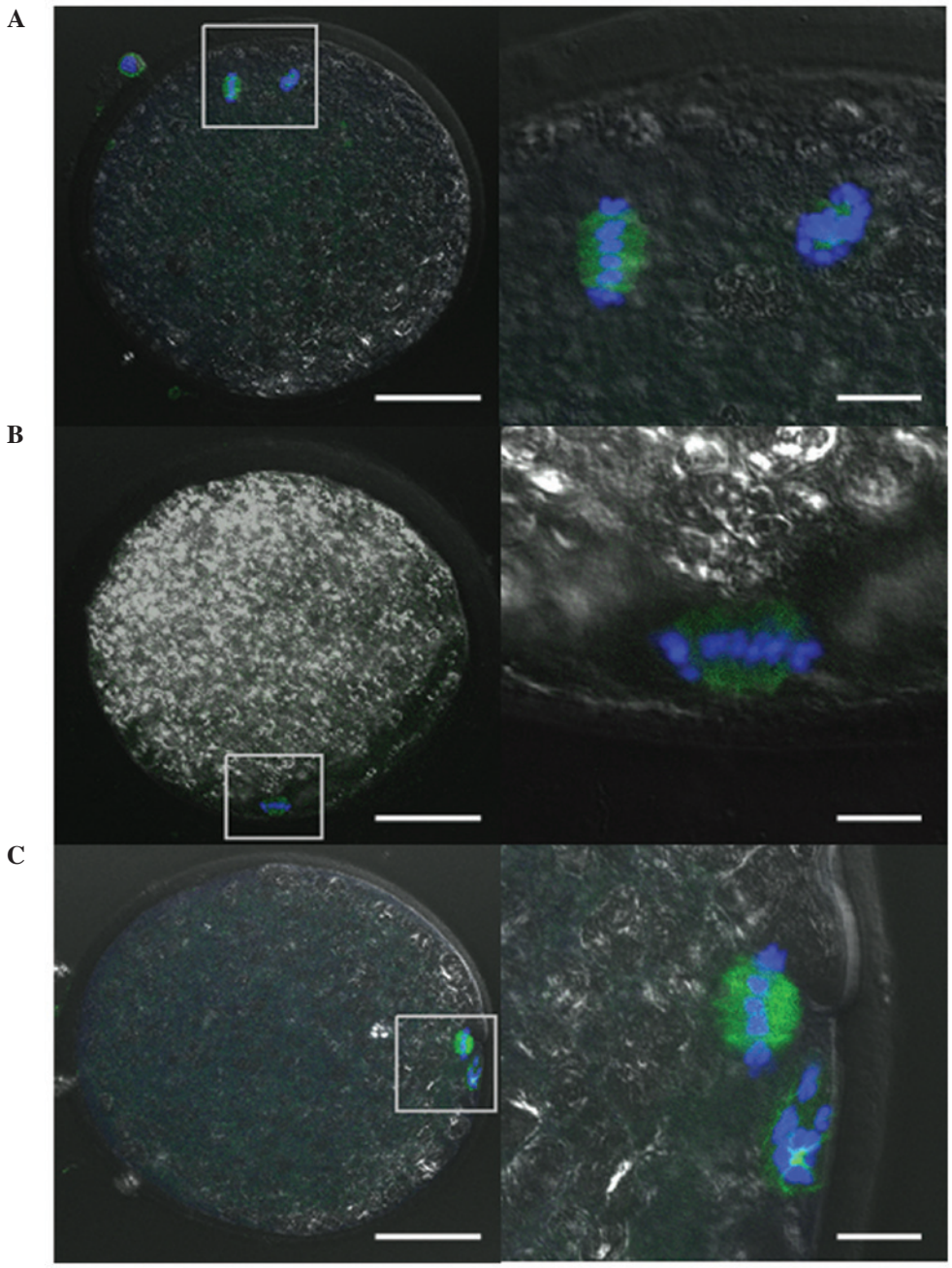

Figure 2. Immunofluorescence localization of microtubules in zinc-deficient porcine oocytes at 42 h after *in vitro* maturation. Microtubules were immunostained with anti-α tubulin (green) and DNA was counterstained with Hoechst (blue). (A) Oocytes of the control group showed metaphase plates and polar bodies at metaphase II stage. (B) TPEN-treated oocytes were arrested at metaphase I. (C) TPEN+zinc-treated oocytes showed metaphase plates and polar bodies at metaphase II stage. Scale bars, 50 and 10 μm in left and right panels, respectively. TPEN, zinc chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylendiamine.

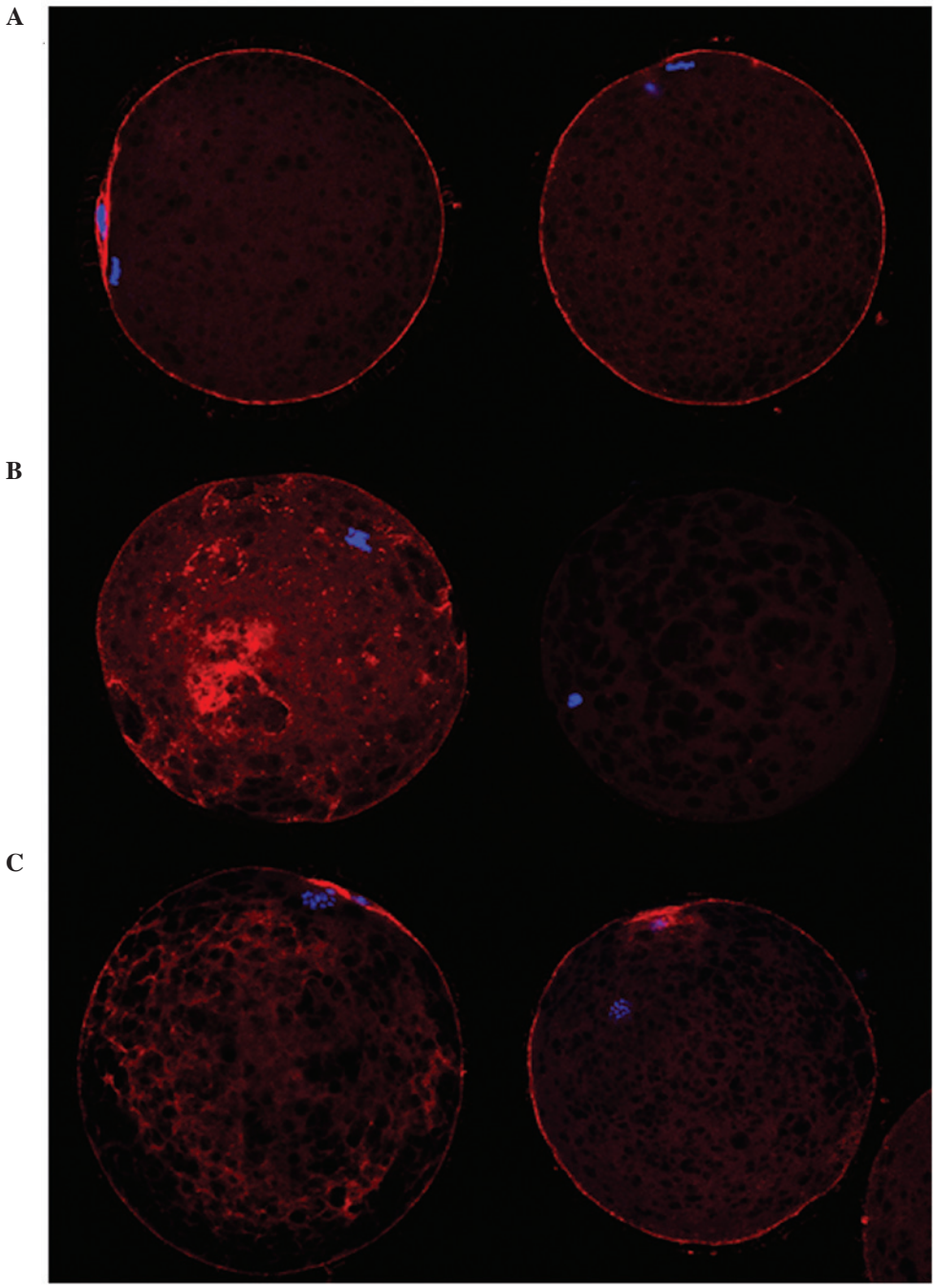

Figure 3. Immunofluorescence localization of microfilaments in zinc deficient porcine oocytes at 42 h after *in vitro* maturation. Microfilaments were immunostained with anti-microfilament (red) and DNA was counterstained with Hoechst (blue). (A) Oocytes of the control group were in metaphase II nuclear stage and showed normal microfilament distributions. (B) TPEN-treated oocytes were arrested at metaphase I and microfilaments were organized in abnormal patterns (left, irregular distribution of microfilament; right, microfilament signals were not present). (C) TPEN+zinc-treated oocytes were in metaphase II nuclear stage. Strong microfilament signals were detected in the cortex, while a number of week microfilament signals were present in cytoplasm. Scale bar, 50 μm. TPEN, zinc chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylendiamine.

TPEN-treated group; the microfilaments were irregularly distributed in the cytoplasm and cortex, which were unevenly shaped. Certain oocytes in the TPEN-treated group did not have a microfilament signal (Fig. 3). Most of the oocytes in the TPEN+zinc-treated group showed a relatively normal microfilament distribution. Weak microfilament signals were detected in the cytoplasm of certain oocytes.

The GSH and ROS levels were significantly altered in the TPEN-treated group; the GSH levels decreased significantly, while the ROS levels increased significantly (Fig. 4). The GSH and ROS levels changed slightly in the TPEN+zinc-treated group, but not significantly.

Subsequent development of PA embryos was also decreased in the TPEN-treated group. On day 2 of IVC, almost all oocytes in the TPEN-treated group were arrested at the one-cell stage (Fig. 5). Although the cleavage patterns on day 2 were slightly different between the control and TPEN+zinc-treated groups, the two groups showed normal cleavage patterns. A total of 40.0 and 42.0% of the embryos developed to blastocysts in the control and TPEN+zinc-treated groups, respectively

Table II. Effect of zinc deficiency on embryonic development after parthenogenetic activation during *in vitro* maturation.

Group	Embryos cultured (n)[a]	Embryos developed to		Total cell number in blastocyst
		≥2-cell (%)	Blastocyst (%)	
Control	157	72.9±2.8	40.0±7.5	51.0±6.2
TPEN	147	0	0	-
TPEN+Zn	144	75.5±2.2	42.0±6.7	47.2±4.8

[a]Three replicates. Values are expressed as the mean ± standard error of the mean. TPEN, zinc chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylendiamine.

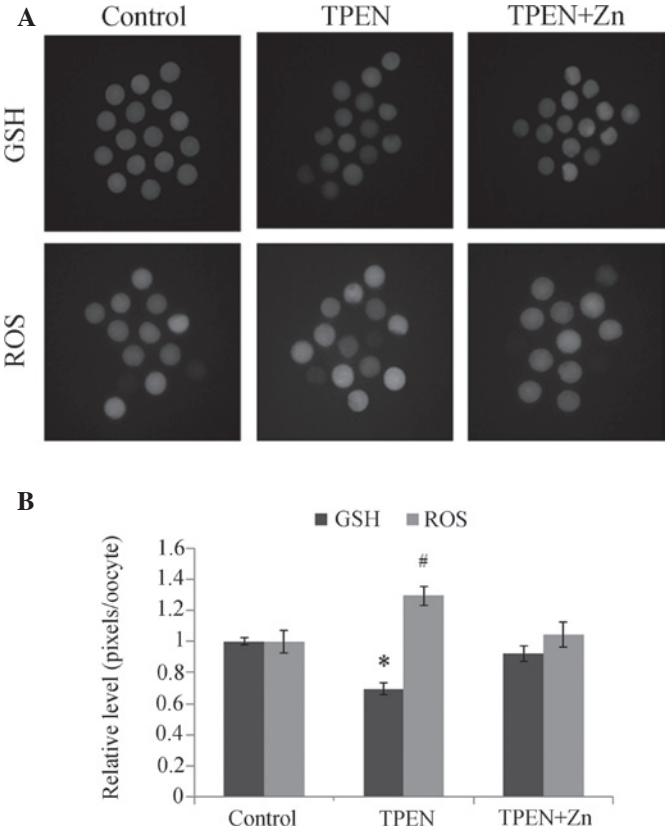

Figure 4. Epifluorescent photomicrographic images of *in vitro* matured porcine oocytes. (A) Oocytes were stained with (top row) CellTracker Blue and (bottom row) 2',7'-dichlorodihydrofluorescein diacetate to detect intracellular levels of GSH and reactive ROS, respectively. Metaphase-II oocytes derived from the control group, TPEN-treated group and TPEN+Zn-treated group. (B) Effect of zinc deficiency on intracellular GSH and ROS levels on porcine oocytes during *in vitro* maturation. Values are expressed as the mean ± standard error of the mean (n=3). $^{*,\#}P<0.05$ vs. control. ROS, reactive oxygen species; GSH, glutathione; TPEN, zinc chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylendiamine.

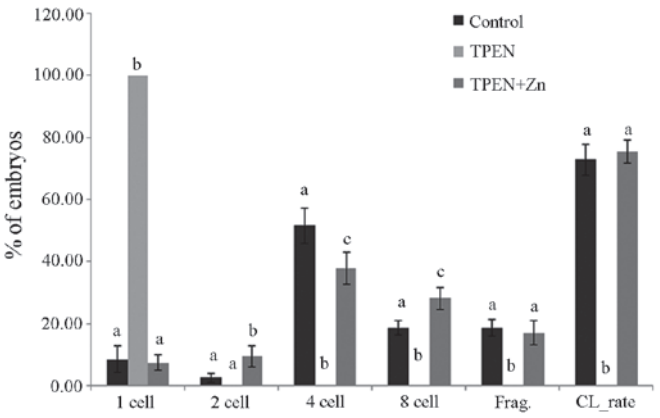

Figure 5. Effect of zinc deficiency in maturation medium on cleavage patterns on day 2. Values are expressed as the mean ± standard error of the mean (n=3). $^{a-c}P<0.05$ between values with different footnotes within a column. Frag, fragmented embryos, which contain unequally-sized blastomeres and multiple cellular fragments; CL_rate, cleave rates, ratio of 2- to 8-cell embryos; TPEN, zinc chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylendiamine.

(Table II). No cleaved embryos or blastocysts were observed in the TPEN-treated group.

Zinc withdrawal inhibits oocyte maturation and subsequent embryonic development in a time-dependent manner. The cumulus cells in the group treated for 7 h were slightly expanded subsequent to IVM, whereas no expansion was observed in the groups treated for 15 and 22 h (Fig. 6).

An assessment of nuclear maturation produced results that were similar to those for cumulus cell expansion. Nuclear maturation rates decreased with increasing TPEN treatment duration. The MI and MII rates in the control group were 11.5 and 83.9%, respectively. The group treated for 7 h had MI and MII rates of 50.4 and 44.8%, respectively. No MII oocytes were observed in the groups treated for 15 and 22 h (Table III). Approximately 98.0 and 97.2% of the oocytes in the groups treated for 15 and 22 h, respectively, were in MI.

Subsequent development was also different with TPEN treatment time. The number of one-cell stage embryos on day 2 increased by increasing TPEN treatment duration. In the group treated for 7 h, the rate of cleaved embryos decreased significantly compared to that in the control group. The numbers of two- and four-cell stage embryos decreased, while the numbers of one-cell stage and fragmented embryos increased. Most embryos did not cleave in the groups treated for 15 and 22 h (Fig. 7). The blastocyst formation rate and total cell number in the blastocysts were highest in the control group. The blastocyst formation rate and total cell number in the blastocysts in the group treated for 7 h decreased significantly (blastocyst formation rate, 10.4%; total cell number, 23.2). Only 2.6 and 3.0% of the embryos developed beyond the two-cell stage in the groups treated for 15 and 22 h, and no blastocysts formed (Table IV).

Table III. Effect of zinc deficiency for various durations on nuclear maturation during *in vitro* maturation.

TPEN treatment time	Oocytes cultured for maturation (n)[a]	Oocytes at various stages (%)			
		Germinal vesicle	Metaphase I	Anaphase and telophase I	Metaphase II
0 h	105	0.9±0.9	11.5±2.9[b]	3.8±0.8[d]	83.9±3.9[d]
7 h	101	1.9±1.9	50.4±1.3[c]	3.0±0.1[c,d]	44.8±3.0[c]
15 h	98	2.0±1.0	98.0±1.0[d]	0.0[b]	0.0[b]
22 h	103	1.9±0.9	97.2±1.7[d]	1.0±1.0[b,c]	0.0[b]

[a]Three replicates. [b-d]P<0.05 between values with different footnotes within a column. Values are expressed as the mean ± standard error of the mean. TPEN, zinc chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylendiamine.

Table IV. Effect of zinc deficiency for various durations on embryonic development after parthenogenetic activation during *in vitro* maturation.

TPEN Treatment time	Embryos cultured (n)[a]	Embryos developed to		
		≥2-cell (%)	Blastocyst (%)	Cell number in blastocyst
0 h	101	65.3±1.4[b]	29.7±1.2[b]	51.4±4.5[c]
7 h	107	42.6±4.8[c]	10.4±1.4[c]	23.2±1.6[d]
15 h	114	2.6±0.1[d]	0.0[d]	-
22 h	103	3.0±1.6[d]	0.0[d]	-

[a]Three times replicates. [b-d]P<0.05 between values with different footnotes within a column. Values are expressed as the mean ± standard error of the mean. TPEN, zinc chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylendiamine.

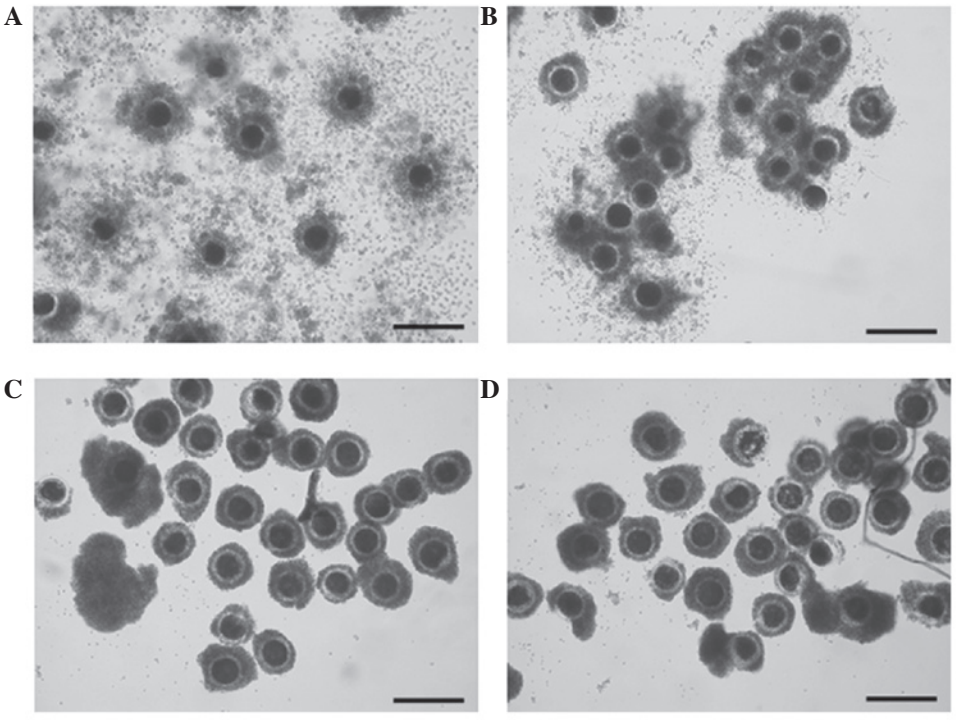

Figure 6. Effect of zinc-deficient time during IVM on cumulus cell expansion at 42 h after IVM. (A) Control (no treatment), (B) 7 h TPEN treatment, (C) 15 h TPEN treatment and (D) 22 h TPEN treatment (magnification, x50; scale bar, 250 μm). The control group shows abundant cumulus cell expansion. Slight cumulus cell expansion was observed in B. However, cumulus cell expansion was inhibited in C and D. IVM, *in vitro* maturation; TPEN, zinc chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylendiamine.

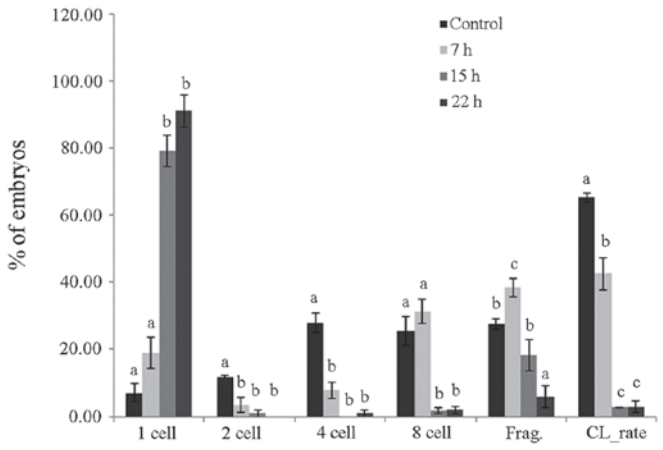

Figure 7. Effect of zinc-deficiency periods during *in vitro* maturation on cleavage patterns on day 2. Values are expressed as the mean ± standard error of the mean (n=3). $^{a-c}P<0.05$ between values with different footnotes within a column. Frag, fragmented embryos, which contain unequally-sized blastomeres and multiple cellular fragments; CL_rate, cleave rates, ratio of 2- to 8-cell embryos.

Discussion

The IVM environment has a prominent effect on oocyte maturation and early embryonic development. Various factors are known to affect oocyte and embryonic development. However, the contribution of zinc to porcine oocyte maturation has yet to be determined. In the present study, the involvement of zinc in porcine oocyte maturation was investigated by inducing zinc deficiency during porcine IVM.

A decrease in the zinc concentration of oocytes was induced by TPEN treatment. TPEN is a lipid-soluble zinc metal chelator that decreases the intracellular levels of zinc (32). Although TPEN has a strong affinity for transition metals, including iron, copper (33) and zinc, while the content of other metals is unchanged relative to that of control cells, TPEN treatment only significantly reduces the zinc content compared with that in the control group (34). TPEN is usually used as a zinc-specific chelator in *in vitro* studies (35,36). The concentration of TPEN was set at 10 μM in accordance with the protocols of previous studies. According to Kim *et al* (34), concentrations <10 μM did not have any effect on meiotic maturation, whereas concentrations ≥20 μM had toxic effects on mice oocytes.

In the present study, a decrease in cumulus cell expansion was observed as the first effect of TPEN treatment. Cumulus cell expansion occurred normally in the control and TPEN+zinc-treated groups but not in the TPEN-treated group. TPEN-induced zinc deficiency was previously shown to markedly increase apoptosis induced by cytokines, lipids and oxidative stress in somatic cells (37-39). Apoptosis caused by zinc deficiency may also inhibit cumulus cell expansion. Cumulus cells have an important role in oocyte maturation. Cumulus cells surround each individual oocyte and are functionally associated to the nuclear or cytoplasmic maturation of oocytes (40). Cumulus cells control nuclear maturation by maintaining a meiotic block at the germinal vesicle stage (41) and trigger the resumption of meiosis by secreting a meiosis-inducing substance (8). Cumulus cells are required for cytoplasmic maturation and developmental competence during IVM as they synthesize and transport GSH to oocytes (42). Thus, poor expansion of cumulus cells has a negative impact on oocyte maturation.

In the present study, nuclear maturation was also affected by TPEN-induced zinc deficiency. MI oocytes increased in number, while MII oocytes decreased in number only in the TPEN-treated group. TPEN treatment causes a meiotic block at telophase I during mouse IVM (34); however, in the present study, the rates of anaphase and telophase did not change in any of the groups, possibly due to differences in the meiotic process or the role of zinc in meiosis in different species. In conventional IVM of porcine oocytes, most incompetent oocytes are arrested at MI (43), and zinc deficiency may be a factor leading to MI arrest. As described above, poor cumulus cell expansion would also have affected nuclear maturation.

Meiotic spindle morphology accurately reflects an oocyte's meiotic status (44,45). Therefore, spindle morphology was investigated by α-tubulin immunofluorescence. The control and TPEN+zinc-treated groups developed a metaphase spindle with a polar body, whereas the TPEN-treated group only developed a metaphase spindle. According to Ueno *et al* (46), spindle morphology indicates oocyte quality. However, in the present study, the shape and size of the spindles were similar in all groups. The spindle length was ~10 μm, the spindle was round, and no significant differences were observed between the experimental groups. Thus, zinc deficiency did not directly affect the meiotic spindle.

Microfilaments are cytoskeletal components with an important role during cell division (47). In the present study, microfilament abnormalities were observed in the TPEN-treated group. A previous study showed that the microfilament area exists at the cortex during metaphase in porcine oocytes (48). However, microfilaments occurred randomly in the cytoplasm and cortex of TPEN-treated oocytes. Abnormal microfilament distribution would affect meiotic maturation. Longo and Chen (49) reported on the role of microfilaments during meiosis in mouse oocytes. Their study showed that the meiotic spindle with chromatin failed to move to the oocyte cortex, and extrusion of the polar body was inhibited by treatment with the microfilament-disrupting agent cytochalasin B. This result provided an explanation for the meiotic block and failure of polar body extrusion in TPEN-treated oocytes. It also suggested that zinc is involved in microfilament distribution during porcine oocyte maturation.

In the TPEN-treated group, the decreased GSH and increased ROS levels indicated poor cytoplasmic maturity. The GSH concentration increases during IVM, reaching its highest level at MII (50,51). Decreased GSH in the TPEN-treated oocytes can be explained by reduced cumulus cell expansion or decreased synthesis due to improper cytoplasmic maturation. The increased ROS levels in the TPEN-treated oocytes implied a lack of anti-oxidant activity of GSH and zinc. The main functions of GSH in oocytes include anti-oxidant activity and protection against the harmful effects of ROS (52). Although the underlying mechanisms have yet to be elucidated, zinc is also involved in protecting oocytes from oxidative stress (53). Zinc acts as an anti-oxidant at multiple cell levels (54) and can induce the synthesis of metallothionein, a protein that chelates redox-active metals and scavenges hydroxyl radicals

via its cysteine groups (55). Zinc is an important constituent of Cu/Zn superoxide dismutase (56), which scavenges free oxygen radicals. Numerous studies have reported that a zinc deficiency induces oxidative stress in *in vitro*-cultured cells (57-59).

Improper cytoplasmic maturation is a major problem in IVM of porcine oocytes. The low developmental potential of porcine oocytes during IVM is the result of improper cytoplasmic maturation. In the present study, improper cytoplasmic maturation due to zinc deficiency led to decreased embryonic development. Thus, the role of zinc in the maturation process is important.

The duration of zinc deficiency also affected porcine oocyte maturation. TPEN treatment fir >7 h inhibited cumulus cell expansion and meiotic maturation. Furthermore, oocytes did not recover with zinc supplementation after >15 h of TPEN treatment. In previous studies on mouse oocytes, normal oocyte meiotic maturation occurred with TPEN treatment for <7 h, whereas meiotic maturation was inhibited by TPEN exposure for >12 h. Although 9 h-exposed oocytes reached MII, they had a large spindle and polar body (34). These results were not observed in porcine oocytes. The effect of zinc on microfilaments may therefore be different between species. In mice, meiotically blocked oocytes due to TPEN treatment displayed a normal microfilament distribution and cleavage, whereas an abnormal microfilament distribution was present and no cleavage occurred in TPEN-treated pig oocytes.

In conclusion, the results of the present study showed that nuclear and cytoplasmic maturation were decreased in zinc-deficient porcine oocytes. Furthermore, zinc deficiency decreased subsequent embryonic development. In addition, zinc deficiency for >1 h caused irreversible damage to oocytes. These results indicated that zinc regulates the meiotic process and has important roles in oocyte maturation. Additional studies are required to identify the mechanism of action of zinc during oocyte maturation.

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