—Original Article—

# Effects of manganese on maturation of porcine oocytes *in vitro* and their subsequent embryo development after parthenogenetic activation and somatic cell nuclear transfer

**Muhammad QASIM[1), Jun-Xue JIN[1, 2), Sanghoon LEE[1, 3), Anukul TAWEECHAIPAISANKUL[1),
Erif Maha Nugraha SETYAWAN[1), Geon A KIM[1) and Byeong Chun LEE[1)**

[1)Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Seoul National University, Seoul 08826, Republic of Korea
[2)Key Laboratory of Animal Cellular and Genetic Engineering of Heilongjiang Province, College of Life Science, Northeast Agriculture University, Heilongjiang 150030, China
[3)Futuristic Animal Resource & Research Center, Korea Research Institute of Bioscience and Biotechnology, Chungcheongbuk-do 28116, Republic of Korea

**Abstract.** This study was carried out to examine the effects of manganese (Mn) on the developmental competence of porcine oocytes during *in vitro* maturation (IVM) after parthenogenetic activation (PA) and somatic cell nuclear transfer (SCNT). Upon treatment of porcine oocytes with different concentrations (0, 3, 6, and 12 ng/ml) of Mn during IVM, PA was performed to determine the optimum concentration. Following PA, the rate of blastocyst formation was higher significantly in treated porcine oocytes at 6 ng/ml of Mn than in other groups (P < 0.05). However, there was no substantial difference in the cleavage rate and total blastocyst cell numbers among all groups. SCNT was performed using the optimal concentration of Mn from PA, which showed an improved blastocyst formation rate in treated oocytes compared to that in control group (P < 0.05). However, the cleavage rate and total cell numbers per blastocyst were not different between the control and the Mn treated groups after SCNT. Additionally, oocyte nuclear maturation, intracellular glutathione (GSH), and reactive oxygen species (ROS) levels were assessed. There was no significant difference observed in nuclear maturation among all the groups. However, enhanced intracellular GSH levels while lower levels of ROS were seen in the Mn treated group compared to the control group (P < 0.05). Thus, these results indicate that Mn supplementation can improve the developmental competence of porcine PA and SCNT embryos by increasing GSH and decreasing ROS levels.
**Key words:** Antioxidant, Embryo development, Manganese (Mn), Porcine oocytes

Humans and pigs share many common characteristics, including diet, body size, and brain size [1]. Pigs are believed to be the preferred disease model when compared with other species [2]. They are an ideal animal model for xenotransplantation, biomedical research [3, 4] and many other specific studies on human diseases [5, 6]. *In vitro* embryo production (IVP) is required to produce the animal models that can possibly reproduce the human pathology [7] such as cardiovascular diseases, cancers, diabetes mellitus, Alzheimer's disease, cystic fibrosis, and Duchenne muscular dystrophy [2]. Therefore, IVP has gained so much importance in recent years. Although there have been many improvements in porcine IVP protocols, the efficiency of embryo production is still much lower than *in vivo* embryo production [8, 9]. The most important reason for this low efficiency is due to the oxidative stress [10] caused by

excessive production of reactive oxygen species (ROS) [11] during *in vitro* oocyte maturation. Therefore, adding free radical scavengers is needed to protect oocytes and to limit the detrimental effects of ROS [12]. Various kinds of antioxidants, for example, melatonin [13], spermine, [14], resveratrol [15], zinc [16], and copper [17] have been used to improve the quality of *in vitro* produced embryos.

Manganese (Mn) is a trace mineral naturally present in food, especially in cereals, nuts, and vegetables [18]. This is required for many physiological processes [19] such as metabolism of lipids, proteins, and carbohydrates [20], bone growth, energy metabolism, reproduction, and antioxidant defenses [21]. Mn is an essential element required to increase the activity of manganese superoxide dismutase (MnSOD), a major antioxidant enzyme located in mitochondria. Moreover, MnSOD activity can be enhanced through the mitochondrial protein influx [22–24] and protects the mammalian cells from the damaging effects of ROS [25]. The MnSOD activity is not only related to levels of superoxide anion but also to the glucose and oxygen consumption in cells [26]. The superoxide dismutase (SOD) activity was increased in cumulus cells when the maturation media was supplemented with Mn [27]. Moreover, the DNA integrity can be preserved and developmental competence of bovine oocytes to blastocyst stage can be increased through the antioxidant effect of Mn

in cumulus oocyte complexes (COCs) [28]. Furthermore, the addition of Mn to maturation medium not only improved the competence of oocytes to be fertilized but also decreased the frequency of apoptotic cumulus cells [29]. Mn functions as a cofactor for several important enzymes such as arginase, pyruvate decarboxylase, and glutamine synthase [30], which are involved in DNA synthesis [31] and DNA protection [25]. Any abnormality in the Mn homeostasis can lead to the misregulation of cell cycle progression [32]. Recently, few studies have been conducted regarding supplementation of Mn in rat [33], cattle [34], and dog [35] *in vivo* and *in vitro* in bovine embryos [28]. It has been reported that the presence of micronutrient such as Mn, not only protects the DNA integrity but also improved the bovine oocyte quality and embryo development to blastocyst stage with increased intracellular levels of glutathione (GSH) [27]. However, no previous study has shown the effects of Mn supplementation during *in vitro* maturation (IVM) of porcine oocytes and their subsequent embryo development after parthenogenetic activation (PA) and somatic cell nuclear transfer (SCNT). Therefore, we designed this study to observe the effects of Mn supplementation during IVM of porcine oocytes on their nuclear maturation, GSH, and ROS levels in mature porcine oocytes, and to observe the development of embryos after performing PA and SCNT.

## Material and Methods

### Chemicals

All chemicals and reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

### Oocyte collection and IVM

Ovaries were collected from a local slaughterhouse in collection vials containing normal saline kept at 32–35ºC and were transported to the laboratory. Aspiration of COCs from antral follicle (3–6 mm in diameter) was performed using an 18-gauge needle. The contents collected were poured into 50 ml conical tube and held at 37ºC until they settle down. The supernatant was discarded, and cellular contents were washed in tissue culture medium-199 (TCM-199; Invitrogen, Carlsbad, CA, USA) containing 2 mM sodium bicarbonate, 10 mM N-(2-hydroxyethyl) piperazine-N'- (2-ethanesulfonic acid; HEPES), 5 mM sodium hydroxide, 1% Pen-Strep (Invitrogen), and 0.3% polyvinyl alcohol (PVA). After washing, COCs with a homogeneous cytoplasm and at least three layers of cumulus cells were examined under a stereomicroscope. COCs were transferred into 500 µl of maturation medium comprising TCM-199 supplemented with 0.91 mM sodium pyruvate, 5 µl/ml insulin-transferrin-selenium solution (ITS-A) 100X (Invitrogen), 0.57 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 10 IU/ml human chorionic gonadotropin (hCG), 10 IU/ml equine chorionic gonadotropin (eCG), 10% porcine follicular fluid (vol/vol), and various concentrations (0, 3, 6, and 12 ng/mL) of Mn (II) sulfate monohydrate (M7899) purchased from Sigma-Aldrich in the treatment groups, after diluting in TCM-199. For IVM, COCs were then cultured at 38.5ºC in 5% $CO_2$ in humidified air for 44 h. Following first 22 h, COCs were cultured for further 22 h in hormone-free IVM medium.

### Evaluation of porcine oocyte maturation

After 44 h of culture in IVM medium, porcine oocytes were denuded by gentle pipetting using 0.1% hyaluronidase in Tyrode's albumin lactate pyruvate (TALP) medium with HEPES buffer. The denuded oocytes were then stained using 5 µg/ml of bisbenzimide (Hoechst 33342) in TALP-HEPES for approximately 10 min. A fluorescence microscope (Nikon, Tokyo, Japan) was used to evaluate the stained oocytes. We repeated the experiment for six times.

### Measurement of intracellular ROS and GSH levels

Denuded oocytes at metaphase II (MII) stage were selected to determine intracellular levels of GSH and ROS after staining with CellTracker Blue $CMF_2HC$ (4-chloromethyl-6.8-difluoro-7-hydroxycoumarin; Invitrogen), and $H_2DCFDA$ (2', 7'-dichlorodihydrofluorescein diacetate; Invitrogen), respectively. TALP containing 10 µM $H_2DCFDA$ and 10 µM CellTracker Blue was used as the incubation medium and 10 MII stage oocytes from each treatment group were transferred to these media and incubated in the dark for 30 min. After incubation, stained oocytes were washed three times in TALP and approximately four oocytes were placed into 4 µl droplet of TALP-HEPES and fluorescence was observed under an epifluorescence microscope (TE2000-S; Nikon) with UV filters (460 nm for ROS and 370 nm for GSH). Image J software (Version 1.49q; National Institutes of Health, Bethesda, MD) was used to observe the fluorescence intensities of oocytes and normalized to control embryos.

### Parthenogenetic activation of oocytes

Denuded oocytes with homogeneous cytoplasm and an extruded first polar body (PB) were selected and gradually equilibrated to avoid the somatic shock [36] in the activation solution containing 0.28 M mannitol, 0.5 mM HEPES, 0.1 mM $CaCl_2$, and 0.1 mM $MgSO_4$. The activation chamber was placed on a heating stage and filled with an activation solution after connecting two electrodes spaced 3.2 mm apart. Oocytes were placed in the chamber and activated with a single direct current (DC) pulse of 1.5 kV/cm for 60 µsec using a BTX Electro-Cell Manipulator 2001 (BTX, San Diego, CA, USA). After activation, oocytes were washed twice with porcine zygote medium-5 (PZM-5; Funakoshi, Tokyo, Japan) and approximately 50–60 electrically activated oocytes were placed in 500 µl of PZM-5 and cultured under a humidified atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$ at 38.5ºC for 7 days.

### Preparation of donor cell

Fibroblast cells derived from the skin of a pig were used as donors. Briefly, the skin was chopped into small pieces and washed in PBS three times. This was cultured in a humidified air atmosphere incubator with 5% $CO_2$ at 38ºC in air in Dulbecco's modified Eagle's medium (DMEM; Gibco, culture medium) containing 10% fetal bovine serum (FBS; Gibco, culture medium; vol/vol), 1 mM sodium pyruvate, and 100 IU/ml each of penicillin and streptomycin. Cells between passages 5 and 7 were used for SCNT. Immediately before SCNT, single cell suspensions were prepared by standard trypsinization.

### Somatic cell nuclear transfer

Denuded oocytes with clear perivitelline space, evenly distributed

cytoplasm, and an extruded PB were selected and washed twice in TALP medium. These oocytes were stained with 5 µg/ml of Hoechst 33342 and kept in the dark for 10 min. Oocytes were transferred into TALP medium containing 7.5 mg/ml of cytochalasin B and observed under an inverted microscope equipped with epifluorescence. An oocyte was held in place with a holding micropipette and the zona pellucida was partially dissected with a fine glass needle to make a slit near the first PB. Then the first PB was aspirated with an aspiration pipette along with a small portion of cytoplasm containing MII chromosomes. After enucleation of an oocyte, a single trypsinized skin fibroblast with a smooth outline was injected into the perivitelline space. All these oocytes were equilibrated in a fusion solution consisting of 0.28 M mannitol solution with 0.5 mM HEPES and 0.01 mM $MgSO_4$ and then fused with a single DC pulse of 1.2 kV/cm for 30 µsec using an electrical pulsing machine (LF101; Nepa Gene, Chiba, Japan). After 30 min, fused couplets were equilibrated in the activation solution (0.28 M mannitol solution containing 0.5 mM HEPES, 0.1 mM $CaCl_2$, and 0.1 mM $MgSO_4$) for 4 min. After activation, they were transferred into a chamber with activation solution and two electrodes and were activated with a single DC pulse of 1.5 kV/cm for 30 µsec using a BTX Electro-Cell Manipulator 2001 (BTX). Electrically activated embryos were washed in PZM-5 and transferred to the culture medium at 38.5ºC in a humidified environment with 5% $O_2$, 5% $CO_2$, and 90% $N_2$ for 7 days. Total blastocyst cell numbers were counted on day 7. Blastocysts were washed in TALP and stained with 5 µg/ml of Hoechst 33342 in TALP-HEPES for 10 min in the dark. After a final wash with TALP, the blastocysts were mounted on a glass slide in a drop of 100% glycerol, compressed gently with a coverslip and observed under a fluorescence microscope.

*Experimental design*

In experiment 1, we evaluated the effects of different concentrations (0, 3, 6, and 12 ng/ml) of Mn treatment during IVM on the oocyte nuclear maturation rate by measuring the frequency of first PB extrusion. In experiment 2, we added Mn during PA to explore the optimal concentration. In experiment 3, we determined the levels of GSH and ROS in the control group and Mn (6 ng/ml) treated groups, to examine the quality of oocytes. In experiment 4, we determined the effects of Mn during IVM on SCNT embryos.

*Statistical analysis*

Each experiment was repeated at least six times. The data are expressed as the mean ± standard error of the mean (SEM). Values were analyzed using univariate analysis of variance (ANOVA) followed by Duncan's multiple range test using SPSS Statistics 17.0 software (SPSS, Chicago, IL, USA). Differences in SCNT blastocyst rates were compared by Student's *t*-test. $P < 0.05$ was considered statistically significant.

## Results

*Effect of Mn supplementation on nuclear maturation of porcine oocytes*

A total of 720 oocytes were used in 6 replicates for the evaluation of nuclear maturation (Fig. 1). The nuclear maturation ranged from 89.4% to 93.3% without showing any significant differences among the groups (Table 1).

*Effect of Mn supplementation during IVM on developmental competence of parthenogenetic embryos*

We attempted to find the optimum concentration of Mn after treating oocytes with different concentrations (0, 3, 6 and 12 ng/ml) of Mn during IVM. The blastocyst formation rate was significantly higher in the group treated with 6 ng/ml Mn compared with the control and 12 ng/ml Mn treated groups (25.85%, 17.78%, and 16.34%, respectively; $P < 0.05$; Table 2). However, no significant difference was found in cleavage rates and total blastocyst cell number among all the groups.

*Effect of Mn supplementation on intracellular levels of GSH and ROS*

The intracellular levels of GSH were significantly higher in the group treated with 6 ng/ml Mn compared with the control group ($1.10 ± 1.01$ pixels/oocyte *vs.* $1.00 ± 0.30$ pixels/oocyte, $P < 0.05$). However, the levels of ROS were lower in the group treated with 6 ng/ml Mn compared with control group ($1.00 ± 0.03$ pixels/oocyte *vs.* $0.78 ± 0.25$ pixels/oocyte, $P < 0.05$) (Fig. 2).

*Effect of Mn supplementation during IVM on embryonic development competence after SCNT*

Based on the optimal concentration from PA, we treated oocytes with 6 ng/ml Mn during IVM and performed SCNT. The embryonic development rate was compared with the control group. As shown in Table 3, the blastocyst formation rate is significantly higher in the group treated with 6 ng/ml Mn than that of the control group. However, there was no effect on cleavage rates and total cell numbers per blastocyst.

## Discussion

For successful *in vitro* production of embryos, the culture conditions during IVM play a significant role. In this study, we demonstrated that Mn treatment during IVM had beneficial effects on the developmental competence of porcine oocytes. GSH levels were increased while ROS levels were decreased by treating the oocytes with 6 ng/ml Mn during IVM. In addition, Mn also improved the *in vitro* developmental competence of PA and SCNT embryos.

*In vitro* culture systems require high oxygen concentration for their maintenance as compared to *in vivo* culture systems, resulting in an increased production of ROS [37]. Oxidative stress from excessive ROS production is the major hindrance to successful porcine embryo production [11]. There is a link between oxygen concentration in *in vitro* culture systems and oxidative stress induced by ROS such as superoxide anion ($O_2^-$) or hydrogen peroxide ($H_2O_2$) [12] that leads to the impairment of oocyte maturation [38]. Oocytes are sensitive to oxidative stress during maturation and the occurrence of oxidative stress is considered to be a very important parameter in evaluating the health of an oocyte [39]. To protect oocytes from the damaging effects of ROS, the use of agents such as antioxidants [40] that can trap free radicles is a prerequisite [11]. Mn is a very important part of various metalloenzymes including MnSOD [41]
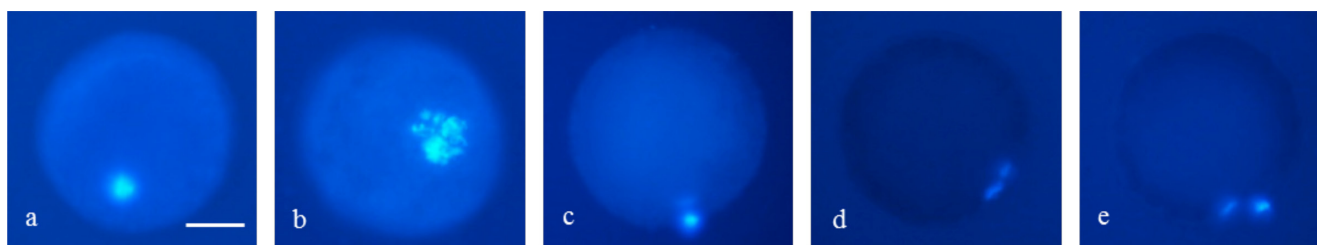
**Fig. 1.** Chromatin configuration of porcine oocytes stained with Hoechst 33342 after 44 h of IVM. (a) Germinal vesicle. (b) Germinal vesicle breakdown. (c) Metaphase I. (d) Anaphase to Telophase I. (e) Metaphase II. Scale bar indicates 100 μm.

**Table 1.** Effect of manganese treatment on nuclear maturation of porcine oocytes during IVM

| Manganese concentration (ng/ml) | No. of oocytes cultured [1] | No. (mean ± SEM, %) of oocytes at the stage of | | | |
|---|---|---|---|---|---|
| | | GV-GVBD [2] | MI [3] | Ana-Telo [4] | MII [5] |
| 0 | 180 | 0 (0.0 ± 0.0) | 9 (5.0 ± 1.1) | 10 (5.5 ± 1.1) | 161 (89.4 ± 2.0) |
| 3 | 180 | 1 (0.1 ± 0.1) | 8 (5.0 ± 1.1) | 7 (3.8 ± 1.0) | 164 (91.1 ± 1.4) |
| 6 | 180 | 0 (0.0 ± 0.0) | 10 (5.5 ± 0.7) | 5 (2.7 ± 1.3) | 165 (91.6 ± 1.6) |
| 12 | 180 | 0 (0.0 ± 0.0) | 6 (3.3 ± 1.7) | 6 (3.3 ± 0.8) | 168 (93.3 ± 2.2) |

[1] Experiment was replicated 6 times. [2] GV-GVBD, Germinal vesicle-Germinal vesicle breakdown. [3] MI, Metaphase I. [4] Ana-Telo, Anaphase-Telophase I. [5] MII, Metaphase II.
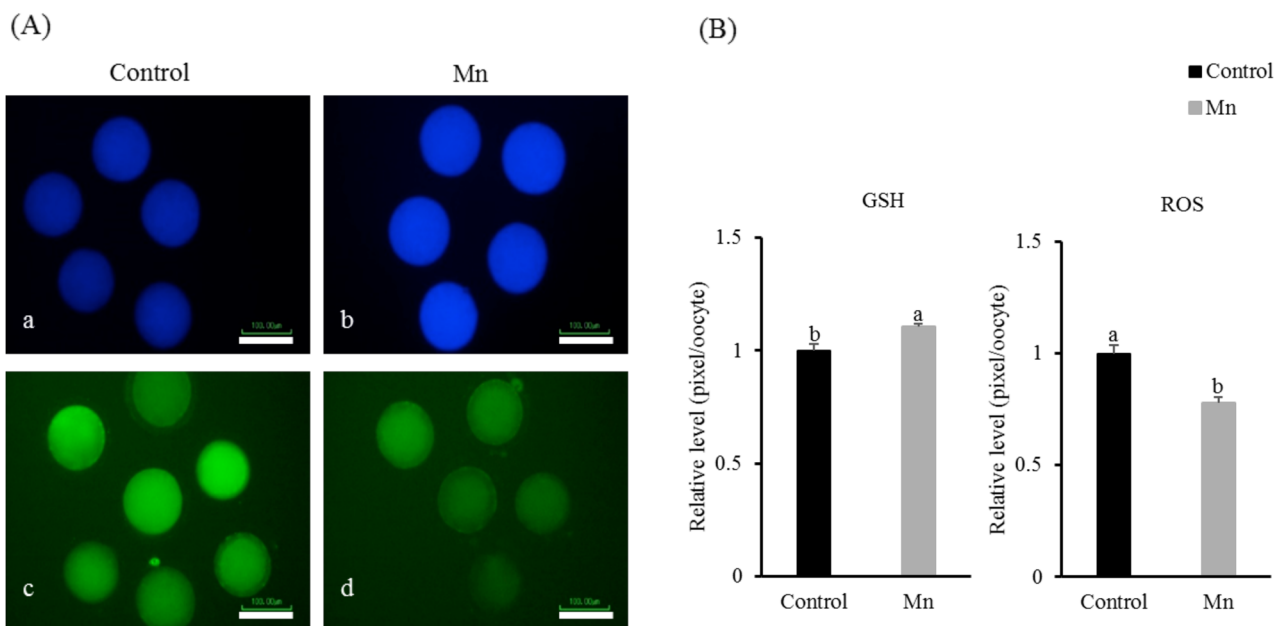


**Fig. 2.** Epifluorescent photomicrographic images of *in vitro* matured porcine oocytes. (A) Matured oocytes are stained with CellTracker Blue (a–b) and 2', 7'–dichlorodihydrofluorescein diacetate ($H_2DCFDA$; c–d) to detect intracellular glutathione (GSH) and reactive oxygen species (ROS) levels, respectively. *In vitro* matured oocytes derived from the control (a and c) and 6 ng/ml of Mn treated group (b and d). (B) Effects of Mn on the intracellular levels of GSH and ROS during IVM of *in vitro* matured porcine oocytes. Bars with different letters (a and b) represent significant differences within the respective endpoint (GSH or ROS; ($P < 0.05$)). Scale bars indicate 100 μm. Error bars show the standard error of the mean.

**Table 2.** Effect of manganese treatment during IVM on embryonic development after PA

| Manganese concentration (ng/ml) | No. of embryos cultured | No. of embryos developed to (mean ± SEM, %) | | Total cell No. (mean ± SEM) in blastocyst |
| --- | --- | --- | --- | --- |
| | | ≥ 2-cells | Blastocyst | |
| 0 | 198 | 145 (74.07 ± 3.75) | 34 (17.78 ± 1.57) [b] | 49.40 ± 1.27 |
| 3 | 194 | 148 (75.99 ± 3.08) | 37 (20.20 ± 2.88) [ab] | 51.04 ± 1.03 |
| 6 | 199 | 162 (81.20 ± 3.23) | 48 (25.85 ± 1.99) [a] | 50.90 ± 1.09 |
| 12 | 201 | 139 (75.12 ± 7.22) | 28 (16.34 ± 3.48) [b] | 49.73 ± 1.22 |

Values are listed as mean ± SEM of six replicates. [a, b] Values within a column with different superscripts differ significantly at $P < 0.05$.

**Table 3.** Effect of manganese treatment during IVM on embryonic development after SCNT

| Manganese concentration (ng/ml) | No. of embryos cultured | No. of embryos developed to (mean ± SEM, %) | | Total cell no. (mean ± SEM) in blastocyst |
| --- | --- | --- | --- | --- |
| | | ≥ 2-cells | Blastocyst | |
| 0 | 197 | 150 (75.93 ± 3.75) | 33 (16.55 ± 2.00) [b] | 52.06 ± 2.05 |
| 6 | 202 | 167 (82.43 ± 2.02) | 46 (22.91 ± 2.18) [a] | 56.85 ± 3.74 |

Values are listed as mean ± SEM of six replicates. [a, b] Values within a column with different superscripts differ significantly at $P < 0.05$.

and acts as a metal binding site [42]. Since Mn increases MnSOD activity, it caused a considerable reduction in ROS levels when porcine IVM media was supplemented with Mn. MnSOD is a very important enzyme that plays a key role in reducing ROS effects [25, 43] and is active only in the presence of manganese [44]. It has been studied that Mn decreases the oxidative stress depending on the cell type and concentration used [43].

GSH is a nonproteinous sulfhydryl compound that plays an important role in protecting the mammalian cells from oxidative damage [45]; it has been shown to improve porcine embryonic development [46]. It is also the major intracellular free thiol that plays a key role in DNA and protein synthesis [47]. Abeydeera *et al*. observed increased fertilization and blastocyst development rate through an increased level of GSH in porcine oocyte during IVM [48]. The maturation competence of porcine oocytes can be predicted by measuring their GSH levels [14]. Porcine embryos are considered to be more sensitive to lipid peroxidation because of their high fatty acid content [49]; therefore, the beneficial effects of antioxidants are comparatively higher in porcine embryos than the other mammalian embryos. ROS generation during maturation of oocytes may lead to cell damage by promoting lipid peroxidation and inactivation of enzymes [50] while Mn lowers lipid peroxidation [51] and accelerates the enzyme activity of the ascorbate-GSH cycle [52]. All these findings indicate that there is a positive correlation between the level of GSH and blastocyst development. When we cultured COCs in IVM media supplemented with 6 ng/ml Mn, intracellular GSH levels in treatment group had increased compared with those in control group.

Besides, increasing levels of GSH, decreasing levels of ROS, and Mn treatment also improved the rate of embryonic development to blastocysts after PA. Based on PA results, we treated the IVM media with Mn and performed SCNT, which resulted in the improved blastocyst formation rate. This showed that the effects of Mn are not limited only to parthenogenetic embryos. However, increase in GSH level improves cytoplasmic maturation in oocytes

[14] though there is a link between the extrusion of first PB and nuclear maturation as it occurs during nuclear maturation (MII) [53]. Supplementation of Mn in IVM media did not improve the nuclear maturation but expressed its beneficial effects on oocytes by improving the cytoplasmic maturation, which in turn enhanced the blastocyst formation rates.

After treating IVM media with different concentrations of Mn, PA and SCNT embryos showed significantly higher blastocyst formation rates at 6 ng/ml of Mn. However, there was no significant difference in the cleavage rates and blastocyst cell numbers among the groups. Ability to develop to blastocyst stage is a good indicator of the developmental capacity of oocytes. Moreover, Mn treatment showed that it improved the capacity of embryos to develop to the blastocyst stage but did not influence the cleavage rate. The differential effects of Mn on cleavage and blastocyst rate might be the reason for zygote development to be dependent on the factors stored in the oocytes before cleavage, which activate the genome of the zygote [54]. Therefore, Mn treatment might show little influence on the cleavage rate. Cattle oocytes matured with Mn supplementation in IVM medium did not improve the cleavage rate [28] and other antioxidants used in porcine and bovine IVM medium showed the same result [16, 17]. Our results are consistent with the previous study with bovine oocytes showing that embryonic development was higher in Mn-treated IVM media [28].

Therefore, in this study, we found the optimal concentration of Mn required during IVM of porcine oocytes. We found that adding 6 ng/ml Mn to porcine IVM media improved the rate of blastocyst formation after PA and SCNT by decreasing ROS levels and increasing the levels of GSH.

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