Effects of Magnetized Medium on *In Vitro* Maturation of Porcine Cumulus Cell-Oocyte Complexes

Yun-Jung Kim[1], Sang-Hee Lee[1], Soo-Jung Jung[2,3], and Choon-Keun Park[1]*

[1]College of Animal Life Sciences, Kangwon National University, Chuncheon 200-701, Korea
[2]Financial Accounting, Seoul National University, Seoul 151-742, Korea
[3]Jung-Jin Pig Farm, Heongseong 225-872, Korea

The objective of this study was to study the effect of magnetized water on porcine cumulus cell-oocyte complexes (COCs). Oocytes obtained from female pig were cultured in a medium magnetized at 0, 2000, 4000, and 6000 Gauss (G) for 5 minutes using the neodymium magnet. Subsequently, intracellular hydrogen peroxide (H₂O₂) concentration, glutathione (GSH) activity, oocyte membrane integrity, anti-apoptosis factor Bcl-xL expression, and nuclear maturation were analyzed. The intracellular H₂O₂ levels in COCs cultured for 44 hours were not significantly different among the variously magnetized samples. However, GSH activity were significantly higher in the magnetized samples compared to the 0 G sample. The Bcl-xL mRNA expression in COCs cultured for 44 hours was higher in the 4000 G sample than other treatment groups. Membrane damage in COCs cultured for 22 and 44 hours was significantly lower in 4000 G group than control group. On the other hand, nuclear stages as maturation indicator significantly increased in 2000, 4000, and 6000 G groups compared to 0 G group. These results indicate that incubation of porcine oocytes and cumulus cells in magnetized medium improves intracellular GSH levels, membrane integrity and nuclear maturation, and inhibits apoptosis *in vitro*.

Keywords : magnetized water, cumulus cell-oocyte complexes, nuclear maturation, *in vitro* maturation, pigs

1. Introduction

Pig is used by many researches due to its physiological similarities to humans [1]. Especially, its use in reproductive research is very important as a model for *in vitro* production of embryos [2]. Maturation of mammalian oocytes is a sequence of events starting with the germinal vesicle (GV) stage and ending with the second meiotic division and formation of the second polar body [3]. In addition to undergoing nuclear modifications (nuclear maturation) to develop fertilization capacity, oocytes store substances and undergo morphological alteration (cytoplasmic maturation), which promote and are essential for the early development of the embryo. Since *in vitro* maturation (IVM) is not as efficient as *in vivo* maturation, new studies are needed to clarify optimal maturation conditions to maximize maturations rates [2]. In addition, the efficiency of IVM techniques is lower in porcine

species than in other species. Four major problems are generally assumed to be the cause of poor results in porcine oocytes [4]. First, during *in vitro* oocyte maturation the nuclear maturation process is separated from the cytoplasmic maturation component, therefore decreasing the maturation rate *in vitro*. Second, the fertilization steps are very complicated *in vitro*, so fertilization rate is lower than *in vivo* [5]. Third, despite recent developments in the *in vitro* maturation of pig oocytes, the production of pig embryos still is inefficient *in vitro*. Finally, oxidative stress appears to be one of the causes of impaired *in vitro* embryo development [6].

Reactive oxygen species (ROS) are formed continuously in cells as a consequence of both oxidative biochemical reactions and external factors. It is widely accepted that ROS play both positive and negative roles *in vivo*. Positive are those related to ROS involvement in energy production, phagocytosis, regulation of cell growth and intercellular signaling, and synthesis of biologically active compounds [7]. While physiological levels of oxygen are necessary for cells to live, the presence of excess ROS, such as superoxide radical (O_2^-), hydroxyl radical (·OH),

*Corresponding author: Tel: +82-33-250-8689
Fax: +82-33-259-5575, e-mail: parkck@kangwon.ac.kr

and hydrogen peroxide (H_2O_2), can also have considerable impact on early embryonic viability, as these compounds have been implied to be involved in nuclear fragmentation and apoptosis, mitochondrial degeneration, developmental block during early cleavage stage and mitotic arrest [8-10].

Glutathione (GSH) is a major non-protein sulfhydryl compound in mammalian cells and is well known for its important role in protecting the cell from oxidative damage [11]. In addition, the intracellular level of GSH is a critical factor that influences oocyte developmental potential after *in vitro* fertilization (IVF) or somatic cell nuclear transfer [12-14]. The high concentration of GSH in M-II stage oocytes is an indicator of oocyte maturation and a storage pool of GSH for pre-implantation stages of embryo development [15-17].

Magnetization of water by passing through a magnetic field is a non-chemical alternative to changing the properties of the water. Structurally, magnetized water molecules form smaller clusters that are more easily absorbed into cells [18]. Solubility is also increased due to smaller clusters, as well as pH changes to slightly alkaline, similar to *in vivo* values. Finally, due to increased electron donor potential, magnetized water has anti oxidative activity. Therefore, we expected that these characteristics of magnetized water will help the *in vitro* maturation of immature porcine oocytes. In the present study, we measured intracellular hydrogen peroxide (H_2O_2) levels and glutathione (GSH) levels, membrane damage of cumulus cell-oocyte complexes (COCs) and expression of an anti-apoptotic gene in porcine COCs incubated in medium magnetized with various magnetic strength.

2. Materials and Methods

2.1. Oocytes collection and *in vitro* maturation

Porcine ovaries were obtained from gilts at a local slaughterhouse and transported to the laboratory within 2 h in physiological saline solution supplemented with 0.9% NaCl at 38.5°C. The cumulus cell-oocyte complexes (COCs) were aspirated from follicles 2 to 6 mm diameter in ovaries using an 18-gauge needle attached to a 10 mL disposable syringe. The COCs with evenly granulated cytoplasm and compact cumulus cells were selected using mouth pipette and washed three times with PBS-PVA. Then, the COCs were placed into each well for maturation in modified Medium-199 (tissue culture medium-199; TCM-199) supplemented with 10% (v/v) porcine follicular fluid (pFF), 10 ng/ml of epidermal growth factor (EGF), 10 IU/ml of human chorionic gonadotropin (hCG), 10% (v/v) luteinzing hormone (LH) and follicle

stimulating hormone (FSH) for 22 h as the first step of IVM. After 22 h of maturation culture, the COCs were subsequently cultured in the same maturation medium without hormones for 22 h as the second step of IVM [19]. The COCs were cultured at 38.5°C in an atmosphere of 5% CO_2/95% air with high humidity.

2.2. Preparation of magnetized water

TCM-199 medium for *in vitro* maturation was circulated through magnetic fields with different gauss (0, 2000, 4000, and 6000 gauss; G) using neodymium magnet for 5 min each. Before the *in vitro* maturation experiment, each treated medium was kept for 3 h for equilibration with the gas phase and temperature in four-well multi-dish.

2.3. Measurement of intracellular ROS and GSH levels in oocytes

The intracellular ROS and GSH levels in oocytes cultured with different magnetic intensity were measured. For the ROS assay, maturated COCs were transferred into four-well multi-dish with culture medium containing 1.5 μM Carboxy-DCFDA (Invitrogen) and 45 nM Hoechst 33342 (Sigma), and then were incubated for 30 min at 38.5°C in the dark. Stained COCs were washed three times in culture medium. The COCs were determined using the CellTracker™Red CMTPX (Invitrogen) for the GSH assay. The COCs were incubated in medium supplemented with 5 nM GSH staining solution and 225 nM Hoechst 33342 (Sigma), and washed in serum-free TC-199 medium. The experiments were replicated three times with a group of 30 COCs in each treatment group. After the GSH assay, the COCs were transferred to 96 well plate (black) for fluorescence intensity measurement with Microplate Spectrofluormeter (Molecular Device) and pictures were taken under the Confocal Laser Microscope (Olympus).

2.4. Evaluation of cumulus cell membrane damage

The integrity of oocyte membrane was estimated by staining with LIVE/DEAD Kit (Molecular Probes). Briefly, the oocytes cultured with magnetized water for different time periods were added 3.5 μL of SYBR-14 and propidium iodide (PI) from a working solution containing 100 nM SYBR-14 and 12 μM PI. They were incubated at 38.5°C for 15 min, and then washed three times in PBS-PVA solution. Stained oocytes were transferred into 96 well plate (black) before analysis and observed under the Microplate Spectrofluormeter (Molecular Device).

2.5. Quantitative reverse transcription PCR analysis

To confirm anti-apoptotic gene expression in COCs

Table 1. Primer sequences used in RT-PCR analysis.

Gene	Primer sequence	Product size (bp)
Bcl-xL	F: 5'-CCCCAGGGACAGCGTATCAG-3' R: 5'-AGAGCGAACCCAGCAGAACC-3'	345
GAPDH	F: 5'-TCGGAGTGAACGGATTTG-3' R: 5'-CCTGGAAGATGGTGATGG-3'	200

Table 2. The PCR conditions.

Gene	Temperature (°C)/ Time (sec)			Cycles
	Denaturation	Annealing	Extension	
Bcl-xL	94/30	56/30	72/30	45
GAPDH	94/30	59/30	72/30	

cultured with magnetized medium for 44 h, the total messenger RNA (mRNA) was extracted using Trizol reagent (TaKaRa) and Chloroform (J. T. BAKER) after centrifugation at 12,000 rpm for 15 min of 150 oocytes per each group. Isolated mRNA was measured with NanoDrop (Thermo Scientific). Calculated total RNA were mixed with reverse transcription (RT) premix kit (Intron biotechnology) for cDNA synthesis. The RT was processed with a program of 45°C for 60 min and 95°C for 5 min. After RT, 10% of the reaction mixture and specific primer for Bcl-xL and GAPDH were used to conduct polymerase chain reaction (PCR, Table 2), and the housekeeping GAPDH gene was used as an internal standard. Subsequent PCR analysis was performed with 95°C for 5 min, 94°C for 30 sec, and 56°C for 30 sec for Bcl-xL; for GAPDH at 59°C for 30 sec, at 72°C for 30 sec, followed by a final extension step at 72°C for 5 min. These primers produced specific 345 and 200 base pair (bp) chains (Table 1). After PCR, the products were analyzed with electrophoresis with 2% agarose gels stained with 0.5μg/ml ethidium bromide (EtBr), visualized with UV light and photographed. Expression of Bcl-xL mRNA was analyzed by a computer system, and relative amount of Bcl-xL mRNA was calculated by normalization with the amount of GAPDH mRNA.

2.6. Assessment of oocytes nuclear maturation

To evaluate nuclear maturation of cultured oocytes at 44 h, the COCs were denuded by gently pipetting in IVM medium containing 0.1% (w/v) hyaluronidase and washed three times in PBS-PVA. The cumulus-free oocytes were mounted on cleaned glass slides and fixed in 3:1 ethanol : acetic acid solution for 2 days before evaluation [20]. Then the oocytes were stained with 1% orcein (Sigma) for 15 min at room temperature and washed three times in

aceto-glycerol solution. Stained oocytes were assessed under phase-contrast microscope (Olympus, BX40) and nuclear stages were classified as germinal vesicle (GV), metaphase-I (M-I), metaphase-II (M-II), and degenerated oocytes [21].

2.7. Statistical Analysis

Statistical analysis was performed with analysis of variance (ANOVA) using SAS (version 9.1, SAS Institute Inc., Cary, NC, USA). Data are presented as mean ± standard error of the mean (SEM). Differences in the mean values of sperm damage after treatment with various treatment conditions were processed using Duncan's multiple range tests. Differences were considered significant when $p < 0.05$.

3. Results

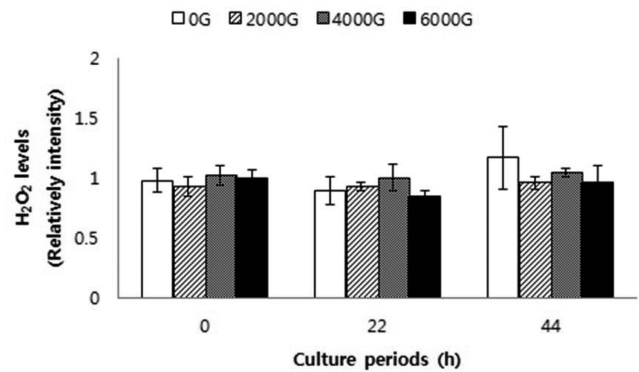The intracellular H_2O_2 levels in oocytes cultured in

Fig. 1. Changes of H_2O_2 levels in porcine cumulus cell-oocyte complexes cultured in magnetized medium for various time periods.
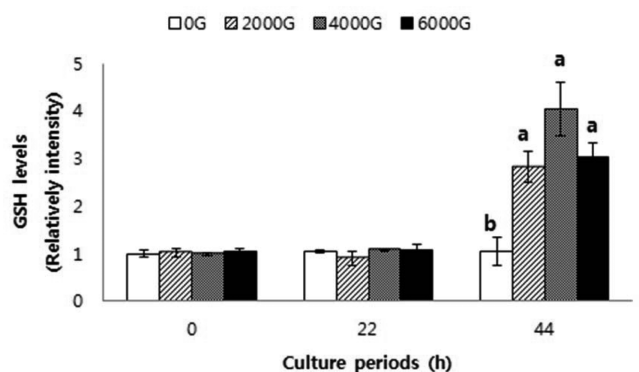

Fig. 2. Changes of GSH levels in porcine cumulus cell-oocyte complexes cultured in magnetized medium for various time periods. Within end point, bars with different letters (a, b) are significantly ($p < 0.05$) different for different intensity of neodymium magnet treatment.
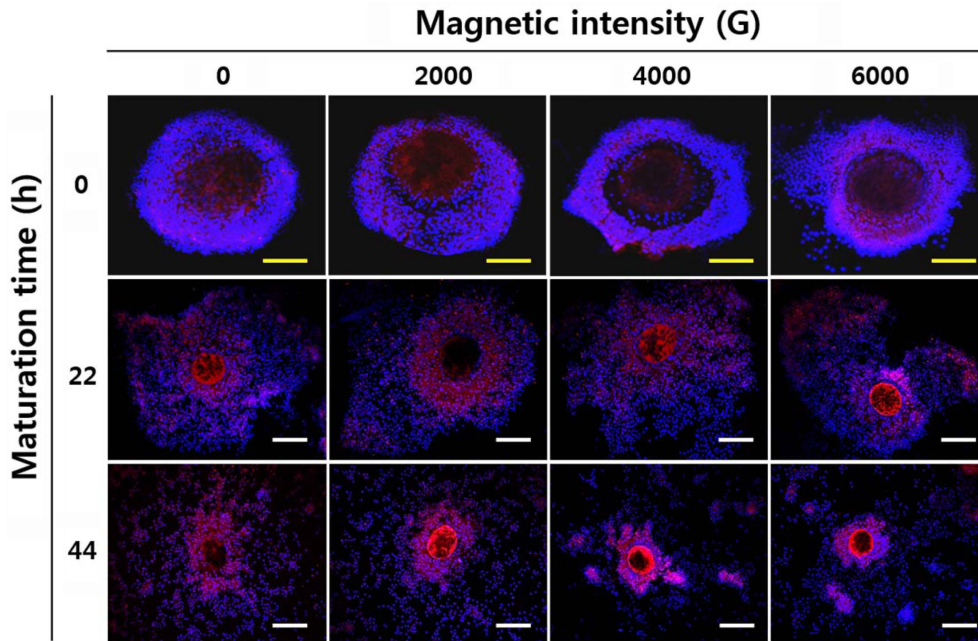
Fig. 3. (Color online) Confocal laser scanning microscopic images of glutathione (GSH) levels during *in vitro* maturation of porcine cumulus cell-oocyte complexes. Red color spot was meant intracellular GSH level by CellTracker[TM]Red CMTPX staining and blue color spot was meant nuclear by Hoechst 33342, and purple color spot was showed CellTracker[TM]Red CMTPX and Hoechst 33342 doubling staining. Yellow scale bar was 50 μm and white scale bar was 100 μm.

magnetized medium are shown in Fig. 1. The fluorescence signal of oocytes incubated in differently magnetized medium did not differ significantly at any time during incubation.

Intracellular GSH levels in oocytes cultured in magnetized medium are shown in Figs. 2 and 3. Between 0 and 22 hours of maturation, the intracellular GSH levels were not significantly different among the groups treated with different magnetic strength. However, at 44 hours,

the GSH levels were significantly higher in oocytes incubated in magnetized medium (2000 G, 2.82 ± 0.33; 4000 G, 4.04 ± 0.56; and 6000 G, 3.04 ± 0.27, respectively) compared to oocytes incubated in regular water (0 G, 1.04 ± 0.30) ($p < 0.05$).

Fig. 4 shows levels of H_2O_2 and GSH after the *in vitro* maturation of oocytes for 44 hours in media magnetized with different magnetic strength. There was no significant difference in H_2O_2 levels between the differ-
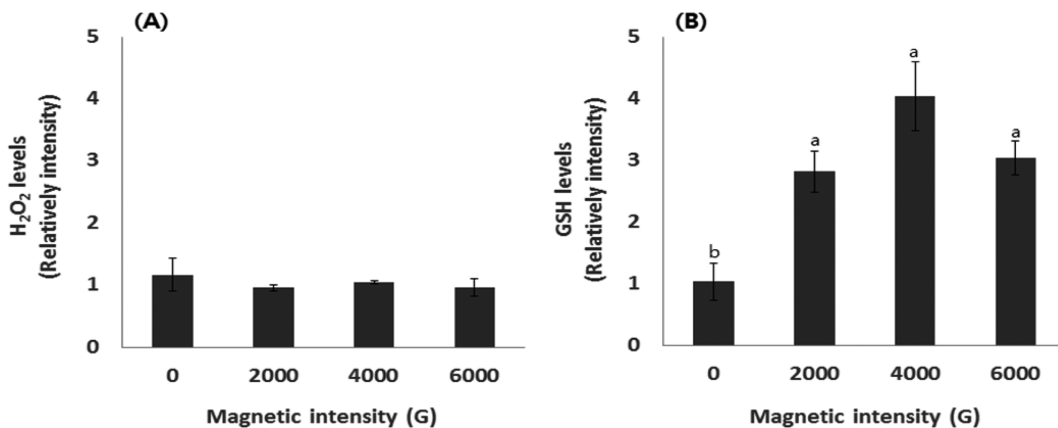

Fig. 4. Changes of H_2O_2 (A) and GSH (B) levels in porcine cumulus cell-oocyte complexes after 44 hours of culture in media magnetized with different magnetic intensity. Within end point, bars with different letters (a, b) are significantly ($p < 0.05$) different for different intensity of neodymium magnet treatment.

Fig. 5. Expression of Bcl-xL mRNA at 44 hours of *in vitro* maturation in porcine oocytes. This data was obtained from duplicate experiments per sample, and 100 oocytes per treatment group were used. Within end point, bars with different letters (a, b) are significantly ($p < 0.05$) different for different intensity of neodymium magnet treatment.
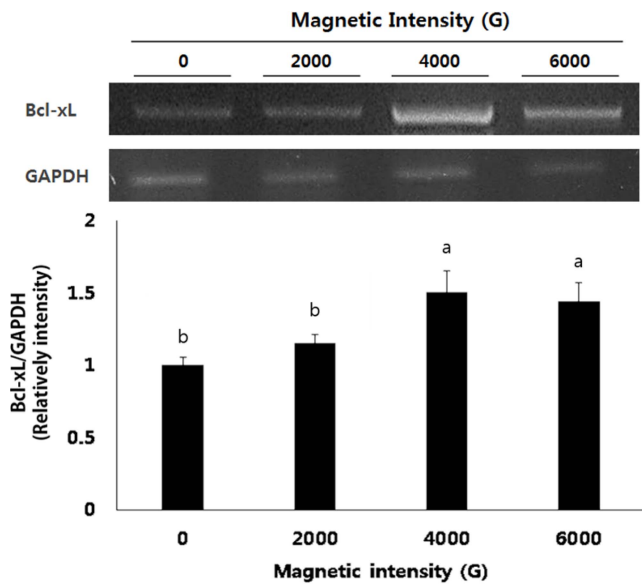
Table 3. Cumulus cell membrane damage in porcine oocytes cultured for different time periods in magnetized medium.

Magnetic intensity (G)	Culture periods of oocytes (h)		
	0	22	44
0	22.058 ± 0.383	25.592 ± 0.494^{ab}	21.577 ± 1.098^{ab}
2000	22.397 ± 1.605	27.646 ± 1.859^{a}	23.863 ± 1.177^{a}
4000	22.335 ± 0.397	21.972 ± 0.377^{c}	19.444 ± 0.873^{b}
6000	22.482 ± 0.628	23.148 ± 0.301^{bc}	21.152 ± 1.917^{ab}

[a,b]Values in the same column with different superscripts are significantly different ($p < 0.05$).

ently magnetized groups. However, GSH levels were significantly higher in groups magnetized with 2000,

4000 and 6000 G compared to control group (0 G) ($p < 0.05$).

Expression of Bcl-xL mRNA in the porcine oocytes after *in vitro* maturation in differently magnetized media is shown in Fig. 5. The RT-PCR was performed with GAPDH housekeeping gene as an internal standard. Expression of Bcl-xL mRNA was increased in oocytes incubated in media treated with higher magnetic intensity.

Cumulus cell membrane damage in porcine oocytes cultured in media treated with various magnetic intensity are shown in Table 3. There was no significant difference among all the groups at 0 hours of culture. However, when oocytes were cultured for 22 and 44 h, cumulus cell membrane damage was significantly lower in oocytes
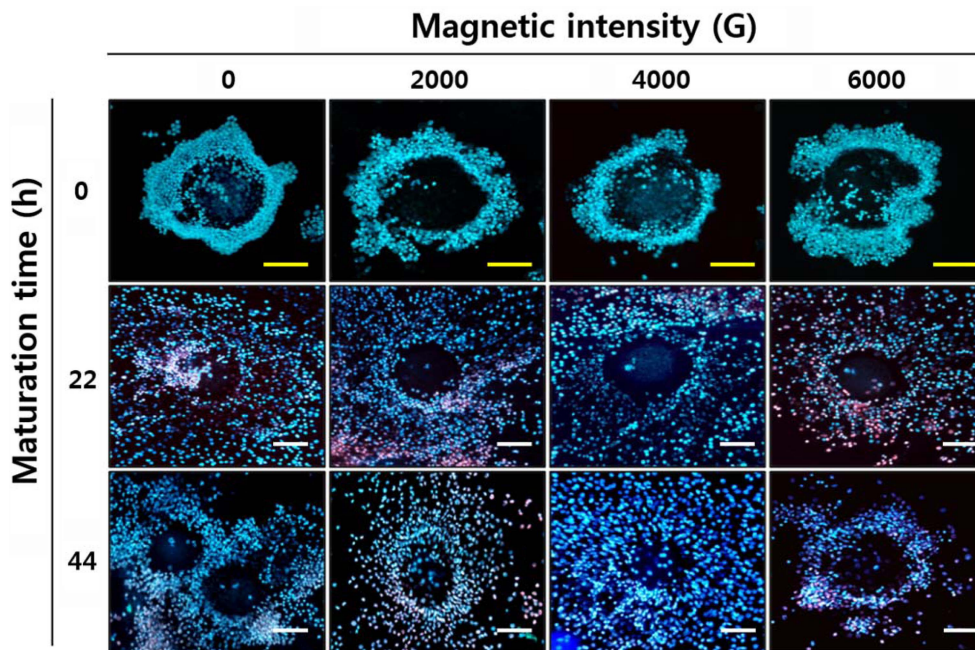

Fig. 6. (Color online) Fluorescent photomicrographic images of cumulus cell membrane damage during *in vitro* maturation of porcine cumulus cell-oocyte complexes incubated in media magnetized with different magnetic intensity. Green color spot was meant intact plasma membrane cumulus cells by SYBR14 staining and red damaged plasma membrane cumulus cells by propidium iodide (PI) staining. Yellow scale bar was 50 μm and white scale bar was 100 μm.

Table 4. Changes of oocyte nuclear stages during *in vitro* maturation of porcine oocytes in magnetized medium.

Magnetic intensity (G)	No. of oocytes examined	No. (%) of oocytes matured to			
		GV	M I	M II	Deg
0	153	15[a] (9.64 ± 1.91)	51[a] (33.25 ± 3.89)	74[b] (48.47 ± 4.91)	13[a] (8.65 ± 1.88)
2000	137	6[ab] (4.40 ± 0.69)	27[b] (19.58 ± 3.93)	91[a] (66.18 ± 3.92)	13[a] (9.84 ± 1.64)
4000	248	4[b] (1.36 ± 0.84)	36[b] (14.45 ± 0.75)	186[a] (75.41 ± 2.32)	22[a] (8.78 ± 1.70)
6000	138	7[ab] (4.94 ± 2.73)	33[b] (23.79 ± 2.24)	93[a] (67.62 ± 4.34)	5[b] (3.65 ± 0.56)

[a,b]Values in the same column with different superscripts are significantly different ($p < 0.05$). GV: germinal vesicle, M-I: metaphase-I, M-II: metaphase-II, Deg: degeneration.

incubated in medium magnetized with 4000 G compared to 2000 G ($p < 0.05$).

The effects of magnetized medium on *in vitro* maturation of porcine oocytes are shown in Table 4. When oocytes were cultured in medium magnetized with various magnetic intensity, the number of nuclear stages was significantly higher at 2000, 4000, and 6000 G compared to control. The highest proportion of oocytes cultured to M-II stage was obtained in the medium magnetized with 4000 G.

4. Discussion

In this study, we investigated the effects of magnetized water on hydrogen peroxide and glutathione levels in the intracellular space, plasma membrane intactness, gene expression of anti-apoptotic factor and nuclear stages of porcine oocytes.

Reactive oxygen species (ROS) are generated during various cellular metabolic reactions as well as indirectly in the cell's environment [22]. In excess concentrations they cause damage to nucleic acids, proteins, and lipids, and to other cellular components [23, 24]. However, magnetic field inhibits H_2O_2 production in cellular systems through changes in the characteristics of cell membrane, effects the cell reproduction and causes certain changes in cell metabolism [23, 25]. Contrary to expectations based on previous studies, which showed that magnetized water inhibits ROS, particularly H_2O_2 in the intracellular space, it did not have an effect on H_2O_2 levels in porcine oocytes [26]. Moreover, magnetized water is characteristic for the balance between free radical production and their clearance controlled by antioxidant compounds [27]. GSH is the major non-protein sulfhydryl antioxidant in mammalian cells and plays a role in many cellular functions, including DNA and protein synthesis, regulation of enzyme activity, both inter- and intracellular transport, and is an electron donor for glutathione peroxidase, which reduces peroxide, thereby protecting the cell from oxidative stress and toxic ROS activity [28-30]. In addition, GSH has a unique function in reproduction and is specifically related to oocyte maturation [17]. As reported previously, GSH concentrations are significantly higher in ovulated MII oocytes than in immature GV stage oocytes [31]. However, it was unclear how magnetized water increases intracellular GSH concentration during oocyte maturation *in vitro*. This study extends the previous observations by showing that GSH concentrations increased significantly at 44 hours of *in vitro* maturation compared to 0 and 22 hours. Moreover, GSH is a key regulator in apoptosis, since cells actively extrude intracellular GSH through specific carriers and a consequent redox disequilibrium triggers the activation of Bax and cytochrome c release [32]. Previous research shows that magnetized water and high levels of GSH reduced apoptosis by interfering with the apoptotic process [33].

Cumulus cells have a close connection with oocytes during the course of maturation in mammals [34, 35]. It is generally accepted that cumulus cells support the maturation of oocytes to the MII stage and greatly enhance cytoplasmic maturation, which is responsible for the capacity to undergo normal fertilization and subsequent embryonic development [11]. Although cumulus cell is very sensitive to apoptosis, the membrane is modified by magnetized water having the anti-apoptotic ability. Therefore, oocyte nuclear maturation also reached MII phase earlier.

This study shows that magnetized medium with increase in GSH levels improves the *in vitro* nuclear maturation in porcine oocytes.

Acknowledgements

References

[1] T. Nagai, Theriogenology **55**, 1291 (2001).

[2] M. G. Marques, C. Nicacio, V. P. de Oliveira, A. B. Nascimento, H. V. Caetano, C. M. Mendes, M. R. Mello, M. P. Milazzotto, M. E. Assumpcao, and J. A. Visintin, Anim. Reprod. Sci. **97**, 375 (2001).

[3] M. R. Blano, S. Demyda, M. M. Mereno, and E. Genero,Biotech. Mol. Biol. Review **6**, 155 (2001).

[4] P. Wongsrikeao, Y. Kaneshige, R. Ooki, M. Taniguchi, B. Agung, M. Nii, and T. Otoi, Reprod. Domest. Anim. **40**, 166 (2005).

[5] L. R. Abeydeera and B. N. Day, Biol. Reprod. **57**, 729 (1997).

[6] C. Matas, P. Coy, R. Romar, M. Marco, J. Gadea, and S. Ruiz, Reproduction **125**, 133 (2003).

[7] A. M. G. Mantovani, C. Madeddu, E. L. Mura, E. Massa, G. Gramignano, M. R. Lusso, V. Murgia, P. Camboni, and L. Ferreli, J. Cell Mol. Med. **6**, 570 (2002).

[8] P. J. Booth, P. Holm, and H. Callesen, Theriogenology. **63**, 2040 (2005).

[9] P. Guerin, S. El Mouatassim, and Y. Menezo, Hum. Reprod. Update. **7**, 175 (2001).

[10] H. Tamura, A. Takasaki, T. Taketani, M. Tanabe, F. Kizuka, L. Lee, I. Tamura, R. Maekawa, H. Aasada, Y. Yamagata, and N. Sugino, J. Ovarian Res. **5**, 5 (2012).

[11] N. S. Hideki Tatemoto, and M. Norio. Biol. Reprod. **63**, 805 (2000).

[12] L. R. Abeydeera, W. H. Wang, T. C. Cantley, R. S. Prather, and B. N. Day, Theriogenology **50**, 747 (1998).

[13] D. G. de Matos and C. C. Furnus, Theriogenology **53**, 761 (2000).

[14] J. Y. You, J. Y. Kim, J. M. Lim, and E. S. Lee, Theriogenology **74**, 777 (2010).

[15] C. C. Furnus, D. G. de Matos, and D. F. Moses, Mol. Reprod. Dev. **51**, 76 (1998).

[16] C. S. Gardiner and D. J. Reed, Biol. Reprod. **51**, 1307 (1994).

[17] K. A. Zuelke, S. C. Jeffay, R. M. Zucker, and S. D. Perreault. Mol. Reprod. Dev. **64**, 106 (2003).

[18] S. Mufarrei, H. A. Batshan, M. I. Shalaby, and T. M. Shafey, Poult. Sci. **4**, 96 (2005).

[19] N. Viet Linh, T. Q. Dang-Nguyen, B. X. Nguyen, N. Manabe, and T. Nagai, J. Reprod. Dev. **55**, 594 (2009).

[20] J. Zhu, E. E. Telfer, J. Fletcher, A. Springbett, J. R. Dobrinsky, P. A. De Sousa, and I. Wilmut, Biol. Reprod. **66**, 635 (2002).

[21] W. Sha, B. Z. Xu, M. Li, D. Liu, H. L. Feng, and Q. Y. Sun, Fertil. Steril. **93**, 1650 (2010).

[22] C. M. Combelles, S. Gupta, and A. Agarwal, Reprod. Biomed. Online. **18**, 864 (2009).

[23] S. Landolfo, H. Politi, D. Angelozzi, and I. Mannazzu, Biochim. Biophys. Acta **1780**, 892 (2008).

[24] C. F. Martino and P. R. Castello, PLoS One **6**, e22753 (2011).

[25] M. Hozayn and A. M. S. A. Qados, Agric. Biol. J. North Am. **1**, 677 (2010).

[26] F. Carlos and P. R. C. Martion, PlLoS ONE **6**, e22753 (2011).

[27] A. H. Hashish, M. A. El-Missiry, H. I. Abdelkader, and R. H. Abou-Saleh, Ecotoxicol. Environ. Saf. **71**, 895 (2008).

[28] D. Biswas and S. H. Hyun, Theriogenology **76**, 153 (2011).

[29] J. Fujii, Y. Iuchi, and F. Okada, Reprod. Biol. Endocrinol. **3**, 43 (2005).

[30] S. Luperchio, S. Tamir, and S. R. Tannenbaum, Free Radical Biol. Med. **21**, 513 (1996).

[31] S. D. Perreault, R. R. Barbee, and V. L. Slott, Dev. Biol. **125**, 181 (1988).

[32] M. de Nicola, S. Cordisco, C. Cerella, M. C. Albertini, M. D'Alessio, A. Accorsi, A. Bergamaschi, A. Magrini, and L. Ghibelli, Ann. N. Y. Acad. Sci. **1090**, 59 (2006).

[33] C. Fanelli, S. Coppola, R. Barone, C. Colussi, G. Gualandi, P. Volpe, and L. Ghibelli, FASEB J. **13**, 95 (1999).

[34] J. J. Eppig. Dev. Biol. **89**, 268 (1982).

[35] N. B. Gilula, M. L. Epstein, and W. H. Beers, J. Cell Biol. **78**, 58 (1978).