The Effects of MEM Vitamins and β-Mercaptoethanol on Porcine Embryonic Development, Reactive Oxygen Species and Apoptosis

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Abstract: The objective of this study was to determine the synergistic effects of beta-Mercaptoethanol (β-ME) and MEM vitamins (MEMv) as antioxidants supplemented into porcine maturation medium on maturation and Reactive Oxygen Species (ROS) of oocytes. In addition, the effects of β-ME and MEMv on subsequent embryonic development, ROS generation by embryos and apoptosis of blastocysts following *In Vitro* Fertilization (IVF), Chemical Activation (CA) and Electrical Activation (EA) were investigated. Cumulus-Oocyte-Complexes (COCs) were cultured in North Carolina State University (NCSU)-23 medium supplemented with $0.05 \times$MEMv, 25 μM β-ME and 25 μM β-ME and $0.05 \times$MEMv, respectively. COCs cultured without β-ME and MEMv were regarded as control. Maturation rate and ROS levels of oocytes at 44 h of culture were assessed. Oocytes were fertilized *in vitro* or activated parthenogenetically and embryonic development and the ROS levels on 2 days of culture and the apoptosis levels of blastocyts were evaluated. Maturation rate of the β-ME and MEMv group was higher than that of the control group (86.0 ± 3.6 vs. $73.3 \pm 1.1\%$; $p < 0.05$) although, ROS levels were not significantly different among the groups. Cleavage rate following IVF was increased in the β-ME and MEMv group compared to those of other groups and ROS levels of the β-ME and MEMv group were lower than those of the control group (6.9 ± 0.2 vs. 8.0 ± 0.2; $p < 0.05$). Blastocyst rate was increased in the β-ME and MEMv group compared to those of the control and MEMv groups ($p < 0.05$). Conversely, the level of apoptosis of the β-ME and MEMv group was lower than others (4.6 ± 1.0 vs. $11.4 \pm 1.9\%$, 12.2 ± 1.8 and $15.6 \pm 3.0\%$, respectively; $p < 0.05$). Cleavage rate of the β-ME and MEMv group following CA was significantly higher compared with control while the ROS level was lower than the β-ME group (19.0 ± 1.9 vs. 25.0 ± 1.7; $p < 0.05$). The blastocyst formation rate of the β-ME and MEMv group was significantly increased than control while the level of apoptosis was lower than those of other groups (5.8 ± 1.2 vs. $17.2 \pm 4.6\%$, 17.0 ± 5.2 and $22.6 \pm 3.2\%$, respectively; $p < 0.05$). Embryonic developments following EA were not significantly different among the groups although, the ROS level of the β-ME and MEMv group was lower ($p < 0.05$). In conclusion, addition of β-ME and MEM vitamins during porcine oocyte maturation was effective on subsequent embryonic development in IVF and CA by alleviating the influences of ROS generation and apoptosis.

Key words: β-mercaptoethanol, MEM vitamins, maturation rate, embryonic development, ROS, apoptosis, porcine oocyte

INTRODUCTION

Recent advances in new technologies to produce cloned and genetically modified pigs involve manipulating oocytes and/or embryos *in vitro*. Although, a great deal of progress has been made, the current *In Vitro* Production (IVP) systems still result in major problem (Gil *et al.*, 2010). Oocyte maturation is a critical component of IVP systems for porcine embryos that have suffered from high incidence of polyspermy and poor quality (Niwa, 1993; Wang and Niwa, 1995; Abeydeera and Day, 1997; Machaty *et al.*, 1998; Gil *et al.*, 2010). In particular, the oxidative stress in porcine oocytes might be caused by longer culture time *in vitro* than that in other mammalian animals (Long *et al.*, 1994; Hunter and Greve, 1997). Many Reactive Oxygen Species (ROS) have a negative intracellular effect and suppress the fusion of sperm and oocyte and obstruct fertility and embryonic development of porcine oocytes (Aitken *et al.*, 1993; De Lamirande *et al.*, 1997; Olson and Seidel, 2000).

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Apoptosis is a process of programmed cell death that is induced by ROS and depletion of glutathione (Franco and Cidlowski, 2009). Apoptosis in pre-implantation embryos can be induced by sub-optimal culture conditions *in vitro* and its incidence in developing embryos can be correlated to embryo quality (Betts and King, 2001; Levy *et al.*, 2001).

Vitamins are important contributors to the development of mammalian embryo *in vitro* (Koo *et al.*, 2008). Vitamins have been shown to improve embryo development in rabbit, hamster and sheep (Kane, 1988; Kane and Bavister, 1988; Gardner *et al.*, 1994). Of the water soluble vitamins, the Vitamin B6 and niacin had the effect to reduce the oxidative stress level (Kamat and Devasagayam, 1996; Gardner *et al.*, 1994; Olson and Seidel, 2000; Kannan and Jain, 2004). Bormann *et al.* (2003) showed that addition of Minimal Essential Medium vitamins (MEMv) during caprine oocyte maturation was beneficial for subsequent blastocyst development and viability. However, the effect of MEMv in porcine oocytes was limited depending on concentration of MEMv and oocyte maturation was not improved at any concentration (Naruse *et al.*, 2007). Therefore, researchers were to seek a synergetic supplement as an antioxidant to improve porcine oocyte maturation and subsequently embryonic development in presence of MEMv.

Beta-Mercaptoethanol (β-ME) has been demonstrated to protect against oxidative damage, particularly lipid peroxidation, both *in vitro* and *in vivo* (Wu *et al.*, 1990; Forrest *et al.*, 1994). This chemical may be effective on porcine oocytes which contain a much higher concentration of fatty acids compared to other species and may be more susceptible to lipid peroxidation (Am-in *et al.*, 2011). Choe *et al.* (2010) demonstrated a synergistic effect of β-ME treatment during *in vitro* maturation on porcine embryonic development in North Carolina State University (NCSU)-23, a culture medium supplemented with cysteine.

Therefore, one of purposes was to determine a possible synergistic effect of the simultaneous addition of β-ME and MEMv on nuclear maturation in relation to ROS generation. In addition, to determine subsequent embryonic development in relation to ROS generations of early stage embryos and apoptosis of blastocyst in parthenogenetic activation and *In Vitro* Fertilization (IVF).

MATERIALS AND METHODS

Culture media: Unless otherwise noted, all chemicals and reagents used were purchased from Sigma-Aldrich (St. Louis, MO, USA). The medium used for collection and washing of porcine Oocyte Cumulus Complexes (COCs)

was modified Tyrode's Lactate-HEPES Polyvinyl Alcohol (TL-HEPES-PVA) composed of 114 mM NaCl, 3.2 mM KCl, 0.4 mM $Na_2H_2PO_4$, 2 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 5 mM $NaHCO_3$, 20 mM HEPES, 16.6 mM sodium lactate (60% syrup), 0.5% PVA, 10 IU mL^{-1} penicillin and 10 µg mL^{-1} streptomycin. Oocyte maturation was performed in NCSU-23 medium (Gil *et al.*, 2007) supplemented with 10% Porcine Follicular Fluid (PFF), 0.6 mM cysteine, 10 ng mL^{-1} Epidermal Growth Factor (EGF), 10 IU mL^{-1} Pregnant Mare Serum Gonadotropin (PMSG) and 10 IU mL^{-1} Human Chorionic Gonadotropin (HCG). PFF was collected from ovarian follicles that ranged from 3-6 mm in diameter by centrifugation at 1,600×g for 30 min at room temperature and filtration through a 1.2 µm syringe filter. The final product was stored at -20 °C until use. Fertilization medium, based on modified Tris-buffered medium (mTBM; 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris (Trizma Base), 11 mM D-glucose and 5 mM sodium pyruvate) was supplemented with 2 mM caffeine and 0.2% Bovine Serum Albumin (BSA). *In Vitro* Culture 1 (IVC 1) was composed of D-glucose-free NCSU-23 supplemented with 0.17 mM sodium pyruvate, 2.73 mM sodium lactate (60% syrup) and 0.4% BSA. *In Vitro* Culture 2 (IVC 2) was NCSU-23 containing 0.4% BSA.

Oocyte collection and *In Vitro* Maturation (IVM): Porcine ovaries were collected from a local abattoir and transported to the laboratory at 34-36°C in 0.9% saline supplemented with 100 IU mL^{-1} penicillin G and 100 µg mL^{-1} streptomycin. COCs were aspirated through an 18-gauge needle. Oocytes with compact cumulus mass and a dark, evenly graduated cytoplasm were washed three times in TL-HEPES-PVA media and maturation media, respectively. COCs were randomly allocated to maturation medium with 0.05×MEMv, 25 µM β-ME and 0.05×MEMv and 25 µM β-ME, respectively. COCs (20~25) were cultured in a 100 µL droplet of maturation medium supplemented with 10 IU mL^{-1} PMSG and 10 IU mL^{-1} HCG for 22 h and then for another 22 h in maturation medium without hormones. COCs cultured without MEMv and β-ME were regarded as control. Oocyte maturation was carried out under sterile mineral oil at 39°C in a humidified atmosphere of 5% CO_2 in air.

Sperm preparation and *In Vitro* Fertilization (IVF): Extended spermatozoa supplied by Irae Yangdon were maintained at 17°C. Percoll solutions and gradients were prepared as described previously (Yu *et al.*, 2002). A two-layer discontinuous gradient was formed by layering 1 mL of a 45% Percoll solution on top of 1 mL of a 90% Percoll

solution in a 15 mL conical tube. Aliquants of extended semen (3 mL) were layered onto the Percoll gradient and centrifuged for 20 min at 850×g at room temperature. The pellet recovered after aspiration of the supernatant was washed two times by centrifugation with 5 mL Dulbecco's Phosphate Buffered Saline (D-PBS) supplemented with 0.1% BSA, 10 IU mL^{-1} penicillin and 10 µg mL^{-1} streptomycin at 350×g for 3 min.

After the supernatant was discarded, motile spermatozoa were collected. Sperm concentration (spermatozoa mL^{-1}) was adjusted to 10×10^5 by dilution with mTBM. Oocytes with expanded cumulus cells were treated with 1 mg mL^{-1} hyaluronidase in TL-HEPES-PVA and were denuded by gentle aspiration with a small-bore glass pipette. The denuded oocytes were washed three times with mTBM and transferred to an mTBM insemination drop (45 µL) overlaid with mineral oil (20-25 oocytes per drop). A 5 µL volume of spermatozoa was added to each insemination drop to give a final concentration of 1×10^5 spermatozoa/mL. Oocytes and spermatozoa were co-cultured for 6 h at 39°C in a humidified atmosphere of 5% CO$_2$ in air.

Parthenogenetic Activation (PA): Following IVM, cumulus cells were removed by repeated pipetting in TL-HEPES supplemented with 0.3% hyaluronidase. For Chemical Activation (CA), oocytes were incubated in IVC 1 medium containing 5 µM calcium ionophore for 5 min, washed twice and incubated for 3 h in NCSU-23 medium supplemented with 2.0 mM 6 D-Methyl Amino Purine (6 D-MAP). For Electrical Activation (EA), oocytes were transferred to pulsing medium consisting of 0.3 M D-mannitol, 0.1 mM MgSO$_4$, 0.05 mM CaCl$_2$ and 0.01% PVA, washed three times and then transferred to a chamber containing two electrodes overlaid with pulsing medium. The oocytes were then stimulated with a direct current-pulse of 1.5 kV cm^{-1} for a duration of 100 µsec using a BTX Electro-Cell Manipulator 2001 (BTX, San Diego, CA, USA). After activation, oocytes were washed and transferred into 500 µL of culture media covered with mineral oil.

In vitro **culture:** Following either PA or IVF, presumptive zygotes were washed three times, transferred to 50 µL roplets (15-20 presumptive zygotes per drop) of IVC 1 medium and incubated for 2 days at 39°C in a humidified atmosphere of 5% CO$_2$ in air. After 2 days of embryo culture, cleavage formation was assessed. Embryos were then washed twice, transferred to 50 µL droplets of IVC 2 medium and incubated for 6 days after which the blastocyst formation was assessed.

Assessment of oocyte meiotic stage: Ocytes denuded by vigorous shaking in 3% sodium citrate solution were fixed in a 4 well culture dish (Nunc, Rochester, NY, USA) containing 500 µL of ethanol:acetic acid (3:1, v/v) for 48 h. They were then dispensed on to a slide to which a coverslip was added. Each sample was stained with aceto-orcein (1% (w/v) orcein in 45% (v/v) acetic acid) and destained with glycerol:acetic acid:distilled water (1:1:3, v/v). Oocytes were evaluated under a light microscope at 400x magnification. Meiotic stages were classified as previously described (Romar and Funahashi, 2006) as germinal vesicle, germinal vesicle breakdown, Metaphase I, Anaphase I, Telophase I, or Metaphase II.

Measurement of reactive oxygen species contents: The levels of ROS in the oocytes and embryos were examined according to the Diclorohydrofuorescein Dacetate (DCHFDA) Method described by Hashimoto *et al.* (2000). Briefly, oocytes after maturation and only cleaved embryos at day 2 of culture were selected, respectively and transferred into IVC 1 medium containing 10 µM DCHFDA. After 30 min of culture, oocytes and embryos were washed in PVA-DPBS. The respective fluorescent emissions from oocytes and embryos were recorded as TIFF files using a cooled CCD camera attached to a fluorescence microscope (Axio, Carl Zeiss, Goettingen, Germany) with filters at 405-435 nm for excitation and at 515 nm for emission. The recorded fluorescent images were analyzed using ImageJ software 1.33 µ (National Institutes of Health, Bethesda, MD, USA) by the intensity of fluorescence in each oocyte and embryo picture.

Assessment of cell numbers in blastocyst: Blastocysts were stained in 500 µL of Hoechst 33342 (5 µg mL^{-1}) in TL-HEPES-PVA for 15 min at room temperature, mounted on slides and covered with cover slips. Cell numbers in blastocyst were determined using a fluorescent inverted microscope (Nikon, Tokyo, Japan).

Detection of apoptosis by terminal deoxynucleotidyl Transferase-Mediated d-UTP Nick End-Labeling (TUNEL) assay: The degree of apoptosis was assessed in the selected blastocysts by the following procedures described previously (Cui and Kim, 2005). Briefly, after washing three times in PBS supplemented with PVA (1 mg mL^{-1}), the embryos at the blastocysts were fixed in 4% paraformaldehyde in PBS without Ca^2 and Mg^2(-) for 1 h at room temperature. Then, the embryos were washed three times in PBS (-)/PVA and permeabilized by incubation in 0.5% Triton X-100 in PBS for 1 h at 4°C.

After permeabilization, blastocysts were washed 3 times in PBS (-)/PVA and incubated with a fluorescein-conjugated TUNEL assay kit (*In Situ* Cell Death Detection kit, Roche; Mannheim, Germany) in the dark for 1 h at 39°C.

The blastocysts were then counterstained with 40 μg mL^{-1} Propidium Iodide (PI) in order to visualize the total cell number. The numbers of TUNEL-positive and total cells in the blastocysts were determined from an optical image of whole-mount embryos under fluorescence microscopy (Axio, Carl Zeiss, Goettingen, Germany). The percentage of TUNEL-positive cells is described as the percentage of TUNEL-positive cells relative to the total number of cells.

Statistical analysis: Four replicates were conducted for each experiment. Percentage data were subjected to an arcsin transformation before analysis. All data and data sets are presented as the mean±SEM and were analyzed by Duncan's multiple range test using the Statistical Analysis System Ver. 8x (SAS, Cary, NC and USA). For all analyses, p<0.05 was considered significant.

RESULTS AND DISCUSSION

Effects of β-ME and MEMv on maturation and ROS generation of oocytes: Maturation rate of oocytes into metaphase II in the group treated with β-ME and MEMv was higher than that of the control (86.0±3.6 vs. 73.3±1.1%; Table 1; p<0.05). However, the levels of ROS were not significantly different among groups.

Effects of β-ME and MEMv on development, ROS generation and apoptosis in porcine embryos following IVF: Cleavage formation, blastocyst formation, ROS generation of cleaved embryos and apoptosis of blastocyst following IVF are shown in Table 2. Cleavage formations were not significantly different among groups, although the β-ME and MEMv group showed the highest cleavage rate (81.7±4.8%). The blastocyst rate of the β-ME and MEMv group was higher than those of the control and MEMv groups (36.1±2.1 vs. 24.9±2.6% and 24.5±3.1%, respectively; p<0.05). Cell numbers of blastocyst were not significantly different among groups. The combination of β-ME and MEMv reduced ROS generation by cleaved embryos compared with the levels in the control group and MEMv group (6.9±0.2 vs. 8.0±0.2 and 7.6±0.1, respectively; Fig. 1a; p<0.05). In addition, the level of ROS in the β-ME and MEMv group was similar to that of the β-ME group (6.9±0.2 vs. 6.8±0.1). The rate of TUNEL-positive cells in the β-ME and MEMv group was lower than those of other groups (4.6±1.0 vs. 11.4±1.9%, 12.2±1.8 and 15.6±3.0%, respectively; p<0.05).

Effects of β-ME and MEMv on development, ROS generation and apoptosis in porcine embryos following CA: Cleavage formation, blastocyst formation, ROS generation of cleaved embryos and apoptosis of blastocyst following CA are shown in Table 3. Cleavage rate in the β-ME and MEMv group was greater than that seen in the absence of antioxidant (80.9±2.7 vs. 71.0±3.1%; p<0.05). Blastocyst rate in the β-ME and MEMv group was higher than that of the control group

Table 1: Effects of β-ME and MEMv in maturation medium on maturation rate and Reactive Oxygen Species (ROS) in porcine oocytes

Groups[a]	Maturation rate[b]		ROS[c]	
	No. of oocytes	Oocytes matured to MII stage (%)	No. of oocytes	Levels (intensity/oocytes)
Control	121	73.3±1.1[b]	40	7.8±0.5
MEMv	142	80.2±6.0[a, b]	44	8.3±0.3
β-ME	104	82.4±5.2[a, b]	63	7.8±0.4
β-ME + MEMv	147	86.0±3.6[a]	68	7.1±0.4

Within the column, values with different letters (a and b) are different (p<0.05). [a]Control: Absence of β-mercaptoethanol and MEM vitamins; MEMv: 0.05× MEM vitamins; β-ME: 25 μM β-Mercaptoethanol; β-ME + MEMv: 25 μM β-Mercaptoethanol and 0.05×MEM vitamins. [b]Maturation rate: the rate of metaphase II after culture of oocytes for 44 h. [c]Levels of ROS were assessed after culture of oocytes for 44 h. Data are expressed as mean±SEM

Table 2: Effects of β-ME and MEMv in maturation medium on developmental competence, generation of Reactive Oxygen Species (ROS) and apoptosis in porcine embryos following IVF

Groups[a]	No. of oocytes	Cleavage rates[b] (%, n)	Blastocyst rates[c] (%, n)	No. of cells in blastocyst (n)	Level of ROS[b] (n)	TUNEL-positive nuclei[c] (%, n)
Control	199	76.5±3.9 (151)	24.9±2.6[b] (49)	33.9±8.1 (49)	8.0±0.2[a] (47)	11.4±1.9[a] (17)
MEMv	200	74.9±4.3 (150)	24.5±3.1[b] (49)	34.4±11.1 (49)	7.6±0.1[a] (46)	12.2±1.8[a] (19)
β-ME	196	75.3±2.8 (148)	30.2±2.5[a, b] (59)	30.6±8.2 (59)	6.8±0.1[b] (42)	15.6±3.0[a] (22)
β-ME + MEMv	205	81.7±4.8 (168)	36.1±2.1[a] (74)	35.0±6.0 (74)	6.9±0.2[b] (31)	4.6±1.0[b] (32)

Within the same column, values with different letters (a and b) are different (p<0.05). [a]Control: Absence of β-mercaptoethanol and MEM vitamins; MEMv: 0.05×MEM vitamins; β-ME: 25 μM β-Mercaptoethanol; β-ME + MEMv: 25 μM β-Mercaptoethanol and 0.05×MEM vitamins. [b]Cleavage rates and levels of ROS (intensity of embryos) were assessed on day 2 of culture. [c]Blastocyst rates and levels of apoptosis in blastocyst were assessed on day 8 of culture. Data are expressed as mean±SEM

Table 3: Effects of β-ME and MEMv in maturation medium on developmental competence, generation of Reactive Oxygen Species (ROS) and apoptosis in porcine embryos following chemical activation

Groups[a]	No. of oocytes	Cleavage rates[b] (%, n)	Blastocyst rates[c] (%, n)	No. of cells in blastocyst (n)	Level of ROS[b] (n)	TUNEL-positive nuclei[c] (%, n)
Control	186	71.0±3.1[b] (131)	13.3±2.4[b] (24)	11.9±5.2 (24)	23.0±2.9[ab] (23)	17.2±4.6[a] (14)
MEMv	217	76.3±2.4[a,b] (165)	16.1±2.1[a,b] (35)	23.8±16.6 (35)	18.0±1.3[b] (31)	17.0±5.2[a] (11)
β-ME	205	74.2±2.7[a,b] (152)	17.8±3.5[a,b] (36)	16.7±7.1 (36)	25.0±1.7[a] (29)	22.6±3.2[a] (17)
β-ME+MEMv	227	80.9±2.7[a] (184)	25.5±4.6[a] (58)	19.1±8.7 (58)	19.0±1.9[b] (22)	5.8±1.2[b] (20)

Within the same column, values with different letters (a and b) are different ($p<0.05$). [a]Control: Absence of β-mercaptoethanol and MEM vitamins; MEMv: 0.05×MEM vitamins; β-ME: 25 µM β-Mercaptoethanol; β-ME + MEMv: 25 µM β-Mercaptoethanol and 0.05×MEM vitamins. [b]Cleavage rates and levels of ROS (intensity of embryos) were assessed on day 2 of culture. [c]Blastocyst rates and levels of apoptosis in blastocyst were assessed on day 8 of culture. Data are expressed as mean±SEM
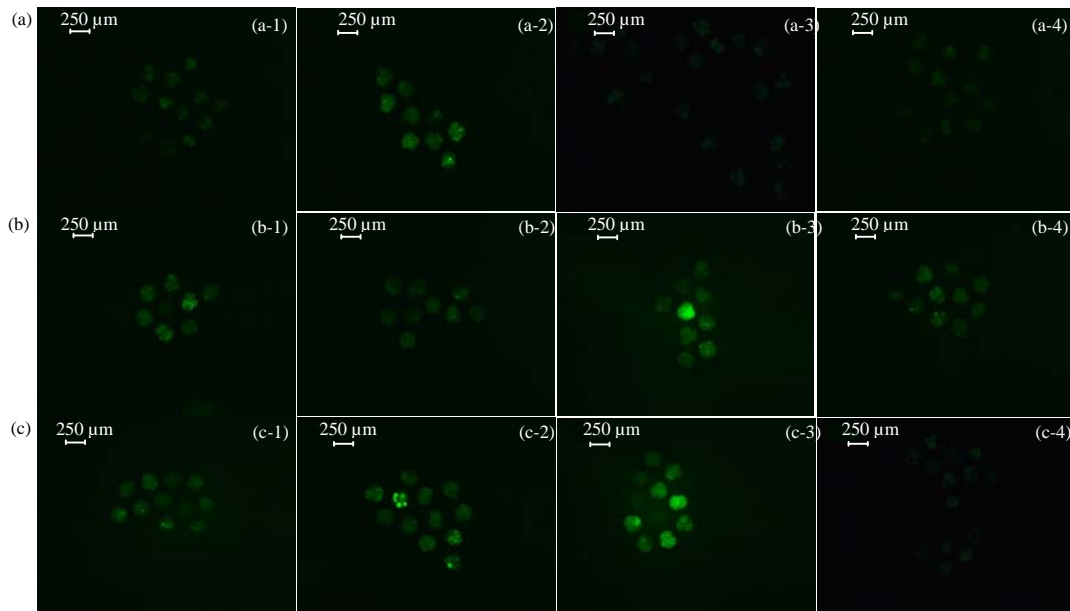

Fig. 1: Images of DCHFDA staining representing the intracellular ROS levels of cleaved embryos on day 2 of culture following *In Vitro* Fertilization (IVF), Chemical Activation (CA) or Electrical Activation (EA). IVF (a): Control (a-1), MEMv (a-2), β-ME (a-3), β-ME+MEMv (a-4). CA (b): Control (b-1), MEMv (b-2), β-ME (b-3), β-ME+MEMv (b-4). EA (c): Control (c-1), MEMv (c-2), β-ME (c-3), β-ME + MEMv (c-4). The embryos showing as brighter green have higher levels of intracellular ROS. Control: absence of β-mercaptoethanol and MEM vitamins; MEMv: 0.05×MEM vitamins; β-ME: 25 µM β-mercaptoethanol; β-ME+MEMv: 25 µM β-mercaptoethanol and 0.05×MEM vitamins. Magnification: 50x

(25.5±4.6 vs. 13.3±2.4%; $p<0.05$). Cell numbers of blastocyst were not significantly different among groups. The combination of β-ME and MEMv reduced ROS generation compared to that in the β-ME group (19.0±1.9 vs. 25.0±1.7; Fig 1b; $p<0.05$). The ROS level of the β-ME and MEMv group was similar to that of the MEMv group (19.0±1.9 vs. 18.0±1.3). The rate of TUNEL-positive cells in the β-ME and MEMv group was lower than those of other groups (5.8±1.2 vs. 17.2±4.6%, 17.0±5.2 and 22.6±3.2%, respectively; $p<0.05$).

Effects of β-ME and MEMv on development, ROS generation and apoptosis in porcine embryos following EA: Cleavage formation, blastocyst formation, ROS generation of cleaved embryos and blastocyst apoptosis

following EA are shown in Table 4. Cleavage formations of oocytes activated electrically were not significantly different among groups. The blastocyst formations and cell numbers were not significantly different among groups. The ROS level in the β-ME and MEMv group was lower than those of the other groups (14.3±1.1 vs. 20.5±1.3, 22.8±1.5 and 20.8±1.4, respectively; Fig 1c; $p<0.05$). The rates of TUNEL-positive cells were not significantly different among groups.

In the present study, one of the purposes was to demonstrate synergetic effects of β-ME and MEMv as antioxidants on the maturation and ROS generation of porcine oocytes. The combination of β-ME and MEMv supplemented into maturation medium significantly increased the maturation rate of oocytes compared to that

Table 4: Effects of β-ME and MEMv in maturation medium on developmental competence, generation of Reactive Oxygen Species (ROS) and apoptosis in porcine embryos following electrical activation

Groups[a]	No. of oocytes	Cleavage rates[b] (%, n)	Blastocyst rates[c] (%, n)	No. of cells in blastocyst (n)	Level of ROS[b] (n)	TUNEL-positive nuclei[c] (%, n)
Control	141	85.9±1.2 (121)	14.8±2.1 (20)	12.9±1.9 (20)	20.5±1.3[a](24)	17.6±7.5 (17)
MEMv	177	85.9±1.6 (152)	18.0±2.0 (31)	16.3±2.2 (31)	22.8±1.5[a](36)	12.0±4.1 (14)
β-ME	176	85.7±2.9 (151)	16.0±3.5 (27)	15.3±2.0 (27)	20.8±1.4[a](31)	9.4±2.2 (15)
β-ME + MEMv	172	90.3±2.9 (157)	20.4±1.6 (35)	15.2±2.3 (35)	14.3±1.1[b](31)	7.2±3.5 (16)

Within the same column, values with different letters (a and b) are different (p<0.05). [a]Control: Absence of β-mercaptoethanol and MEM vitamins; MEMv: 0.05×MEM vitamins; β-ME: 25 μM β-Mercaptoethanol; β-ME+MEMv: 25 μM β-Mercaptoethanol and 0.05×MEM vitamins. [b]Cleavage rates and levels of ROS (intensity of embryos) were assessed on day 2 of culture. [c]Blastocyst rates and levels of apoptosis in blastocyst were assessed on day 8 of culture

of the control in this study. Naruse *et al.* (2007) presented that the maturation rate of porcine oocytes treated with MEMv was similar to that of the control group without MEMv. Kobayashi *et al.* (2006) also demonstrated that the MII rate of porcine oocytes in a β-ME group was similar to those of groups without β-ME. On the other hand, maturation rates of oocytes were not directly related to ROS reduction by antioxidants. *In vitro* culture results in higher oxygen concentration than *in vivo* environments, leading to an increased level of ROS (Luvoni *et al.*, 1996). The antioxidant requirements are varied during IVM, IVF and IVC although, antioxidants are beneficial additives to synthetic culture media as ROS scavengers (Choe *et al.*, 2010). Researchers infer that the process of oocyte maturation *in vitro* might generate less ROS compared to *in vitro* fertilization and *in vitro* culture of embryos. The levels of ROS might not be as influenced by antioxidants as researchers had expected. In addition, maturation rate was determined by assessing MII rate while ROS of oocytes were assessed after culture for 44 h. Researchers can also speculate that the levels of ROS in the β-ME and MEMv group might be diluted by other oocytes at different nuclear stage which might generate more or less ROS.

Researchers understood that nuclear maturation rate according to antioxidants was consecutively related to cleavage rate of oocytes following IVF or CA. β-ME and MEMv treatment during oocyte maturation increased cleavage formation of oocytes following IVF or CA. In addition, ROS generation in cleaved embryos was related to cleavage rate depending on the antioxidant used.

Namely, β-ME and MEMv treatment during oocyte maturation increased cleavage formation in IVF and CA by reducing ROS generation of cleaved embryos while a single antioxidant did not show a constant effect on cleavage formation in relation to ROS. Researchers suggest that the combination of β-ME and MEMv might be more effective for *in vitro* maturation of porcine oocytes which are to be used for IVF or CA. It has been reported that the presence of β-ME in IVM media enhances the intracellular GSH concentration in embryos and improves *in vitro* fertilization (Nagai, 2001; De Matos *et al.*, 2002; Takahashi *et al.*, 2002). Vitamin B6 and niacin in MEMv have been reported to reduce the level of ROS (Kamat and Devasagayam, 1996; Kannan and

Jain, 2004). Researchers speculate that β-ME addition to the IVM medium supplemented with MEMv might have contributed to reduce ROS by producing GSH and improve cleavage formation in the present study. On the other hand, cleavage rates of oocytes following EA were not influenced by antioxidants although, ROS generation in cleaved embryos was significantly reduced by β-ME and MEMv compared to treatment with a single antioxidant. In electrical activation, an electric pulse induces temporary pore formation in the mammalian oocyte membrane. When Ca^{2+} ions are present in the pulsing medium, an influx of Ca^{2+} occurs through the temporary pore (Sun *et al.*, 1992). Calcium ion concentrations present in pulsing medium affected ROS generation (Lee *et al.*, 2005; Miyoshi *et al.*, 2005). Koo *et al.* (2008) indicated that electrical activation induced ROS in porcine embryos and generation of ROS should be considered for optimizing electrical activation. Researchers infer that oxidative stress by ROS upon electrical activation might be more severe compared to *in vitro* fertilization and chemical activation and may be an obstacle to subsequent embryonic development of electrically activated oocytes.

The relationship between cleavage rate and ROS generation in cleaved embryos subsequently influenced blastocyst formation and blastocyst apoptosis. Yang *et al.* (1998) suggested that ROS are a detriment to embryo development causing apoptosis. In this study, the combination use of β-ME and MEMv supplemented into maturation medium improved the rate of blastocyst formation by reducing apoptosis during IVF and CA. Addition of β-ME and MEMv during oocyte maturation may enhance normal embryo development by suppression of ROS concentration and apoptosis level in embryos. However, single use of β-ME or MEMv showed blastocyst rates similar to the absence of antioxidant. Percentages of TUNEL-positive nuclei were not significantly decreased in either group. Kobayashi *et al.* (2006) demonstrated β-ME supplemented into maturation medium did not improve blastocyst formation after Intracytoplasmic Sperm Injection (ICSI).

However, β-ME treatment during *in vitro* maturation increased blastocyst formation following IVF (Han *et al.*, 2003). Naruse *et al.* (2007) demonstrated that MEMv during IVM increased

blastocyst rate following parthenogenetic activation. The results of the studies mentioned above cannot be directly compared to the results due to the variety of concentrations and antioxidants as well as the use of different oocyte manipulation methods after antioxidant treatment. However, the combination of β-ME and MEMv in the present study produced a constant effect on blastocyst formation as well as cleavage formation in both IVF and CA. Overall, the capacity of a single antioxidant such as MEMV or β-ME might be limited compared to the combination of β-ME and MEMv. The level of ROS in oocytes activated electrically following β-ME and MEMv treatment during IVM was significantly reduced at an early embryo stage although, the level of apoptosis was not significantly decreased in late stage embryos. Researchers suspect that the antioxidants added during maturation might be consumed to reduce many ROS generated in early stage embryos by electrical activation compared to IVF and CA. As a result, the combination use of β-ME and MEMv might not improve late stage embryonic development in electrically-activated oocytes. In a future study, researchers might consider a higher concentration of β-ME and MEMv or another effective antioxidant that might improve blastocyst formation by decreasing ROS and preventing apoptosis due to electrical activation.

Kobayashi *et al.* (2006) reported that the cell number of porcine blastocysts produced by ICSI was not increased by β-ME treatment. MEM vitamins also did not affect cell number of porcine blastocysts (Naruse *et al.*, 2007). In the present study, no antioxidants improved blastocyst cell numbers. Researchers suggest that antioxidant supplementation during oocyte maturation might not affect embryo mitosis following blastocyst formation.

CONCLUSION

In this study, the combination use of β-ME and MEMv during oocyte maturation improved embryonic development from oocytes fertilized *in vitro* or activated chemically by alleviating ROS generation and apoptosis.

Researchers suggest that the synergistic effect of β-ME and MEMv during IVM of porcine oocytes may help to improve the sub-optimal conditions within *in vitro* culture systems.

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