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Effect of Antioxidant Treatment during Parthenogenetic Activation Procedure on the Reactive Oxygen Species Levels and Development of the Porcine Parthenogenetic Embryos

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ABSTRACT

The present study was conducted to examine the effect of antioxidant treatment during parthenogenetic activation procedure on the reactive oxygen species (ROS) levels and *in vitro* development of porcine parthenogenetic embryos. Porcine *in vitro* matured oocytes were activated by a combination of electric stimulus and 2 mM 6-dimethylaminopurine (6-DAMP) before *in vitro* culture. During the activation period, oocytes were treated with 50 μM β-mercaptoethanol (β-ME), 100 μM L-ascorbic acid (Vit. C) or 100 μM L-glutathione (GSH). To examine the ROS level, porcine parthenogenetic embryos were stained in 10 μM dichlorohydrofluorescein diacetate (H_2DCFDA) dye 20 h after culture, examined under a fluorescence microscope, and the fluorescence intensity (pixels) were analyzed in each embryo. The parthenogenetic embryos were cultured for 6 days to evaluate the *in vitro* development. The apoptosis was measured by TUNEL assay. The H_2O_2 levels of parthenogenetic embryos were significantly lower in antioxidant treatment groups (26.9±1.6～29.1±1.3 pixels/embryo, $p<0.05$) compared to control (33.2±1.7 pixels/embryo). The development rate to the blastocyst stage was increased in antioxidant treatment groups (32.0～32.5%) compared to control (26.9%, $p<0.05$), although, there was no difference in apoptosis among groups. The result suggests that antioxidant treatment during parthenogenetic activation procedure can inhibit the ROS generation and enhance the *in vitro* development of porcine parthenogenetic embryos.

(Key words : Antioxidant, ROS level, *In vitro* development, Parthenogenetic activation, Pig)

INTRODUCTION

The cellular stress during somatic cell nuclear transfer (SCNT) procedures could generate excessive reactive oxygen species (ROS) in the bovine embryos (Bae *et al.*, 2011; Hwang *et al.*, 2012). The excessive level of ROS generated during micromanipulation or parthenogenetic activation procedures can alter the structures of cellular molecules, which may result in serious damages of the mitochondria and nuclear DNA, and restrict embryonic development (Halliwell and Aruoma, 1991; Rhoads *et al.*, 2006; Kim *et al.*, 2011). Therefore, it was important to inhibit the excessive ROS generation during the SCNT procedure later than during *in vitro* culture period.

Various antioxidants have been used to ROS scavengers in various species. Recently, we reported that antioxidant treatment with β-mercaptoethanol (β-ME) and vitamin C (Vit. C) during SCNT procedures reduced the ROS generation (Bae *et al.*, 2012). Besides, a lot of antioxidants such as ascorbic acid (Tatemoto *et al.*, 2004), α-tocopherol (Olson and Seidel, 2000), β-ME (Nedambale *et al.*, 2006), and L-glutathione (GSH, Luvoni *et al.*, 1996), have been used to overcome ROS in various species such as cattle, pig, and mice.

Parthenogenetically activated embryos were used as a model for the SCNT study because *in vitro* development characteristics of parthenogenetic embryos resembled those of SCNT embryos and could be considered as a good model to analyze the influence of exogenous factors on the embryonic development of SCNT

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embryos. Artificial activation of oocytes is a crucial step in SCNT. During porcine parthenogenetic activation procedure, ROS also generated in the parthenogenetic embryos by exogenous stress such as electric stimulation and 6-dimethylaminopurine (6-DMAP) treatment (Hwang *et al.*, 2011). The present study was conducted to examine the effect of antioxidant treatment during parthenogenetic activation procedure on the ROS levels and *in vitro* development of porcine parthenogenetic embryos.

MATERIALS AND METHODS

In Vitro Maturation of Oocytes

Porcine cumulus-oocyte complexes (COCs) were aspirated from follicles (3～6-mm diameter) of ovaries and subsequently washed in Tyrode's lactate-Hepes buffer containing 0.1% (w/v) polyvinyl alcohol (PVA; Sigma, St. Louis, MO, USA). Matured oocytes were prepared by *in vitro* maturation of COCs for 40～44 h, at 39℃, 5% CO_2, and cumulus cells were removed by vortexing before use. The culture medium for *in vitro* maturation was tissue culture medium 199 (TCM199; Gibco-BRL, Grand Islannd, NY, USA) supplemented with 0.5 μg/ml, FSH (Sigma), LH (Sigma) and 10 ng/ml EGF (Sigma).

Parthenogenesis and *In Vitro* Culture

Porcine oocytes were placed between two wire electrodes (1-mm apart) of a fusion chamber that was overlaid with 0.3 M mannitol solution containing 0.5 mM Hepes, 0.1 mM $MgCl_2 \cdot 6H_2O$, 0.5 mM $CaCl_2 \cdot 2H_2O$. Electroactivation was induced with a two direct current pulse of 1.5 kV/cm for 30 μsec using BTX Electro Cell Manipulator 200 (BTX, San Diago, CA, USA), followed by treatment with 2 mM 6-DMAP (Sigma) for 3 h before *in vitro* culture. After parthenogenesis, the embryos were cultured in PZM-3 (Yoshioka *et al.*, 2002) at 39℃, 5% CO_2 in air for 20 h or 6 days to evaluate the ROS generation levels, *in vitro* development, and apoptosis in blastocysts.

Antioxidant Treatment

Three kinds of antioxidant were treated during the parthenogenesis procedures. For the antioxidant treatment groups, 50 μM β-ME (Sigma), 100 μM Vit. C (Sigma) or 100 μM GSH (Sigma) were added to activation medium during the parthenogenesis; electroactivation and 6-DMAP treatment. The above concentrations of antioxidants were selected by a separated experiment (unpublished data). Antioxidant treated parthenogenetic embryos were culture in above culture condition.

Measurement of Intercellular ROS Content

The ROS levels of embryos were measured according to the method described previously (Hwang *et al.*, 2012). Briefly, parthenogenetic embryos after 20 h of culture were stained in 10 μM 2'7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes, Eugene, OR, USA) dye for 30 min in the dark at 39℃ to measure the H_2O_2 levels. After incubation, the embryos were washed with phosphate buffered saline (PBS) and immediately examined under a fluorescence microscope (BX-50, Olympus, Tokyo, Japan) with filters at 450～480 nm for excitation and at 515 nm for emission. The fluorescent images were recorded as JPEG files using a digital camera (ESO 600D, Canon, Tokyo, Japan) and analyzed the intensity of fluorescence in each embryo using ImageJ software 1.37 (NIH). Five replicates were performed for each of the groups (total 90～100 embryos in each group).

TUNEL Assay

TUNEL assay was carried using *in situ* Cell Death Detection Kit (TMR red, Roche, Mannhaim, Germany). The parthenogenetic blastocysts were washed in PBS supplemented with 0.3% polyvinyl pyrrolidone (PVP) and fixed with 3.7% (v/v) paraformaldehyde in PBS for 1 h at 4℃. Fixed embryos were permeabilized by incubation in 0.5% Triton X-100 for 30 min at room temperature (RT), washed and incubated with TUNEL mixture (TMR red in a ratio of 1:9) in the dark for 1 h at 37℃. Embryos were then washed and in 0.5% Triton X-100 for 5 min at RT. Embryos were washed and transferred into 40 μg/ml Hoechst 33342 (Sigma) in the dark for 30 min at RT. Embryos were then mounted onto a slide glass in mounting solution (Vectashield, Vector Laboratories, Inc, CA). Slides were analyzed for TUNEL positive staining under a fluorescence microscope (BX-40, Olympus, Japan) at 100× magnification. Experiments were repeated at least 3 times using total 40～50 embryos in each group.

Statistical Analysis

ROS levels, cell number and apoptosis data were analyzed using ANOVA, followed by Duncan's multiple range tests using the Statistical Analysis System (SAS Institute, Inc, Cary, NC, USA), and development rates were analyzed by Chi-square test.

RESULTS

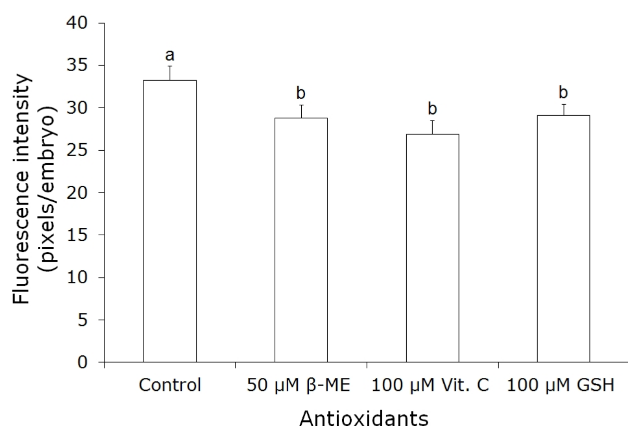

Fig. 1. H_2O_2 levels of parthenogenetic porcine embryos treated with different antioxidants of 50 μM β-ME, 100 μM Vit. C and 100 μM GSH. [a~c] Values with different letters are significantly different (p<0.05).

H_2O_2 Levels of Parthenogenetic Embryos

H_2O_2 levels were significantly lower in antioxidant treatment groups with 50 μM β-ME, 100 μM Vit. C and 100 μM GSH (28.8±1.5, 26.9±1.6, and 29.1±1.3 pixels/embryo, respectively) than that of control (33.2±1.7 pixels/embryo, p<0.05) (Fig. 1).

In Vitro Development of Parthenogenetic Embryos

There was no difference between control (90.3%, 289/320) and antioxidant treatment groups, and among treatment groups in the cleavage rate (92.9~95.0%). The developmental rate to the blastocyst stage was significantly increased (p<0.05) in antioxidant treatment groups [50 μM β-ME; 32.2% (109/339), 100 μM Vit. C; 32.0% (108/338), and 100 μM GSH; 32.5% (105/323)] compared to control (26.9%, 86/320). There were no differences among groups in the mean cell number of blastocysts (Table 1).

Apoptosis in Blastocysts

TUNEL assay revealed that there were no differences in the proportion of apoptotic cells among antioxidant groups (50 μM β-ME; 1.4±0.2%, 100 μM Vit. C; 1.1±0.2%, and 100 μM GSH; 1.3±0.4%) and control group (1.7±0.3%), but, slightly lower apoptosis was occurred in Vit. C treatment group compared to control (Fig. 2).

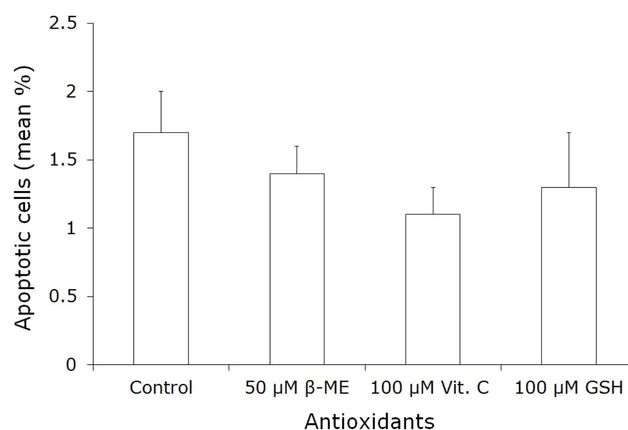

Fig. 2. Effect of antioxidant treatment during activation procedures on the apoptosis of porcine parthenogenetic embryos.

DISCUSSION

The stresses derived from various endogenous and exogenous factors in organisms and cells generate the ROS (David *et al.*, 2007). The stress of embryos during micromanipulation procedures, one of the factors affecting the development of SCNT embryos has been overlooked until now. The cellular stresses during SCNT procedures such as enucleation, electrofusion, and activation treatments could generate ROS (Hwang *et al.*, 2012), which result in serious damages of the mitochondria and DNA of SCNT embryos and subsequently restrict the reprogramming of SCNT embryos. In our previous study, the H_2O_2 level of porcine oocytes was reduced immediately after electric stimulation, but H_2O_2 level and ˙OH radical product levels of parthenogenetic embryos were increased with time elapsed after electric activation from 0 h to 3 h, and after 6-DMAP culture (Hwang *et al.*, 2011). Koo *et al.* (2008) reported that

Table 1. Effect of antioxidant treatments during activation procedures on the development of porcine parthenogenetic embryos*

Antioxidant treatment	No. of embryos cultured	No. of embryos developed to (%)		Cell number (mean±SE)
		≥2-Cells	Blastocysts	
Control	320	289(90.3)	86(26.9)[a]	45.8±1.7
50 μM β-ME	339	315(92.9)	109(32.2)[b]	44.9±1.7
100 μM Vit. C	338	319(94.4)	108(32.0)[b]	42.8±1.5
100 μM GSH	323	310(96.0)	105(32.5)[b]	44.2±2.4

[*] Experiments were repeated 8 times in each treatment group.
[a,b] Values with different superscripts are significantly different (p<0.05).

greater ROS were induced in porcine two-cell embryos activated by electrical stimulation in comparison to IVF embryos.

Goto *et al.* (1993) and Nagao *et al.* (1994) showed ROS generation can be reduced by controlling the oxygen concentration in the air during *in vitro* culture of mouse and bovine oocytes, which could promote the embryonic development. Many researchers either controlled the O_2 tensions in *in vitro* culture conditions (Orsi and Leese, 2001) or supplemented the antioxidants to culture medium (Ali *et al.*, 2003) in order to prevent the ROS generation. A lot of antioxidants such as Vit. C, α-tocopherol, β-ME, catalase, GSH and SOD enhanced the antioxidant systems within embryo and stimulate embryonic development (Olson and Seidel, 2000; Orsi and Leese, 2001). In this study, oocytes were treated by one of the three antioxidants of 50 μM β-ME, 100 μM Vit. C or 100 μM GSH during activation procedure of parthenogenesis. An excessively high concentration of antioxidant in *in vitro* culture system induced apoptosis of embryos by breaking of redox balance (Kitagawa *et al.*, 2004). Thus, we selected the optimum concentrations of each antioxidant, which were effective to inhibit the ROS generation in the parthenogenetic embryos through the preliminary experiment (unpublished data).

In the present study, we just focused the stresses induced by parthenogenetic activation treatment. Thus, antioxidant treatment was applied during only parthenogenetic activation procedure, but not during *in vitro* culture period, which is the different point of the present study compared to other previous study (Olson and Seidel, 2000; Orsi and Leese, 2001; Ali *et al.*, 2003). The ROS level of parthenogenetic one-cell embryos was reduced by antioxidant treatment during only parthenogenetic procedure. Furthermore, the development rate to the blastocyst stage was enhanced by antioxidant treatment during parthenogenetic activation procedure. ROS can mediate the mitochondria dependant apoptotic response to extracellular factors (Herrera *et al.*, 2001). Uhm *et al.* (2007) reported that the addition of antioxidants to the culture media can decrease apoptosis in embryos by preventing oxidant mediated damage. Our data revealed that although, there was no significant difference, slightly lower apoptosis were occurred in antioxidant treatment groups, especially, Vit. C treatment group compared to control group.

In conclusion, the result of this study suggests that antioxidant treatment during parthenogenetic activation procedure can reduce the ROS generation levels and enhance the *in vitro* development of porcine parthenogenetic embryos.

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