Generation of Reactive Oxygen Species in Porcine Parthenogenetic Embryos

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ABSTRACT

The present study was conducted to examine the reactive oxygen species (ROS) generation levels in porcine parthenogenetic embryos. Porcine *in vitro* matured oocytes were activated by the combination of electric stimulus and 6-DMAP before *in vitro* culture. Porcine oocytes and parthenogenetic embryos were stained in 10 μM dichlorohydrofluorescein diacetate (DCF) or 10 μM hydroxyphenyl fluorescein (HPF) dye each for 30 min at 39℃. The fluorescent emissions from the samples were recoded as JPEG file and the intensity of fluorescence in oocytes and embryos were analyzed. H_2O_2 and ·OH radical levels of porcine oocytes were reduced immediately after electric stimulation. However, H_2O_2 and ·OH radical levels of parthenogenetic embryos were increased with time elapsed after electric stimulation from 0 h to 3 h and after DMAP culture. During *in vitro* culture, H_2O_2 and ·OH radical levels were gradually increased from the one-cell stage to the two- and four-cell stages. The result of the present study suggests that the ROS was not increased by electric pulse in porcine embryos. Rather than it seems to be associated with the stage of development and the culture condition.

(Key words : Reactive oxygen species, H_2O_2, ·OH radical, Parthenogenesis, Porcine)

INTRODUCTION

The efficiency of somatic cell nuclear transfer (SCNT) is still low and have many serious abnormal problems (Garry *et al.*, 1996; Hill *et al.*, 1999; Kubota *et al.*, 2000). Some epigenetic modifications were considered as a reason to these problems (Kang *et al.*, 2001a,b; Xue *et al.*, 2002; Nolen *et al.*, 2005; Yang *et al.*, 2005). However, some other basic reasons might be responsible to the abnormality and low SCNT efficiency.

The development of SCNT embryos can be influenced by cellular stress which can be occurred by the manipulations during SCNT procedures would also affect the reprogramming of SCNT embryos. The cellular stress during micromanipulation procedures could generate reactive oxygen species (ROS), which result in serious damages of the mitochondria and DNA of SCNT embryos and subsequently restrict the reprogramming of SCNT embryos.

ROS includes oxygen ions, free radicals, and peroxides, both inorganic and organic. They are generally very small molecules and are highly reactive due to the presence of unpaired valence shell electrons. ROSs form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling. However, under the an environmental stress, ROS levels can increase dramatically, which can result in significant damage to cell structures.

Parthenogenetically activated (PA) embryos were used as a model for the study because *in vitro* development characteristics of PA embryos resembled those of SCNT embryos and could be considered as a good model to analyze the influence of exogenous factors on embryonic development of SCNT embryos. Artificial activation of oocytes is a crucial step in SCNT embryos. The present study was conducted to examine the ROS generation levels in porcine parthenogenetic embryos.

MATERIALS AND METHODS

In Vitro Maturation (IVM) of Oocytes

Porcine cumulus-oocyte complexes (COCs) were aspirated from follicles (3- to 6-mm diameter) of ovaries and washed in Tyrode's lactate-Hepes buffer containing 0.1% (w/v) polyvinyl alcohol (PVA; Sigma, St. Louis, MO, USA). About 100 COCs were transferred into 500 μl droplets of maturation medium overlaid with paraffin oil and cultured for 40~42 h at 39℃, 5% CO_2 in air. The culture medium for IVM was Tissue Culture

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Medium 199 (TCM199 ; Gibco-BRL, GrandIsland, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 0.5 μg/ml LH, FSH and 10 ng/ml EGF.

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 0.1 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $MgCl_2 \cdot 6H_2O$ and 0.5 mM Hepes. Electro-activation was induced with a two direct-current pulse of 1.2 kV/cm for 30 μsec using a BTX Electro Cell Manipulator 200 (BTX, San Diago, CA, USA), followed by treatment with 2 mM 6-dimethylaminopurine (6-DMAP, Sigma) for 3 h before *in vitro* culture. After parthenogenesis, the embryos were cultured in PZM-3 (Yoshioka *et al.* 2002) supplemented with 3 mg/ml BSA for 2 days at 39℃ under an atmosphere of 5 % CO_2 in air.

Analysis of ROS Products

Porcine metaphase-II (MII) oocytes and parthenogenetic embryos at the activation procedures and early developmental stages were stained in 10 μM dichlorohydrofluorescein diacetate (DCF, Molecular Probes, Eugene, OR, USA) or 10 μM hydroxyphenyl fluorescein (HPF, Molecular Probes) each for 30 min at 39℃ to measure the H_2O_2 level (Hashimoto *et al.*, 2000) or ˙OH radical level (Setsukinai *et al.*, 2003). After washing in PBS, oocytes and embryos were mounted onto the slide glass. The fluorescent emissions from the samples were recorded as JPEG files using a digital camera (F5.0, 4 sec; Coolpix, Nikon, Japan) attached to a fluorescent microscope (BX-50, Olympus, Japan) with filters at 450~480 nm for excitation and at 515 nm for emission. The images were analyzed using ImageJ software 1.37 (NIH) by the intensity of fluorescence in each oocyte and embryo.

Experimental Designs

Experiment 1

MII oocytes and parthenogenetic embryos were stained immediately after vortexing-induced denudation, electric pulse or 6-DMAP treatment, and the H_2O_2 and ˙OH radical levels were analyzed.

Experiment 2

Parthenogenetic embryos were stained at 0, 1, 2, and 3 h after electric stimulation, and the H_2O_2 and ˙OH radical levels were analyzed.

Experiment 3

H_2O_2 and ˙OH radical levels of porcine parthenogenetic embryos were analyzed at the 1-, 2-, and 4-cell stages.

Statistical Analysis

Data were analyzed by Duncan's multiple-range tests using the General Linear Model procedure of the software package Statistical Analysis System (SAS Institute, Inc., Cary, NC, USA).

RESULTS

ROS Levels at Activation Procedures

During manipulation, H_2O_2 levels were extremely decreased by electric pulse (13.0±1.0 pixels/egg) but reincreased after treatment of 6-DMAP (42.6±4.4 pixels/egg, $p<0.05$) (Fig. 1). ˙OH radical levels, however, did not differ among groups (Fig. 2).

ROS Levels by the Time Elapsed after Electric Stimulation

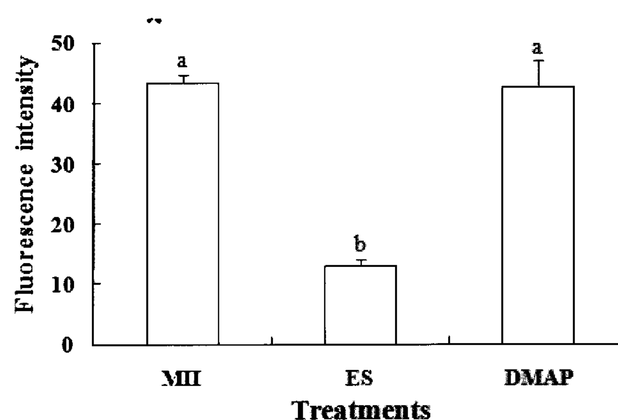

Fig. 1. Level of H_2O_2 in oocytes after different treatments. MII, metaphase II oocytes; ES, electric stimulus. [a,b] Values with different letters differ significantly ($p<0.05$).

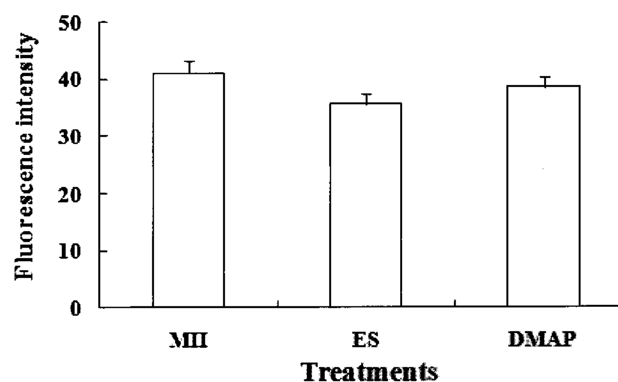

Fig. 2. Level of ˙OH radical products (B) in oocytes after different treatments. MII, metaphase II oocytes; ES, electric stimulus.

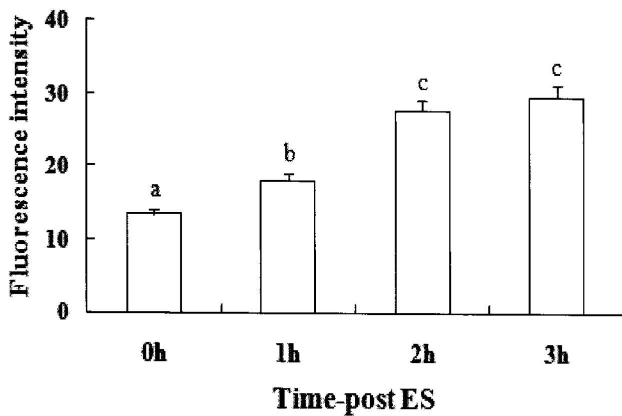

Fig. 3. Level of H_2O_2 in parthenogenetic oocytes by the time elapsed after electric stimulation (ES). [a~c] Values with different letters differ significantly ($p<0.05$).

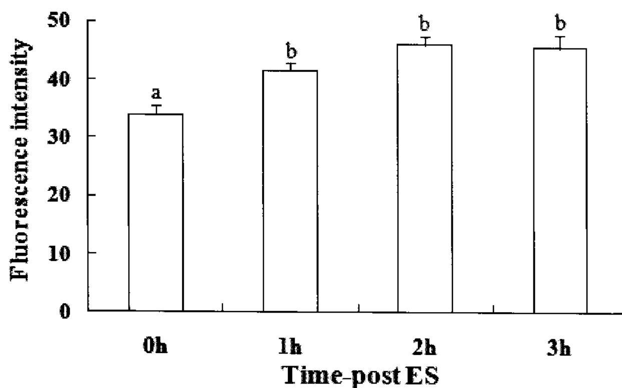

Fig. 4. Level of ·OH radical products in parthenogenetic oocytes by the time elapsed after electric stimulation (ES). [a,b] Values with different letters differ significantly ($p<0.05$).

H_2O_2 and ·OH radical levels of porcine parthenogenetic oocytes were low immediately after electric stimulation (13.5±0.6 and 34.0±1.6 pixcels/egg, respectively). However, H_2O_2 (18.1±1.0, 27.7±1.6 and 29.5±1.8) and ·OH radical levels (41.6±1.3, 46.0±1.5 and 45.5±2.3) were increased with the time elapsed after electric stimulation from 0 h to 1, 2 and 3 h ($p<0.05$, Fig. 3 and Fig. 4).

ROS Levels of Parthenogenetic Embryos during *In Vitro* Culture

During *in vitro* culture of parthenogenetic porcine embryos, H_2O_2 level (28.7±1.3, 45.0±3.3 and 52.8±3.2, respectively, Fig. 5) and ·OH radical product level (36.6±1.2, 46.9±1.4 and 54.2±1.6, respectively, Fig. 6) were gradually increased from the one-cell stage to the two- and four-cell stages ($p<0.05$).

DISCUSSION

The level of ROS generation in embryos is particu-

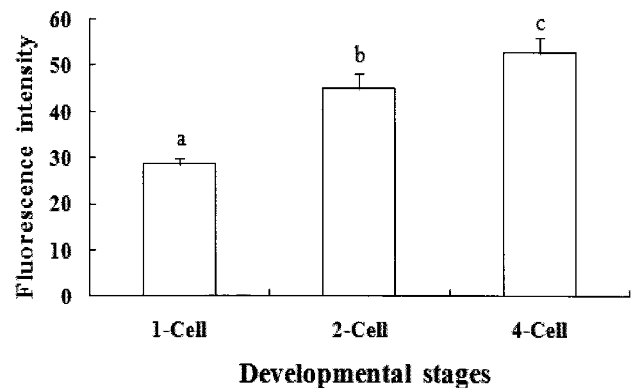

Fig. 5. Level of H_2O_2 in parthenogenetic embryos during early development. [a~c] Values with different letters differ significantly ($p<0.05$).

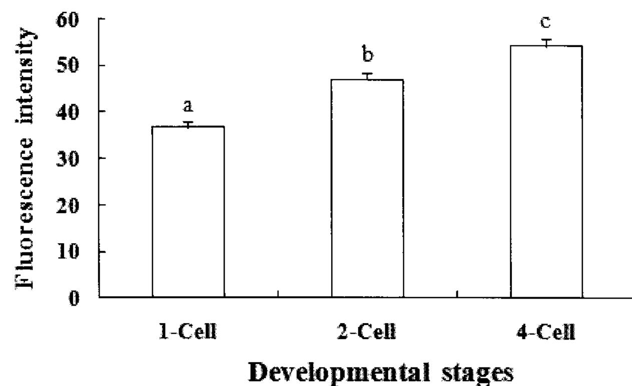

Fig. 6. Level of ·OH radical products in parthenogenetic embryos during early development. [a~c] Values with different letters differ significantly ($p<0.05$).

larly important during *in vitro* culture at various stages of development. During *in vitro* culture, embryos are exposed to relatively high oxidative stress compared to the environment *in vivo*, thus the generation of ROS within embryos is increased. Oxidative stress can cause numerous types of embryo damage due to the fact that ROS easily pass through cell membranes. ROS can alter most kinds of cellular molecules such as lipids, proteins and nucleic acids, which results in mitochondrial damage, embryo cell block, ATP depletion, and apoptosis (Guerin *et al.*, 2001). However, the stress of embryos during micromanipulation procedures, one of the factors affecting the development of nuclear transfer embryos has been overlooked until now. The cellular stress during micromanipulation procedures could generate ROS, which result in serious damages of the mitochondria and DNA of SCNT embryos and subsequently restrict the reprogramming of SCNT embryos.

Also, an electric pulse induced ROS generation in many types of cells (Bobanovic *et al.*, 1992; Gabriel and Teissie 1994; Sabri *et al.*, 1996). Artificial activation, using various stimuli, mimics these transient increases in Ca^{2+}. Although several agents, including Ca^{2+} iono-

mycin (Che *et al.*, 2007), ethanol (Yi and Park, 2005), thimerosal (Machaty *et al.*, 1997), and 6-DMAP (Im *et al.*, 2007) have been used to activate porcine oocytes, an electrical pulse (Prochazka *et al.*, 1992; Zhu *et al.*, 2002) is most frequently used for this purpose. Influx of Ca^{2+} across the plasma membrane following an electrical pulse directly initiates activation of porcine oocytes. Koo *et al.* (2008) reported that greater ROS were induced in porcine embryos after electrical activation of oocytes compared to IVF embryos. Furthermore, ROS generation in embryos after electrical activation was significantly increased by higher intensity and longer duration electrical pulses and by higher exogenous Ca^{2+} concentrations. Increase of intracellular calcium concentration by electrical stimulation induces the generation of large amounts of ROS (Banfi *et al.*, 2001; Brookes *et al.*, 2004). In this study, H_2O_2 level of porcine oocytes was reduced immediately after electric stimulation, but H_2O_2 level and $^{\cdot}OH$ radical product level of parthenogenetic embryos were increased with time elapsed after electric activation from 0 h to 3 h, and after 6-DMAP culture. Vortexing-mediated cumulus free of oocytes can also give a physical stress to the oocytes. In the present study, H_2O_2 level of porcine oocytes were reduced immediately after electric stimulation. We supposed that the generated ROS in the oocytes have been released out of the cell through the temporary formed large pores or holes of the oocyte membrane by the electrical stimulation (Zimmermann, 1982; Sun *et al.*, 1992).

ROS are metabolites of oxygen, and in small amounts of them are necessary to maintain the normal cell function. However, in excessive levels, these free radicals may peroxidate polyunsaturated fatty acids in the cell membranes, resulting in poor embryo development (Enkhmaa *et al.*, 2009). In this study, during *in vitro* culture of parthenogenetic porcine embryos, H_2O_2 level and $^{\cdot}OH$ radical product level were gradually increased from the one-cell stage to the two- and four-cell stages.

The result of the present study suggests that the ROS was not increased by electric pulse in porcine embryos. Rather than it seems to be associated with the stage of development and the culture condition, and physical or chemical stress can affect the development of embryos.

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