Generation of Reactive Oxygen Species in Bovine Somatic Cell Nuclear Transfer Embryos during Micromanipulation Procedures

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ABSTRACT

The present study was conducted to examine the generation of reactive oxygen species (ROS) during micromanipulation procedures in bovine somatic cell nuclear transfer (SCNT) embryos. Bovine enucleated oocytes were electrofused with donor cells, activated by a combination of Ca-ionophore and 6-dimethylaminopurine culture. Oocytes and embryos were stained in dichlorodihydrofluorescein diacetate or 3'-(p-hydroxyphenyl) fluorescein dye and the H_2O_2 or ˙OH radical levels were measured. *In vitro* fertilization (IVF) was performed for controls. The samples were examined with a fluorescent microscope, and fluorescence intensity was analyzed in each oocyte and embryo. The H_2O_2 and ˙OH radical levels of reconstituted oocytes were increased during manipulation (37.2~49.7 and 51.0~55.2 pixels, respectively) as compared to those of mature oocytes ($p<0.05$). During early *in vitro* culture, the ROS levels of SCNT embryos were significantly higher than those of IVF embryos ($p<0.05$). These results suggest that the cellular stress during micromanipulation procedures can generate the ROS in bovine SCNT embryos.

(Key words : Somatic cell nuclear transfer, Micromanipulation procedures, ROS generation, Cattle)

INTRODUCTION

Somatic cell nuclear transfer (SCNT) technique is an efficient tool to produce clone animals (Wilmut *et al.*, 1997), transgenic animals (Schnieke *et al.*, 1997), and xenograftic animals (Lai *et al.*, 2002), however, its efficiency is still low and have many serious problems such as, high abnormality and prenatal and postnatal death (Garry *et al.*, 1996). Some epigenetic modifications are considered to be reasons for these problems (Kang *et al.*, 2001; Inoue *et al.*, 2002; Xue *et al.*, 2002). However, other basic reasons might be responsible to these cytogenetic abnormalities as well as the low SCNT efficiency.

The development of SCNT embryos can be influenced by a number of factors, such as recipient cytoplasm, donor cell cycle stage, activation condition, and so on (Choi *et al.*, 2004). Further, the cellular stress that can be caused by the manipulations during SCNT procedures would also affect the reprogramming of SCNT embryos. Various cellular stresses generate reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide (˙O2-), and hydroxyl radical (˙OH) (Rhoads *et al.*, 2006). This might result in serious damages of the cells (Aitken *et al.*, 1989; Halliwell and Aruoma, 1991; Yang *et al.*, 1998; Rhoads *et al.*, 2006). However, cellular stresses that were induced by SCNT procedures have not been noted. Recently, we reported that the ROS generation level of porcine parthenogenetic embryos induced by activation treatment-derived stresses (Hwang *et al.*, 2011). The present study was aimed at examining the ROS generation level during nuclear transfer procedures in bovine SCNT embryos.

MATERIALS AND METHODS

In Vitro Maturation of Oocytes

Bovine cumulus-oocyte complexes (COCs) were aspirated from follicles (2- to 7-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). About ten COCs were transferred into 50 µl droplets of maturation medium overlaid with paraffin oil and cultured for 20~22 h at 39℃ and 5% CO_2 in air. The culture medium for *in vitro* maturation was Tissue Culture Medium 199 (TCM199; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 0.02 U/ml follicle-stimulating hormone (Sigma), 1 µg/ml estradiol (Sig-

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ma), 50 μg/ml gentamicin (Sigma), and 0.2 mM Na-pyruvate (Sigma).

Culture of Somatic Cells

Bovine ear skin fibroblast cells (4~6 passaged) from a Korean native cow were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% FBS, 0.2 mM Na-pyruvate (Sigma), and 1% penicillin/streptomycin for 2~3 days to achieve about 70% confluency. Subsequently, the cells were further cultured for 5 days in DMEM containing 0.5% FBS. Prior to use, the cells were trypsinized and then centrifuged in TCM199 medium supplemented with 3 mg/ml bovine serum albumin (BSA; Sigma).

Nuclear Transfer

SCNT was carried out in Hepes-buffered TCM199 (Gibco-BRL) supplemented with 3 mg/ml BSA and 5 μg/ml cytochalasin B (Sigma). After the *in vitro* maturation of COCs, the cumulus cells were removed by vortexing for 5 min in phosphate-buffered saline (PBS) supplemented with 0.1% (w/v) hyaluronidase (Sigma) and 0.1% (w/v) PVA (Sigma). Prior to the enucleation, oocytes were cultured in TCM199 containing 0.4 μg/ml demecolcine (Sigma) for 40 min in order to extrude their metaphase II (MII) chromosome mass. The enucleation of oocytes was done by removing the MII chromosome mass and the 1st polar body. A serum starved donor cell was injected into the perivitelline space of an enucleated recipient oocyte.

Electrofusion and Activation

Reconstructed 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 $MgSO_4$, 0.05 mM $CaCl_2$, and 0.1% BSA. Fusion was induced with a single direct-current pulse of 1.3 kV/cm for 30 μsec using a BTX Electro Cell Manipulator 200 (BTX, San Diago, CA, USA). Subsequent to the fusion treatment, the reconstituted oocytes were placed in CR1-aa (Rosenkrans and First, 1991) containing 3 mg/ml BSA and checked for fusion. The fused oocytes were then activated using 10 μM Ca-ionophore (A23187; Sigma) for 5 min and subsequently cultured in CR1aa containing 3 mg/ml BSA and 2 mM 6-dimethylamino-purine (DMAP, Sigma) for 3 h.

***In Vitro* Fertilization (IVF)**

Bovine COCs matured for 22 h were inseminated with frozen-thawed spermatozoa (2×10^6 spermatozoa/ml) in a 50 μl drop of BO medium (Brackett and Oliphant, 1975) containing 5 mM caffeine (Sigma), 10 μg/ml heparin (Sigma), and 3 mg/ml BSA at 39℃ and 5% CO_2 in air for 6 h.

***In Vitro* Culture of Embryos**

After activation or insemination culture, the SCNT and IVF embryos were further cultured in 50 μl drop (about 10 embryos per each drop) of CR1aa containing 3 mg/ml BSA and 50 μg/ml gentamicin at 39℃ and 5% CO_2 in air prior to the analysis of ROS levels at the one-(12 h post fusion or insemination), two- (26 h post fusion or insemination), and four-cell (42 h post fusion or insemination) stages

Analysis of ROS Products

The recipient oocytes and reconstituted eggs in various micromanipulation steps, and the SCNT and IVF embryos at the early developmental stages were stained in 10 μM dichlorodihydrofluorescein diacetate (H_2-DCFDA, Molecular Probes, Eugene, OR, USA) or 10 μM 3'-(p-hydroxyphenyl) fluorescein (HPF, Molecular Probes) each for 30 min at 39℃ for measuring the H_2O_2 level (Hashimoto *et al.*, 2000) or the ˙OH radical level (Setsukinai *et al.*, 2003). After washing in PBS, the oocytes and embryos were mounted onto the slide glass. The fluorescent images from the samples were recorded as JPEG files using a digital camera (Coolpix, Nikon, Japan) that was 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.

Statistical Analysis

Data were analyzed using ANOVA, followed by Duncan's multiple-range tests using the Statistical Analysis System (SAS Institute, Inc., Cary, NC, USA).

RESULTS

ROS Generation Levels during Manipulation Procedures

During micromanipulation, the H_2O_2 levels in recipient oocytes and SCNT embryos were increased by enucleation (37.2±0.4 pixels/oocyte), electrofusion (49.7±1.3 pixels/oocyte), and activation treatments (40.6±1.3 pixels/oocyte) in comparison with MII oocytes (33.1±0.7 pixels/oocyte $p<0.05$), and the level of H_2O_2 was made extremely high immediately after electrofusion (Fig. 1). The ˙OH radical level was significantly high during the manipulation procedures (51.0±0.6 to 55.2±1.4 pixels/oocyte) in comparison with MII oocytes (46.8±1.3 pixels/oocyte, $p<0.05$, Fig. 2).

ROS Generation Levels during Early *In Vitro* Culture of SCNT and IVF Embryos

During early *in vitro* culture, the H_2O_2 level of SCNT embryos was significantly high ($p<0.05$) at the one-

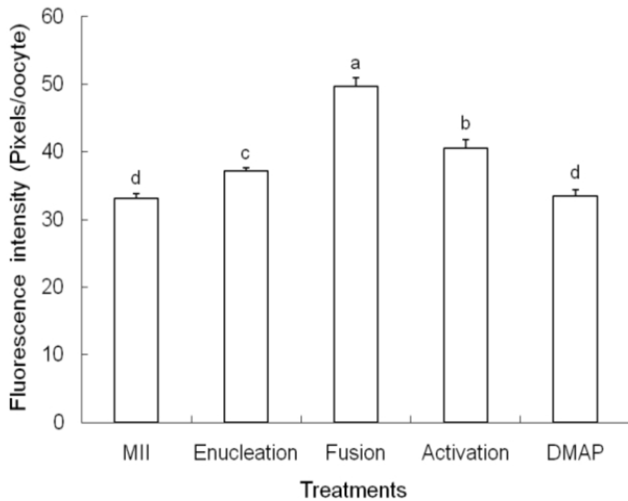

Fig. 1. Levels of H_2O_2 in bovine oocytes and SCNT eggs during micromanipulation. MII, metaphase II oocytes; DMAP, 2 mM 6-dimethylaminopurine treatment. Seven replicates were performed for each of the groups (total 45～50 eggs in each group) used on the same day, thereby allowing direct comparisons between groups. Data are presented by mean±SEM (bars). [a～d] Values with different letters differ significantly (p<0.05).

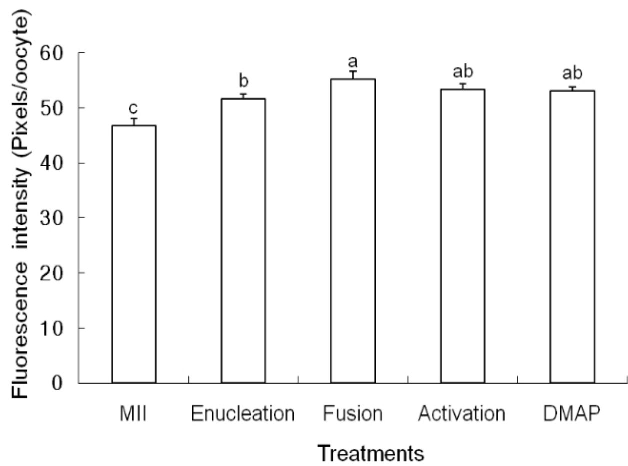

Fig. 2. Levels of ˙OH radical in bovine oocytes and SCNT eggs during micromanipulation. MII, metaphase II oocytes; DMAP, 2 mM 6-dimethylaminopurine treatment. Seven replicates were performed for each of the groups (total 45～50 eggs in each group) used on the same day, thereby allowing direct comparisons between groups. Data are presented by mean±SEM (bars). [a～c] Values with different letters differ significantly (p<0.05).

(32.4±1.1 pixels/embryo), two- (27.7±1.2 pixels/embryo), and four-cell stages (25.1±1.4 pixels/embryo) in comparison to IVF embryos (17.3±0.9, 22.0±1.5 and 16.5±1.2 pixels/embryo, respectively, Fig. 3). In addition, the ˙OH radical levels were also significantly high (p<0.05) in SCNT embryos (52.0±1.3, 33.4±1.0, and 26.9±1.1 pixels/embryo, respectively) in comparison to IVF embryos (29.6±0.8, 26.0±0.8, and 20.7±2.7 pixels/embryo, respectively) at the one-, two-, and four-cell stages (Fig. 4).

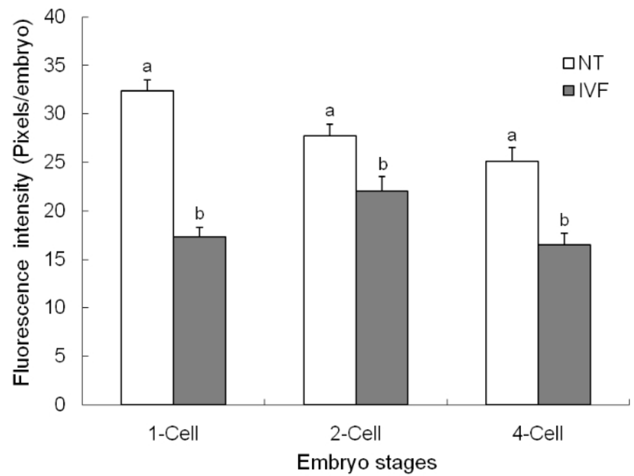

Fig. 3. Levels of H_2O_2 in bovine SCNT and IVF embryos during early *in vitro* development. Five replicates were performed for each of the embryonic stage (total 50～55 embryos in each group). The SCNT and IVF embryos were analyzed on the same day, thereby allowing direct comparisons between SCNT and IVF groups. Data are presented by mean±SEM (bars). [a,b] Values with different letters within each stage differ significantly (p<0.05).

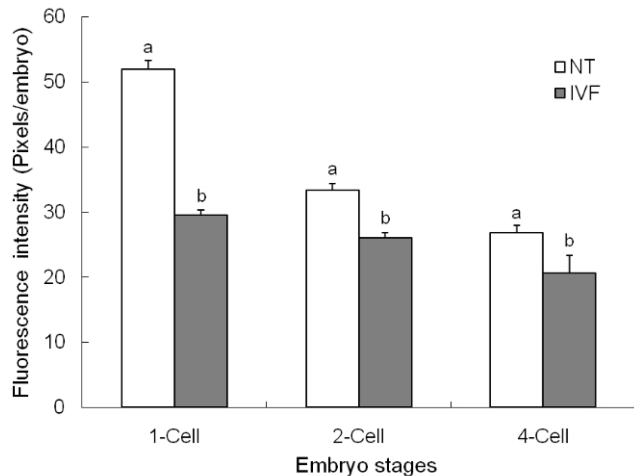

Fig. 4. Levels of ˙OH radical in bovine SCNT and IVF embryos during early *in vitro* development. Five replicates were performed for each of the embryonic stage (total 50～58 embryos in each group). The SCNT and IVF embryos were analyzed on the same day, thereby allowing direct comparisons between SCNT and IVF groups. Data are presented by mean±SEM (bars). [a,b] Values with different letters within each stage differ significantly (p<0.05).

DISCUSSIONS

Reactive oxygen species (ROS) are metabolites of oxygen. ROS generated under normal respiratory conditions; however, it can be enhanced in response to a range of abnormal conditions, including exposure to various stresses (Rhoads *et al*., 2006). The H_2O_2 and ˙OH radicals are some typical ROS. In general, ROS levels

are difficult to measure accurately (Halliwell and Whiteman, 2004). In the present study, oocytes and embryos were treated with two fluorescence dyes for detecting intracellular ROS. The fluorescence dye H_2-DCFDA (Hashimoto *et al.*, 2000) was used for detecting H_2O_2. The fluorescence dye HPF (Setsukinai *et al.*, 2003), which is mainly used for detecting ˙OH radicals, was also used in this study. HPF yields a fluorescein selectively when reacting with ˙OH radicals but not other ROS (Indo *et al.*, 2007).

In the present study the ROS levels of MII oocytes were found to be high, which was probably owing to the high oxygen tension and glucose (Ali *et al.*, 2003; Kitagawa *et al.*, 2004). However, it has been suggested that the MII oocytes normally have a high level of ROS. During *in vitro* maturation of oocytes, ROS plays a role in the induction of oocyte nuclear and/or cytoplasmic maturation near ovulation (Blondin *et al.*, 1997). It has also been suggested that high levels of ROS might produce oocyte meiotic arrest (Downs and Mastropolo, 1994). Micromanipulation procedures, such as enucleation, electrofusion, and activation can cause extreme stress to the oocytes and generate ROS in the cytoplasm. In the present study, the ROS generation levels were increased during micromanipulation procedures, especially by electrofusion and activation, and reduced gradually during DMAP treatment and early *in vitro* development. Regardless of the reduction of ROS level, the ROS levels of SCNT embryos were as high as ever in comparison to IVF embryos. In the present study, we did not evaluate the development and ROS level in the later stage embryos, because we placed the focus on the evaluation of the effects of mechanical stresses during SCNT procedure on the ROS generation and cellular damages. As the same reason, we also used the 20% O_2 for experiments, which conventional O_2 tension in SCNT experiments. ROS levels in the later stage of embryos can be affected by *in vitro* culture system (Ali *et al.*, 2003; Kitagawa *et al.*, 2004).

An electric pulse induced ROS generation in various types of cells (Bobanović *et al.*, 1992; Gabriel and Teissié, 1994) and embryos (Koo *et al.*, 2008). Koo *et al.* (2008) reported that greater ROS were induced in porcine embryos subsequent to the electrical activation of oocytes in comparison to IVF embryos. Furthermore, ROS generation in embryos after electrical activation was found to be significantly increased by higher intensity and longer duration electrical pulses. Nevertheless, the mechanism of ROS generation induced by electric pulse is unclear, and increased lipid peroxidation in membranes by electric pulse is regarded as an important cause of ROS generation (Maccarrone *et al.*, 1995). Further, calcium ion can affect ROS generation. Elevated intracellular calcium has been shown to enhance ROS production in intact cultured cells (Przygodzki *et al.*, 2005). In the mammalian cells it was found that when the cytosolic Ca^{2+} concentration was elevated, NADPH oxidase 5 generated large amounts of ROS (Banfi *et al.*, 2001). Also, excessive mitochondrial Ca^{2+} accumulation has been extensively associated with mitochondrial oxidative stress and can increase mitochondrial ROS formation (Brookes *et al.*, 2004). Ca^{2+} could act directly on the mitochondrial membrane, changing its properties and leading to enhanced ROS generation (Grijalba *et al.*, 1999).

In conclusion, the result of the present study suggests that the cellular stress during micromanipulation procedures can generate the ROS in bovine SCNT embryos, which may lead the cellular damages in bovine SCNT embryos. Further study is needed to estimate the mitochondrial and DNA damages of SCNT embryos induced by micromanipulation-derived ROS generation.

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