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Effect of L-Glutathione Treatment during Somatic Cell Nuclear Transfer Procedures on the Subsequent Embryonic Development and DNA Methylation Status of Cloned Bovine Embryos

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ABSTRACT

We investigate the effect of L-glutathione (GSH), an antioxidant, treatment during the somatic cell nuclear transfer (SCNT) procedures on the *in vitro* development and DNA methylation status of bovine SCNT embryos. Bovine *in vitro* matured (IVM) oocytes were enucleated and electrofused with a donor cell, then activated by a combination of Ca-ionophore and 6-dimethylaminopurine. The recipient oocytes or reconstituted oocytes were treated with 50 μM GSH during these SCNT procedures from enucleation to activation treatment. The SCNT embryos were cultured for 7 days to evaluate the *in vitro* development, apoptosis and DNA methylation in blastocysts. The apoptosis was measured by TUNEL assay and caspase-3 activity assay. Methylated DNA of SCNT embryos at the blastocyst stages was detected using a 5-methylcytidine (5-MeC) antibody. The developmental rate to the blastocyst stage was significantly higher (P<0.05) in GSH treatment group (32.5±1.2%, 78/235) than that of non-treated control SCNT embryos (22.3±1.8%, 50/224). TUNEL assay revealed that the numbers of apoptotic cells in GSH treatment group (2.3±0.4%) were significantly lower (P<0.05) than that of control (3.8±0.6%). Relative caspase-3 activity of GSH treated group was 0.8±0.06 fold compared to that of control. DNA methylation status of blastocysts in GSH treatment group (13.1±0.5, pixels/embryo) was significantly lower (P<0.05) than that of control (17.4±0.9, pixels/embryo). These results suggest that antioxidant GSH treatment during SCNT procedures can improve the embryonic development and reduce the apoptosis and DNA methylation level of bovine SCNT embryos, which may enhance the nuclear reprogramming of bovine SCNT embryos.

(Key words : somatic cell nuclear transfer, GSH, *in vitro* development, apoptosis, DNA-methylation)

INTRODUCTION

The physical cellular stress derived from micromanipulation is regarded as one of the most critical factors affecting the low efficiency of somatic cell nuclear transfer (SCNT) embryos generating excessive reactive oxygen species (ROS) (Hwang *et al.*, 2012). The excessive level of ROS can alter the cellular molecules, which may result in serious damages of the mitochondrial alterations, DNA fragmentation, abnormal metabolism, apoptosis, embryo cell block and epigenetic abnormality (Halliwell and Aruoma, 1991; Raha and Robinson, 2000; Kitagawa *et al.*, 2004; Rhoads *et al.*, 2006).

Antioxidant enzymes such as L-ascorbic acid (Vit. C, Tatemoto *et al.*, 2004), α-tocopherol (Vit. E, Olson and Seidel, 2000), β-mercaptoethanol (β-ME, Nedambale *et al.*, 2006), GSH (Salmen *et al.*, 2005) and superoxide dismutase (SOD, Tatemoto *et al.*, 2004) have been used for reduction of ROS in various species. Out of these, GSH is intracellular free thiol compound, and protects cells from ROS toxicity and regulates the intracellular redox balance (Lafleur *et al.*, 1994). Intracellular level of GSH was known to be an important factor that influences oocyte maturation and the subsequent development of embryos after IVF or SCNT (Yoshida *et al.*, 1993). Recently, we reported that 50 μM GSH during manipulation procedures reduced the ROS generation in bovine parthenogenetic and SCNT embryos (Bae *et al.*, 2012). Moreover, the antioxidant treatment during SCNT procedures could inhibit the mitochondrial and DNA damages at the one-cell stage of bovine SCNT

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embryos (Kim *et al.*, 2012).

The epigenetic reprogramming of donor cells was needed to successful cloning by SCNT. DNA methylation level of normal embryos during preimplantation development is relatively low. However, DNA methylation patterns of SCNT embryos are not fully demethylated, the level of DNA methylation in SCNT embryos is higher than normal embryos and is similar to somatic cells (Kang *et al.*, 2001). Thus, this hypermethylation status in SCNT embryos is regarded one of the reasons for abnormal gene expression and low cloning efficiency (Armstrong *et al.*, 2006).

The present study was conducted to examine the effect of an antioxidant, GSH treatment during SCNT procedures on the *in vitro* development, apoptosis and DNA methylation status of cloned bovine embryos.

MATERIALS AND METHODS

1. *In Vitro* Maturation of Oocytes

Bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory at 30～35℃. Bovine cumulus-oocyte complexes (COCs) were aspirated from ovary follicles (2～7 mm diameter), and washed in Tyrode's lactate (TL)-Hepes buffer containing 0.1% (w/v) polyvinyl alcohol (PVA; Sigma-Aldrich, St. Louis, MO, USA). The COCs with compact several layers of cumulus cells and homogeneous ooplasm were selected for *in vitro* maturation (IVM). Approximately 50～100 COCs were cultured in 500 μl of Tissue Culture Medium 199 (TCM-199; Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco), 0.2 mM Na-pyruvate (Sigma-Aldrich), 50 μg/ml gentamicin (Sigma-Aldrich), 0.02 IU/ml FSH (Sigma-Aldrich) and 1 μg/ml 17β-estradiol (Sigma-Aldrich) at 39℃, 5% CO_2 in air for 20～22h..

2. Preparation of Somatic Cells

Bovine somatic cells were obtained from an ear tissue of Korean native cow (Hanwoo). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FBS (Gibco) and 1% antibiotic (Sigma-Aldrich) at 39℃, 5% CO_2 in air. The cells were passaged 2～5 times, and then frozen by using DMEM supplemented with 10% dimetylsulfoxide (DMSO; Sigma-Aldrich) and stored in liquid nitrogen. Before SCNT, cells were thawed and cultured in DMEM supplemented with 10% FBS and 1% antibiotic until they reached confluence to synchronize the cell cycleat the G0/G1 phase at 39℃, 5% CO_2 in air. Cells were trypsinized with 0.05% trypsin-EDTA (Gibco) and centrifuged at 500×g for 5 min in TCM-199 (Gibco) supplemented with 3 mg/ml BSA (Sigma-Aldrich) (TCM-BSA). Cells were cultured in TCM-BSA containing 10 μg/ml phytohemagglutinin-P (PHA-P; Sigma-Aldrich) for 5 min prior to SCNT.

3. Nuclear Transfer, GSH Treatment and *In Vitro* Culture

The cumulus cells of IVM oocytes were removed by vortexing in PBS (Gibco) supplemented with 0.1% hyaluronidase (Sigma-Aldrich) and 0.1% polyvinyl pyrrolidone (PVP; Sigma-Aldrich) for 3 min. Prior to enucleation, cumulus-free oocytes were cultured in TCM-BSA containing 0.4 μg/ml demecolcine (Sigma-Aldrich) for 40 min to extrude their metaphase II (MII) chromosome mass. The enucleation of oocytes was performed by removing the MII chromosome mass and first polar body using injection pipette in Hepes-buffered TCM-BSA containing 5 μg/ml cytochalasin B (Sigma-Aldrich). Then a donor cell was injected into the perivitelline space of an enucleated recipient oocyte. The reconstructed oocytes were placed between two wire electrodes (1 mm apart) of a fusion chamber overlaid with fusion medium (0.3 M mannitol, 0.1 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $MgCl_2 \cdot 6H_2O$ and 0.5 mM Hepes). For the fusion, a single direct current (DC) pulse of 1.25 kV/cm was applied for 30 μs using a BTX Electro Cell Manipulator 200 (BTX, San Diago, CA, USA). After fusion treatment, there constructed oocytes were cultured in TCM-BSA for 40 min and checked for fusion. The fused embryos were further activated by 10 μ M Ca-ionophore (A23187; Sigma-Aldrich) for 5 min and followed by an incubation in CR1aa supplemented with 3 mg/ml BSA and 2 mM 6-dimethylaminopurine (DMAP; Sigma-Aldrich) for 3 h. For GSH treatment group, 50 μM GSH (Sigma-Aldrich) was added to each medium for micromanipulation, fusion, holding and activation culture. The above concentrations of antioxidants were selected by our previous experiment (Bae *et al.*, 2012). After activation, SCNT embryos were cultured in CR1aa (Rosenkrans and First, 1994) supplemented with 3 mg/ml BSA (Sigma-Aldrich) for 2 days and then cultured in CR1aa supplemented with 10% FBS (Gibco) for further 5 days at 39℃, 5% CO_2 in air.

4. TUNEL Assay

Terminal deoxynucleotidyl transferase dUTP nick end la-

beling (TUNEL) assay was perfoemed using *In Situ* Cell Death Detection Kit (TMR red, Roche, Mannhaim, Germany). The SCNT blastocysts were fixed with 4% (v/v) paraformaldehyde (Sigma-Aldrich) in PBS for 1 h at 4℃. Fixed blastocysts were permeabilized by incubation in 0.5% Triton X-100 (Sigma-Aldrich) for 30 min at room temperature (RT), followed by incubation with TUNEL mixture (TMR red in a ratio of 1:9) for 1 h at 37℃ in the dark and by incubation with 0.5% Triton X-100 for 5 min at RT. Embryos were then stained with 40 μg/mL Hoechst 33342 (Sigma-Aldrich) for 30 min at RT in the dark and mounted on a slide glass with mounting solution (Vectashield, Vector Laboratories, CA, USA). Slides were analyzed for TUNEL positive staining under a fluorescence microscope (BX-40, Olympus, Tokyo, Japan). Experiments were repeated at least 4 times using total 20～25 blastocysts in each group.

5. Caspase-3 Activity Assay

Intracellular caspase-3 activities in blastocysts were analyzed using the Caspase-3 Colorimetric Activity Assay Kit (CHEMICON, Billerica, MA, USA). Briefly, 3 blastocysts from each group were transferred to a microcentrifuge tube containing 50 μL of 1× lysis buffer and lysed for 15 min on ice. Lysates were centrifuged at 500 ×g for 5 min and the supernatants were incubated with assay mixture (5× assay buffer 20 μl, distilled water 20 μl and caspase-3 substrate 10 μl) for 2 h at 37℃ in the dark. The optical density (OD) values were obtained using a microplate reader (Power Wave XS, BioTek, Winooski, VT, USA) at 405 nm, 37℃. Experiments were repeated 5 times using total 15 blastocysts in each group.

6. Immunofluorescence Staining of Methylated DNA

Methylated DNA of SCNT embryos at the blastocyst stage was detected using a 5-methylcytidine (5-MeC) antibody. Embryos were fixed with 4% (v/v) paraformaldehyde (Sigma-Aldrich) in PBS for 1 h at 4℃ and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) for 15 min at RT. Embryos were then washed and further treated with 4 N HCl for 1 h at RT to denature DNA. After washing with PBS supplemented with 0.3% PVP (Sigma-Aldrich), embryos were treated with 3% BSA in PBS (Sigma-Aldrich) for 1 h at RT to preclude unspecific binding of primary antibody. Embryos were incubated with primary antibody (monoclonal antibody 5-methylcitidine; Eurogenetec, BI-MECY-0100, Fremont, CA, USA) for 1 h at 37℃ for the immunostaining of methylated DNA. Embryos were then incubated with Alexa Fluor 488 goat-anti-mouse IgG (Invitrogen, A10680, Karlsruhe, Germany) for 1 h at 37℃ in the dark. Stained embryos were mounted on a slide glass with PBS supplemented with 0.3% PVP (Sigma-Aldrich) and slides were analyzed for green fluorescence intensity under a fluorescence microscope (BX-40, Olympus). The fluorescence intensity of each blastocyst in images was measured using ImageJ software (National Institute of Mental Health, Bethesda, MD, USA). Experiments were repeated 4 times using total 20 blastocysts in each group.

7. Statistical Analysis

Data were pooled and statistically expressed as mean ± standard error (SEM). Significant differences between data were analyzed by Student's *t*-tests using Statistical Analysis System (SAS Institute, Inc., Cary, NC, USA).

RESULTS

1. *In Vitro* Development of SCNT Embryos

There were no significant differences between control and GSH treatment groups, and among treatment groups in the cleavage rate (72.3 ± 2.5% vs. 71.9 ± 3.5%) and developmental rate to the morular stage (40.6 ± 3.2% vs. 49.3 ± 2.5%). However, the developmental rates to the blastocyst stage were significantly higher ($P<0.05$) in GSH treatment groups (32.5 ± 1.2%, 78/235) than that of control (22.3 ± 1.8%, 50/224) (Fig. 1). There was no difference in blastocyst cell number between both groups (data not shown).

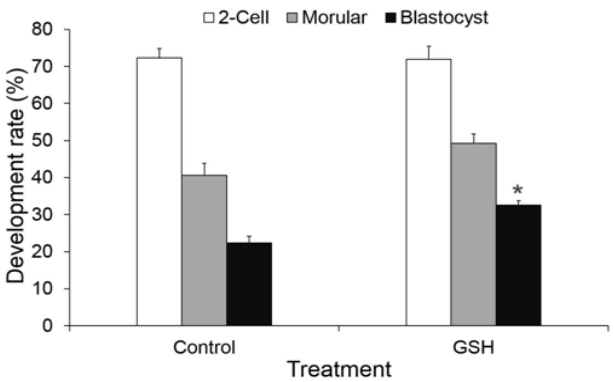

Fig 1. *In vitro* development of SCNT embryos. Experiments were repeated 10 times in each treatment group. Percentage data are presented as mean ± S.E. * Significantly different from the SCNT control within the same development stage ($P<0.05$).

2. Apoptosis

TUNEL assay revealed that the percentage of apoptotic cells in blastocysts from GSH treatment group (2.3 ± 0.4%, 49/2096 cells) were significantly lower (P<0.05) than that of control (3.8 ± 0.6%, 73/1,912 cells, Fig. 2). Although there was no difference, the relative intracellular caspase-3 activity of GSH treatment group at the blastocyst stage was 0.79 ± 0.06 fold compared to control (defined as 1 fold) (Fig. 3).

3. DNA Methylation Status

DNA methylation level at the blastocyst stage in GSH treatment groups (13.1 ± 0.5, pixels/embryo) were significantly lower (P<0.05) than that of control (17.4 ± 0.9, pixels/embryo) (Fig. 4).

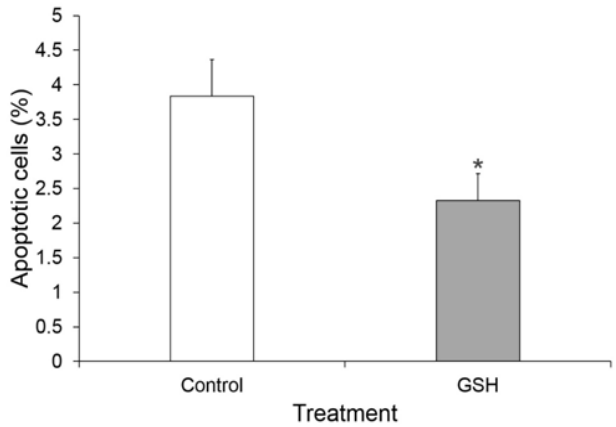

Fig. 2. TUNEL assay of the blastocyst stage SCNT embryos. Data are presented as mean ± S.E. * Significantly different from the SCNT control (P<0.05).

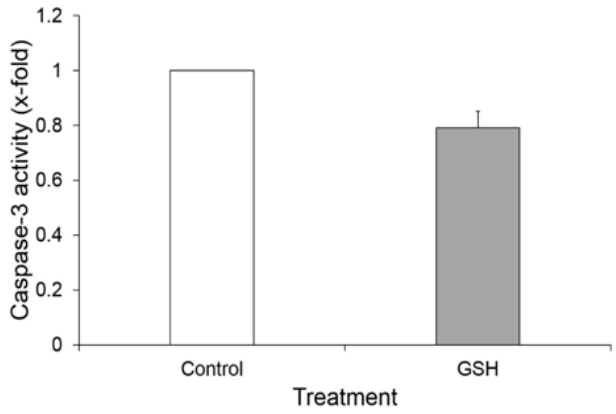

Fig. 3. The relative intracellular caspase-3 activity of SCNT embryos. Datum in GSH is presented as mean ± SE.

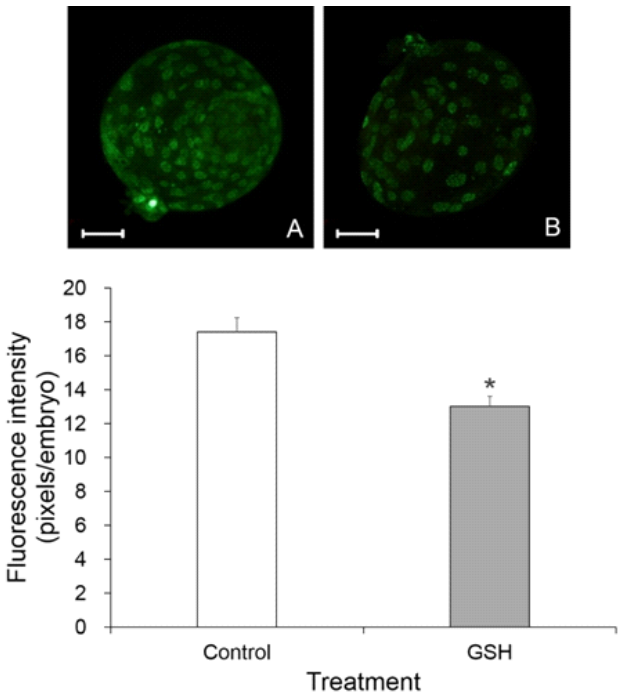

Fig. 4. DNA methylation of SCNT embryos at the blastocyst stage. (The upper part) Fluorescence images of SCNT embryos stained with 5-MeC. (A) SCNT control embryo, (B) GSH-treated SCNT embryo. Scale bar = 50 μm. (The lower part) DNA methylation status of SCNT embryos at the blastocyst stage. Data are presented as mean ± S.E. * Significantly different from the SCNT control (P<0.05).

DISCUSSION

ROS such as hydrogen peroxide (H_2O_2), superoxide anions ($O_2^{-}\cdot$) and hydroxyl radicals (OH·), are produced in cells during the normal aerobic metabolism. A suitable amount of ROS are necessary to maintain the normal cell function (Nasr-Esfahani and Johnson, 1991; Guerin *et al.*, 2001), and maintained by the balance between the rate of production and scavenging by intracellular antioxidant systems (Cui *et al.*, 2012). However, if ROS are not effectively removed from cells, the high level of ROS in the cells can induce oxidative stress, which may damage the mitochondrial function, nuclear DNA and viability of embryos (Halliwell and Aruoma, 1991).

The cellular stresses during SCNT procedures such as enucleation, cell injection, fusion and activation, could generate the excessive ROS (Hwang *et al.*, 2012). ROS can induce the several dysfunctions including mitochondria and DNA damages and subsequently inhibit the reprogramming of SCNT embryos

(Hwang *et al.*, 2013). However, the cellular stress of embryos during micromanipulation procedures has been overlooked until now.

Many researchers have been used various methods such as the application of low O_2 tension (Iwamoto *et al.*, 2005) and addition of antioxidants (Ali *et al.*, 2003) to prevent the excessive ROS. The addition of antioxidants in culture medium such as Vit. C, Vit. E, β-ME, GSH and SOD enhanced the antioxidant systems within embryo and embryonic development (Olson and Seidel, 2000; Orsi and Leese, 2001). However, there has been limited information about the effect of antioxidant treatment during micromanipulation procedures. Thus, in the present study, we focused the antioxidant treatment during SCNT procedures, but not during *in vitro* culture period, which is the different point compared to other previous study. In our previous studies, we determined the optimal concentrations of GSH in the bovine parthenogenetic embryos, and we confirmed that GSH treatment during SCNT procedures is effective to inhibit the ROS generation in bovine SCNT embryos (Bae *et al.*, 2012). Moreover, the antioxidant treatment during SCNT procedures could inhibit the mitochondrial and DNA damages of bovine SCNT embryos (Kim *et al.*, 2012).

The blastocyst stage is a crucial period during preimplantation development, and quality of blastocysts portends their ability to implant (Fabian *et al.*, 2005). Apoptosis is one of the main factors determining the quality of blastocyst. Apoptosis in blastocyst might lead to either early embryonic death or fetal anomalies (Brill *et al.*, 1999). In this study, TUNEL assay result showed that GSH treatment reduced the number of apoptotic cells in blastocysts compared to SCNT control. The caspase-3 activity, however, was similar in both control and GSH-treated groups, which was different from TUNEL result. The reason for this difference is unclear, but might be due to the small number of blastocysts used for caspase-3 assay rather than for TUNEL assay.

DNA methylation status is an indicator of the occurrence of appropriate epigenetic reprogramming in SCNT embryos. Many studies reported that genomic hypermethylation in cloned embryos induce transcriptional abnormalities, (Bourćhis *et al.*, 2001; Dean *et al.*, 2001; Kang *et al.*, 2001). DNA methylation status in SCNT blastocysts was significantly higher than IVF blastocysts (Yamanaka *et al.*, 2011). Similarly, the present results showed that the level of DNA methylation at the blastocyst stage in SCNT control group was hypermethylated. GSH treatment during the SCNT procedures could reduce the DNA methylation level of SCNT bovine embryos. It was suggested that the protection of cells from cellular damage during manipulation procedures by an antioxidant, GSH, resulted in more normal methylation in SCNT embryos during *in vitro* development.

In conclusion, the present study shows that an antioxidant, GSH treatment during SCNT procedures could improve the embryonic development and reduce the apoptosis and DNA methylation level of bovine SCNT embryos, which may enhance the nuclear reprogramming of bovine SCNT embryos and improve the efficiency of SCNT.

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