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Generation of Reactive Oxygen Species and Subsequent DNA Fragmentation in Bovine Cultured Somatic Cells

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ABSTRACT

The present study was conducted to examine the reactive oxygen species (ROS) generation levels and subsequent DNA damage in the bovine cultured somatic cells. Bovine ear skin cells were classified by serum starvation, confluence and cycling cells. Cells were stained in 10 μM dichlorohydrofluorescein diacetate (H_2DCFDA) or 10 μM hydroxyphenyl fluorescein (HPF) dye to measure the H_2O_2 or ˙OH radical levels. The samples were examined with a fluorescent microscope, and fluorescence intensity was analyzed in each cell. H_2O_2 and ˙OH radical levels of cultured somatic cells were high in confluence group (7.1±0.7 and 8.4±0.4 pixels/cell, respectively) and significantly low in serum starvation group (4.9±0.4 and 7.0±0.4 pixels/cell, respectively, p<0.05). Comet tail lengths of serum starvation (148.3±5.7 μm) and confluence (151.1±5.0 μm) groups were found to be significantly (p<0.05) increased in comparison to that of cycling group (137.1±7.5 μm). These results suggest that the culture type of donor cells can affect the ROS generation, which leads the DNA fragmentation of the cells

(Key words : ROS generation, Bovine somatic cells, Culture type, DNA fragmentation)

INTRODUCTION

The efficiency of somatic cell nuclear transfer (SCNT) can be influenced by a number of factors (Choi *et al.*, 2004). The culture condition or culture type of donor cells also influences on the development of SCNT embryos by affecting the donor cell cycle stage (Cheong, 2003). During *in vitro* culture of donor cells, cells can be received various cellular stresses by the culture condition or culture type, such as duration of culture period, nutrition, concentration of the cells, and exposure to high O_2 tension. Cellular stresses generate reactive oxygen species (ROS) in the cells (Rhoads *et al.*, 2006).

H_2O_2 and ˙OH radicals are the typical ROS. H_2O_2, a compound of relatively low toxicity, can react with intra-mitochondrial Fe^{2+} and Cu^{+}, to produce highly toxic hydroxyl (˙OH) radicals (Sweetlove & Foyer, 2004). ROS levels in general are difficult to measure accurately (Halliwell & Whiteman, 2004). The fluorescence dye dichlorohydrofluorescein diacetate (H_2DCFDA) was used to detect H_2O_2 (Hashimoto *et al.*, 2000) and the fluorescence dye hydroxyphenyl fluorescein (HPF) was used to detect mostly ˙OH radicals (Setsukinai *et al.*, 2003).

Generation of ROS in the cells may result in serious damages of the mitochondria and DNA of the cells (Halliwell & Aruoma, 1991; Tatemoto *et al.*, 2000; Rhoads *et al.*, 2006) including mitochondrial dysfunction (Rhoads *et al.*, 2006), apoptosis (Yang *et al.*, 1998) and abnormal metabolism (Raha & Robinson, 2000). DNA fragmentation is also one of the cellular damage induced by ROS (Henle & Linn, 1997; Kitagawa *et al.*, 2004). OH radical derived from H_2O_2 attacks the deoxyribose moiety of DNA to cause the strand breaks, and these breaked DNA strands can be detected by the comet assay (Takahashi *et al.*, 1999).

The cellular stresses in the donor cells that were induced during *in vitro* culture have not been noted in related with culture type. The present study was conducted to examine the ROS generation levels and subsequent DNA damage in the bovine somatic cells cultured in different culture condition or type.

MATERIALS AND METHODS

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, Grand Island,

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NY, USA) supplemented with 10% FBS, 0.2 mM Na-pyruvate (Sigma, St. Louis, MO, USA) and 50 μg/ml gentamicin for 2~3 days to reach at about 70% confluency. After then, cells were classified by three groups; 1) immediately use (cycling), 2) use after culture for 5 days in DMEM containing 0.5% FBS (serum starvation), and 3) use after culture for more than 10 days to confluent state (confluence). Before use, the cells were trypsinized and then centrifuged in TCM199 medium supplemented with 3 mg/ml bovine serum albumin (BSA; Sigma).

Analysis of ROS Products

Somatic cells were stained in 10 μM H_2DCFDA (Molecular Probes, Eugene, OR, USA) or 10 μM 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, cells were mounted onto the slide glass. The fluorescent emissions from the samples were recorded as JPEG files using a digital camera (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 softwere 1.37 (NIH) by the intensity of fluorescence in each cell (70~80 cells in each group).

Comet Assay

The DNA damages in bovine cultured somatic cells were analyzed by comet assay at the end of the culture period. Comet assay was performed using the OxiSelect™ Comet Assay Kit (Cell Biolabs, San Diego, CA, USA). Prior to assay, OxiSelect™ comet agarose (Cell Biolabs, Assay kit no. 235002) was melted at 90℃ for 20 min and cooled at 37℃ for 20 min. About 75 μl of agarose dropped onto an OxiSelect™ 3-well comet slide (Cell Biolabs, Assay kit no. 235001) and about twenty cells were transferred to agarose drops and subsequently chilled at 4℃ for 20 min. The samples were lysed at lysis buffer [250 mM NaCl, 20% EDTA solution (Cell Biolabs, Assay kit no. 235004), 10% DMSO, 10% 10× lysis solution (Cell Biolabs, Assay kit no. 235005), pH ~10.0] at 4℃ for 1 h. The slides were carefully transferred to chilled alkaline solution (300 mM NaOH, 1 mM EDTA) and immersed for 30 min at 4℃. Subsequently, the slides were transferred to the horizontal electrophoresis chamber filled with cold TAE buffer (ELPIS Biotech, Daejeon, Korea). Electrophoresis was conducted for 20 min at 50 V, after which the slides were stained with 1× Vista Green DNA Dye (Cell Biolabs, Assay kit no. 235003) for 20 min. The slides were examined using a fluorescent microscope with FITC filter. All steps after agarose treatment were conducted in the dark in order to prevent additional DNA damage. The comet tail lengths were measured in individual cells using CASP (ver 1.2.2, Zbigniew Koza, Poland).

Statistical Analysis

Data were analyzed using ANOVA, followed 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 Generation Levels in Cultured Somatic Cells

ROS levels were analyzed by counting of pixels per each cell from the fluorescence images (Fig. 1). H_2O_2 level of cultured somatic cells was significantly high (p<0.05) in serum starvation (7.6±0.4 pixels/cell) and confluence (8.0±0.2 pixels/cell) groups compared to cycling group (5.8.1±0.7 pixels/cell, Fig. 2A). ·OH radical level was high in confluence group (9.0±0.4 pixels/cell, p<0.05) compared to cycling group (7.1±0.4 pixels/cell). ·OH radical level in serum starvation group was not different from those of other groups (Fig. 2B).

DNA Fragmentation of Cultured Somatic Cells

Comet assay results revealed that three types of cultured somatic cells exhibited a clearly defined comet tail (Fig. 3A~C). However, the length of migrated DNA fragments in serum starvation (148.3±5.7 μm) and confluence (151.1±5.0 μm) groups were found to be significantly (p<0.05) increased in comparison to that of cycling group (137.1±7.5 μm, Fig. 4).

DISCUSSIONS

Reactive oxygen species (ROS) are metabolites of oxygen, and either reactive anions containing oxygen atoms, or molecules containing oxygen atoms which can produce free radicals. ROS generation takes place un-

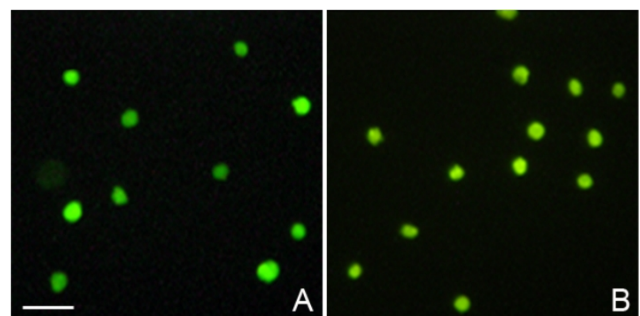
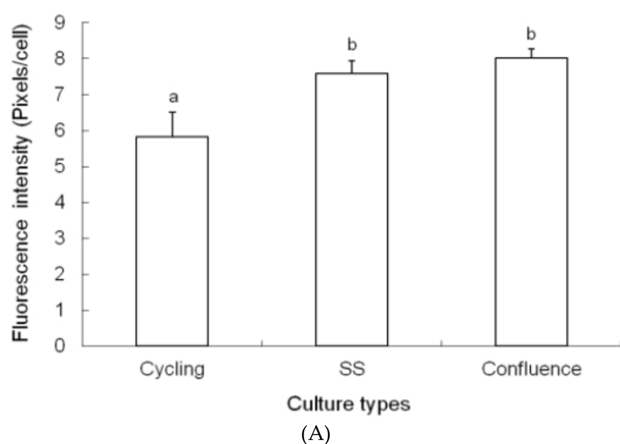
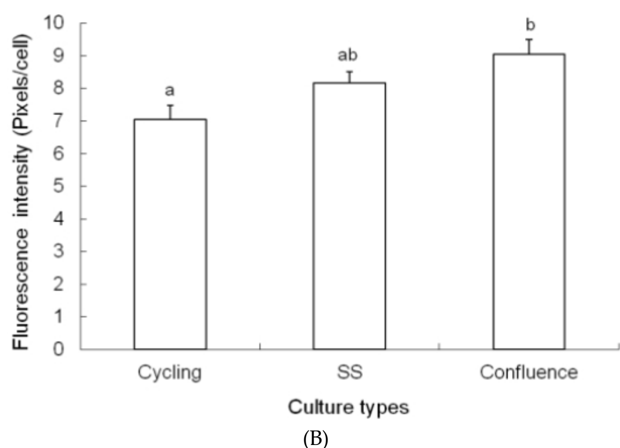


Fig. 1. Fluorescence images of donor cells stained with H_2DCFDA (A) or HPF (B). Scale bar=50 μm.



(A)



(B)

Fig. 2. Levels of reactive oxygen species (ROS) in bovine somatic cells with different culture types. Data are presented by mean± SEM (bars). SS, serum starvation. A) H_2DCFDA, B) HPF. [a,b] Values with different letters differ significantly (p<0.05).

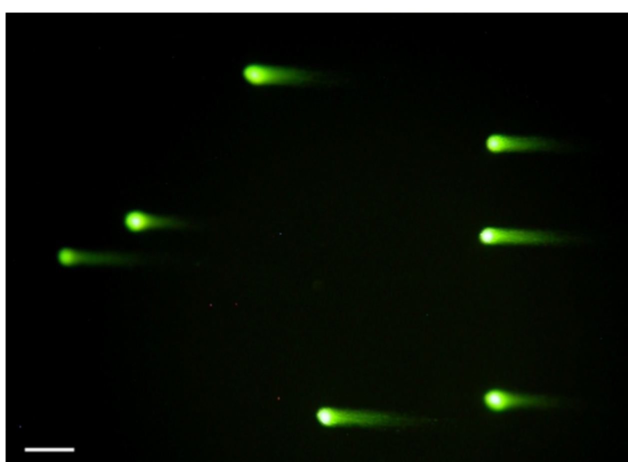


Fig. 3. Comet images of fragmented DNA migration of bovine somatic cells (cycling). Scale bar=50 μm.

der normal respiratory conditions but can be enhanced in response to a range of abnormal conditions, including exposure to various cellular stresses (Rhoads *et al.*,

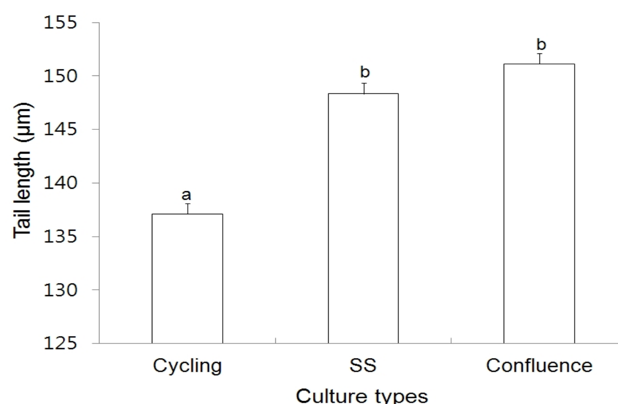


Fig. 4. Comet tail moment length of bovine somatic cells with different culture types. Data are presented by mean±SEM (bars). SS, serum starvation. [a,b] Values with different letters differ significantly (p<0.05).

2006).

Culture conditions of bovine somatic cells for SCNT affected intracellular ROS. Serum starvation is one of cellular stress, which results in the gradual production of endogenous ROS (Han *et al.*, 2005). In this study, serum starvation of the cells increases the ROS level of the cells. Serum starvation provided reduced proliferating time and less cell-to-cell contact to the cells, however, nutritional deficiency may be an extreme stress to somatic cells. More increased ROS level was observed in confluence culture condition. In the confluence culture, cells might be in extreme stresses by closed cell-to-cell contact, increased proliferating time or aging, and long term culture under high O_2 tension. Cycling cells may be received less cellular stress due to the short culture period, reduced proliferating time and less cell-to-cell contact.

Oxygen tension is a most important factor affecting on the generation of ROS. In the present study, somatic cells were cultured in a condition of 20% O_2. The culture of cells with a high O_2 tension (20%) *in vitro* may produce more free radicals than cells culture under 5% O_2 (Liu & Foote, 1995). In conventional *in vitro* cell culture, somatic cells were exposure this high O_2 condition all the period of culture. Calcium ion in the culture medium may affect ROS generation. Elevated intracellular calcium enhances ROS production in various intact cultured cells (Goldman *et al.*, 1998; Przygodzki *et al.*, 2005). In the mammalian cells, excessive mitochondrial Ca^{2+} accumulation may increase mitochondrial ROS formation by association with mitochondrial oxidative stress (Brookes *et al.*, 2004; Kowaltowski, 2009). The longer culture time of somatic cells in the medium with Ca^{2+} ion, the more ROS products could be generated.

In this study, comet tail movement was observed in the all type of cells cultured with different condition.

Comet tail lengths of serum starved and confluent cells were significantly longer than that of cycling cells, which reveals that DNA fragmentations of serum starved and confluent cells are induced with high frequency. It was suggested that increased DNA fragmentation in serum starved and confluent cells is related to increased ROS generation levels in these cell groups. This DNA fragmentation in the cells would result in more increase in irreversible strand breaks of DNA through the DNA replications during cell proliferation. (Cox *et al.*, 2000).

In conclusion, the result of the present study reveals that the culture type of bovine somatic cells can affect the ROS generation level and lead the subsequent DNA fragmentation, which may result in the abnormal reprogramming of bovine SCNT embryos after nuclear transfer.

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