Inhibition of Reactive Oxygen Species Generation by Antioxidant Treatments during Bovine Somatic Cell Nuclear Transfer

Hyo-Kyung Bae[1], Ji-Ye Kim[1], In-Sun Hwang[1], Choon-Keun Park[2], Boo-Keun Yang[2] and Hee-Tae Cheong[1,†]

[1]*College of Veterinary Medicine and Institute of Veterinary Science, Kangwon National University, Chuncheon 200-701, Korea*
[2]*College of Animal Life Sciences, Kangwon National University, Chuncheon 200-701, Korea*

ABSTRACT

This study was conducted to examine the optimal concentration and treatment time of antioxidants for inhibition of the ROS generation in bovine somatic cell nuclear transfer (SCNT) embryos. Bovine oocytes were activated parthenogenetically, during which oocytes were treated with various antioxidants to determine the optimal concentrations and kind of antioxidants. Determined antioxidants were applied to oocytes during *in vitro* maturation (IVM) and/or SCNT procedures. Finally, antioxidant-treated SCNT embryos were compared with *in vitro* fertilized (IVF) embryos. H_2O_2 levels were analyzed in embryos at 20 h of activation, fusion or insemination by staining of embryos in 10 μM 2'7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) dye, followed by fluorescence microscopy. H_2O_2 levels of parthenogenetic embryos were significantly lower in 25 μM β-mercaptoethanol (β-ME), 50 μM L-ascorbic acid (Vit. C), and 50 μM L-glutathione (GSH) treatment groups than each control group (24.0±1.5 vs 39.0±1.1, 29.7±1.0 vs 37.0±1.2, and 32.9±0.8 vs 36.3±0.8 pixels/embryo, $p<0.05$). There were no differences among above concentration of antioxidants in direct comparison (33.6±0.9～35.2±1.1 pixels/embryo). Thus, an antioxidant of 50 μM Vit. C was selected for SCNT. H_2O_2 levels of bovine SCNT embryos were significantly lower in embryos treated with Vit. C during only SCNT procedure (26.4±1.1 pixels/embryo, $p<0.05$) than the treatment group during IVM (29.9±1.1 pixels/embryo) and non-treated control (34.3±1.0 pixels/embryo). Moreover, H_2O_2 level of SCNT embryos treated with Vit. C during SCNT procedure was similar to that of IVF embryos. These results suggest that the antioxidant treatment during SCNT procedures can reduce the ROS generation level of SCNT bovine embryos.

(Key words : Somatic cell nuclear transfer, Reactive oxygen species, Antioxidants, Cattle)

INTRODUCTION

The efficiency of somatic cell nuclear transfer (SCNT) technique in mammals is low because of the many serious problems such as high abnormality and embryonic death (Garry *et al*., 1996). The reasons for these problems might be due to the abnormal nuclear reprogramming of reconstituted embryos (Kang *et al*., 2001; Inoue *et al*., 2002) as well as technical problems. Various factors such as nucleo-cytoplasmic interaction, activation condition (Choi *et al*., 2004), and physical cellular stress derived from micromanipulation (Hwang *et al*., 2012) can influence on the reprogramming of SCNT embryos. It was reported that the physical cellular stress during the SCNT procedures generated excessive ROS in the bovine embryos (Hwang *et al*., 2012). The excessive level of ROS can alter the most kinds of cellular molecules, which may result in the serious damages of the mitochondria and nuclear DNA in the cells including mitochondrial dysfunction, ATP depletion, abnormal metabolism and apoptosis (Halliwell and Aruoma, 1991; Raha and Robinson, 2000).

Antioxidant supplementation to the medium may reduce or inhibit the ROS generation, then suppress the ROS damage and improve the developmental capacity of the embryos (Olson and Seidel, 2000; Ali *et al*., 2003; Wongsrikeao *et al*., 2007). Various antioxidants, such as α-tocopherol (Olson and Seidel, 2000; Dalvit *et al*., 2005), L-ascorbic acid (Vit. C: Tatemoto *et al*., 2004; Dalvit *et al*., 2005), β-mercaptoethanol (β-ME: Nedambale *et al*., 2006), glutathione (GSH: Luvoni *et al*., 1996), and superoxide dismutase (SOD: Tatemoto *et al*., 2004) have been used to ROS scavengers in various species.

In the present study, we examined whether the antioxidant treatment during SCNT procedures can inhibit

* This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0007198) and Institute of Veterinary Science, Kangwon National University, Korea.

† Corresponding author : Phone: +82-33-250-8659, E-mail: htcheong@kangwon.ac.kr

the ROS generation in the cytoplasm of SCNT embryos. Three kinds of antioxidant, β-ME, Vit. C and GSH, were employed to examine the optimal concentration and treatment time of antioxidants.

MATERIALS AND METHODS

In Vitro Maturation of Oocytes

Bovine cumulus-oocyte complexes (COCs) were aspirated from the follicles of 2~7 mm in diameter using a 10 ml syringe with an 18-gauge needle. COCs were washed in Tyrode's lactate (TL)-Hepes containing 0.1% (w/v) polyvinyl alcohol (PVP; Sigma, St. Louis, MO, USA). They were then cultured with *in vitro* maturation (IVM) medium [TCM-199 (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% FBS (Fetal bovine serum, Gibco-BRL), 0.2 mM Na-pyruvate (Sigma), 50 μg/ml gentamicin (Sigma), 0.02 IU/ml FSH (Sigma), and 1 μg/ml 17β-estradiol (Sigma)] at 39℃, 5% CO_2 in air for 20~22 h.

Parthenogenesis

After IVM, cumulus cells of COCs were removed by vortexing in TCM-199 with 0.1% hyaluronidase, and metaphase II (MII) oocytes with a first polar body were selected for parthenogenesis. Parthenogenetic embryos were produced by exposing oocytes to the same electrical pulses and chemicals as those used for the reconstituted oocytes (see below). Activated oocytes were placed in the CR1aa (Rosenkrans and First, 1994) supplemented with 3 mg/ml BSA at 39℃, 5 % CO_2 in air for further 16 h.

Preparation of Donor Cells

Ear fibroblast from a Korean Native Cow (Hanwoo) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% FBS (Gibco-BRL) and 1% antibiotic (Sigma) at 39℃, 5% CO_2 in air, and passaged two times before freezing. Prior to SCNT, cells were thawed and resuspanded in DMEM supplemented with 10% FBS and 1% antibiotics, and cultured to confluence to synchronize the cell cycle at the G_0/G_1 phase. After then, cells were retrieved from the monolayer by trypsinization and transferred to TCM-199 medium (Gibco-BRL) containing 3 mg/ml BSA (Sigma) before the SCNT.

Nuclear Transfer

Nuclear transfer was carried out in a 50 μl drop of Hepes-buffered TCM-199 (Gibco-BRL) supplemented with 3 mg/ml BSA (Sigma) and 5 μg/ml cytochalasin B (CB; Sigma). A cumulus-free oocyte were cultured in TCM-199 containing 0.4 μg/ml demecolcine (Sigma) for 40 min to extrude their MII chromosome mass. Enucleation was performed by aspirating the MII chromosome mass and the first polar body using injection pipette. Then donor cell with a smooth surface was injected into the perivitelline space of an enucleated recipient oocyte. Prior to injection, the donor cells were cultured in TCM-199 medium (Gibco-BRL) containing 3 mg/ml BSA (Sigma) and 10 μg/ml phytohemagglutinin-P (PHA-P; Sigma) for 10 min.

Electrofusion and Activation

Reconstituted oocytes were places between 1 mm diameter wire electrodes in a fusion chamber overlaid with fusion medium (0.3 M mannitol, 0.05 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $MgCl_2 \cdot 6H_2O$, and 0.5 mM HEPES). A single direct current (DC) pulse of 1.25 kV/cm was charged for 30 μsec using a BTX Electro Cell Manipulator 200 (BTX, San Diego, CA, USA). After fusion treatment, the reconstituted oocytes were placed in TCM-199 supplemented with 3 mg/ml BSA for 30~40 min and checked for fusion. The fused embryos were further activated by 10 μM Ca-ionophore (A23187; Sigma) for 5 min and subsequently incubated in CR1aa containing 2 mM 6-dimethylaminopurine (DMAP; Sigma) for 3 h. After activation, SCNT embryos were cultured in the CR1aa supplemented with 3 mg/ml BSA at 39℃, 5% CO_2 in air for further 16 h.

Antioxidant Treatment

Three antioxidants such as β-ME, Vit. C and GSH were used for the experiments. At first, various concentrations of antioxidants (0, 25 and 50 μM β-ME, 0, 50 and 100 μM Vit. C, or 0, 50 and 100 μM GSH) were added to parthenogenetic activation medium during the whole parthenogenetic procedures of the oocytes to determine the optimal concentration of antioxidants. Next, determined concentrations of antioxidants were added to parthenogenetic activation medium to make the direct comparison among the antioxidants. Finally, a determined antioxidant of optimal concentration was added to IVM medium and/or medium for whole SCNT procedures from the enucleation to activation.

In Vitro Fertilization

After IVM, about ten COCs 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 0.03 mg/ml BSA (Sigma) at 39℃, 5% CO_2 in air for 6 h. After insemination, fertilized embryos were cultured in CR1aa supplemented with 3 mg/ml BSA for further 14 h.

Measurement of Intercellular ROS Content

The ROS levels of embryos were measured according to the method described previously (Hwang *et al.*, 2012). Briefly, parthenogenetic, SCNT and IVF embryos at 20 h of parthenogenetic activation, electrofusion or insemination, respectively, were stained in 10 μM 2'7'-dichlorodihydrofluorescein diacetate (H2DCFDA) dye for 30 min in the dark at 39℃ to measure the H_2O_2 levels. After incubation, the embryo were washed three times 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).

Statistical Analysis

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

RESULS

The H_2O_2 Levels of Parthenogenetic Embryos following Treatment with Various Concentrations of Antioxidants

The H_2O_2 level of parthenogenetic embryos was significantly lower in 25 μM β-ME treatment group (24.0±1.5 pixels/embryo, $p<0.05$) than those of 0 and 50 μM β-ME treatment groups (39.0±1.1 and 29.4±1.5 pixels/embryo, respectively, Fig. 1A). The H_2O_2 level in the 50 μM Vit. C treatment group (29.7±1.0 pixels/embryo, $p<0.05$) was significantly lower than that of 0 μM Vit. C treatment group (37.0±1.2 pixels/embryo, Fig. 1B). The 50 μM GSH treatment group showed significantly decreased H_2O_2 level (33.9±0.8 pixels/embryo, $p<0.05$) compared with the 0 and 100 μM GSH treatment group (37.9±1.1 and 36.3±0.8 pixels/embryo, respectively, Fig. 1C).

The H_2O_2 Levels of Parthenogenetic Embryos following Treatment with Different Antioxidants

As shown in Fig. 2, H_2O_2 levels were significantly lower in antioxidant treatment groups with 25 μM β-ME, 50 μM Vit. C and 50 μM GSH (33.8±0.8, 33.6±0.9, and 35.2±1.1 pixels/embryo, respectively) than that of control (39.1±1.4 pixels/embryo, $p<0.05$). However, there was no significant difference among the antioxidant treatment groups in direct comparison. We selected an antioxidant of 50 μM Vit. C for the SCNT experiment.

The H_2O_2 Levels of SCNT Embryos with Different Treatment Time of Antioxidant

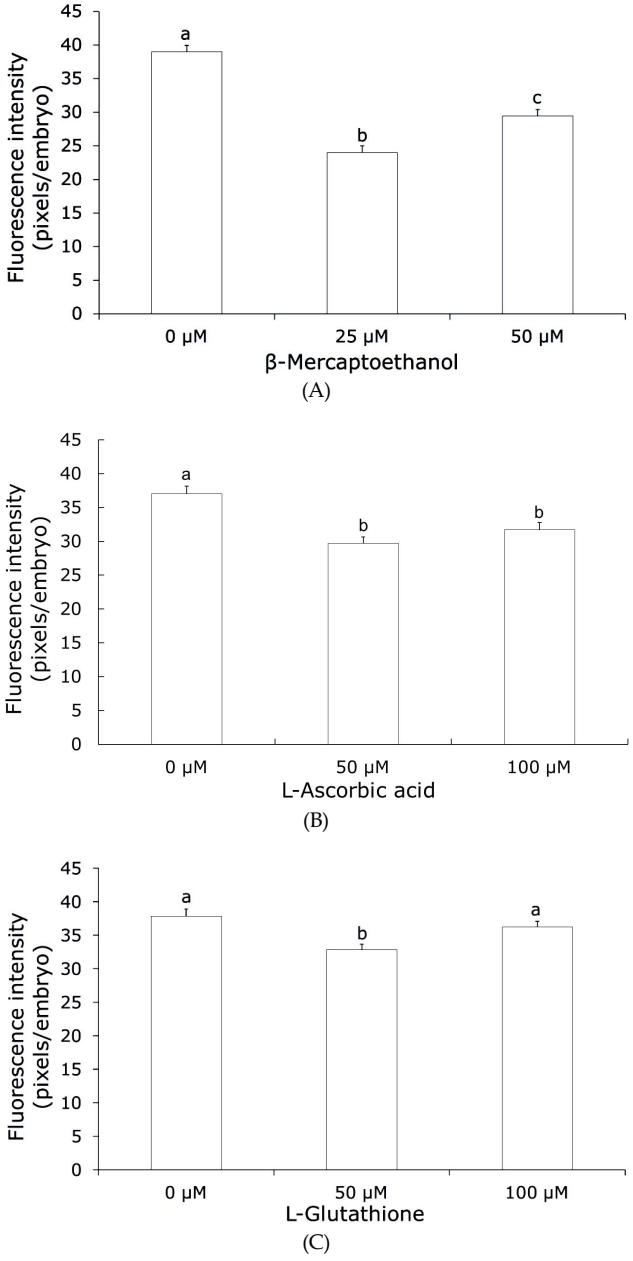

Fig. 1. H_2O_2 levels in bovine parthenogenetic embryos treated with different concentrations of various antioxidants. Five replicates were performed for each of the groups (total 90~100 embryos in each group). $^{a\sim c}$ Values with different letters differ significantly ($p<0.05$).

It was observed that the H_2O_2 generation level of bovine SCNT embryos was significantly lower in embryos treated with a 50 μM Vit. C during only SCNT procedure (26.4±1.1 pixels/embryo, $p<0.05$) than SCNT embryos that non-treated (34.3±1.0 pixels/embryo) and treated during only IVM (29.9±1.1 pixels/embryo). There was no synergistic effect with antioxidant treatment during the both IVM and SCNT procedures (27.8±1.1 pixels/embryo, Fig. 3).

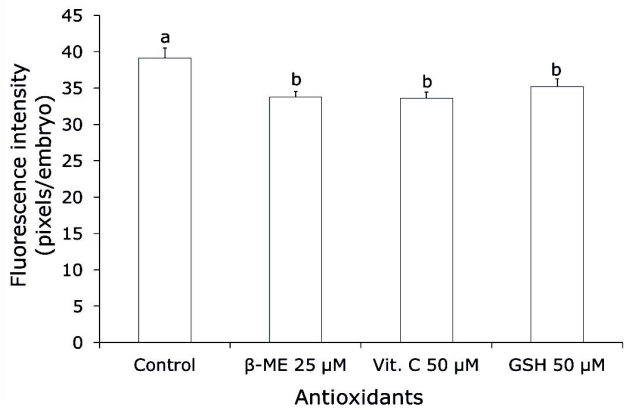

Fig. 2. H_2O_2 levels in bovine parthenogenetic embryos treated with different antioxidants. Five replicates were performed for each of the groups (total 90~100 embryos in each group). [a,b] Values with different letters differ significantly (p<0.05).

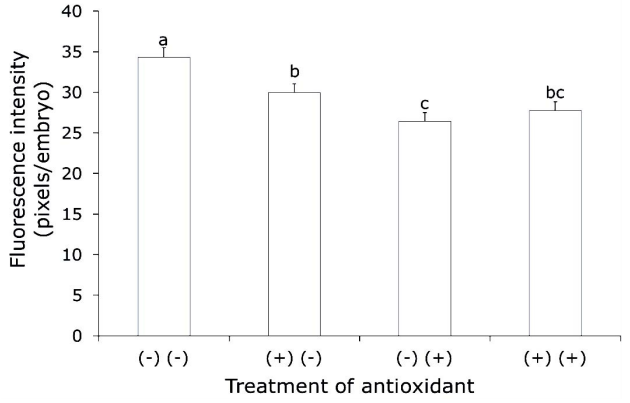

Fig. 3. H_2O_2 levels in bovine somatic cell nuclear transfer (SCNT) embryos treated with antioxidant (Vit. C) during *in vitro* maturation (IVM) and/or SCNT procedures. Ten replicates were performed for each of the groups (total 75~80 embryos in each group). [a~c] Values with different letters differ significantly (p<0.05). (−)(−), Control; (+)(−), Treated during IVM; (−)(+), Treated during SCNT; (+)(+), Treated during both IVM and SCNT.

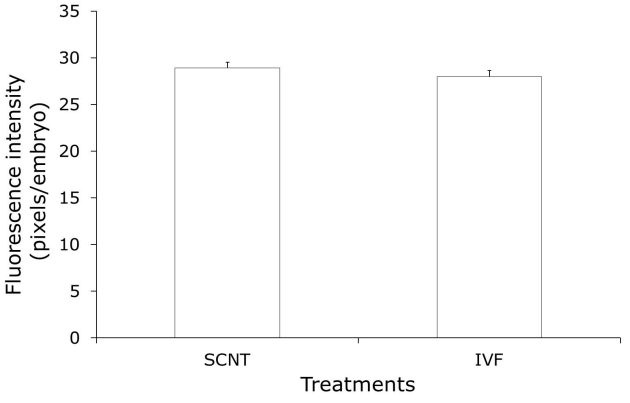

Fig. 4. H_2O_2 levels in *in vitro* fertilized (IVF) and antioxidant (Vit. C)-treated somatic cell nuclear transfer (SCNT) bovine embryos. SCNT embryos were treated with Vit. C during SCNT procedures. Seven replicates were performed for each of the groups (total 80~90 embryos in each group).

Comparison of H_2O_2 Levels between the SCNT Embryos Treated with Antioxidant and IVF Embryos

As shown in Fig. 4, the H_2O_2 level of SCNT embryos treated with 50 μM Vit. C during SCNT procedures was similar to that of IVF embryos (28.9±0.6 vs 28.0±0.7 pixels/embryo). There was no significant difference in the H_2O_2 level between the both groups.

DISCUSSION

ROS such as hydrogen peroxide, superoxide anions, and hydroxyl radicals, are produced in low quantities (approximately 2%) as by-products of normal aerobic metabolism. However, increase in the generation of ROS and/or a decrease in cellular antioxidant capacity can induce oxidative stress (Fridovich, 1999). According to Crosby *et al.* (1988), the high level of ROS in the cells can inhibit the mitochondrial respiration and damage the nuclear DNA and ion transporters of cell membrane, and inactivate various enzyme and cellular metabolism.

According to our previous findings, during the SCNT procedures including enucleation, cell injection, fusion and activation, an excessive ROS can generate by physical cellular stresses that were induced by oocyte manipulation (Hwang *et al*., 2012). Of course, normally produced ROS-induced oxidative stress will minimize the cellular damages as an intracellular antioxidant system is activated. However, the excessive generation of ROS by the physical or chemical impact increased the oxidative stress, which might beyond the protection capability of intracellular antioxidant system, then might cause the several dysfunctions such as chromosomal abnormalities and low developmental ability of embryos (Almeida and Bolton, 1998; Silva *et al*., 2007).

During *in vitro* culture, cells are exposed to higher concentrations of oxygen compared to *in vivo* environment, which causes the constant production of free radicals (Cordova *et al*., 2010). 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. The supplementation of antioxidants to culture medium, such as Vit. C, α-tocopherol, β-ME, catalase, GSH, and SOD enhanced the antioxidant systems within embryo and stimulate embryonic development (Nonogaki *et al*., 1991; Olson and Seidel, 2000; Orsi and Leese, 2001).

β-ME acts as an antioxidant by itself and indirectly by increasing intracellular GSH and cysteine activity. Nedambale *et al.* (2006) showed that the supplementation of β-ME, low molecular weight substances, into *in vitro* culture medium protects cell against oxidative

stress and promotes the amino acid transport and DNA synthesis, which enhanced the development of embryos and increase the viability of vitrified bovine blastocysts. An adequate dose of β-ME had a beneficial effect on the quality of sperm and normal pronuclear formation and blastocyst formation of IVF embryos (Gonçalves *et al*., 2010).

Vit. C is essential nutrient and well known as ROS scavengers *in vivo* and *in vitro* (Padayatty *et al*., 2003). The supplementation of Vit. C to culture medium improved mouse blastocyst production (Eppig *et al*., 2000). Also, the development capacity of IVM bovine oocytes was improved when oocytes were matured in the presence of Vit. C for the first 12 h of IVM (Cordova *et al*., 2010). Jeong *et al.* (2006) reported that the blastocyst formation rate and the blastocyst quality of embryos were improved when 100 μM Vit. C supplemented to culture medium.

GSH as a cysteine containing peptide plays a critical role in protecting the cell from oxidative stress (Lafleur *et al*., 1994). Also, GSH promotes the detoxification of toxic substances as a substrate for transferases and GSH peroxidase. In the present study, although ROS level was significantly reduced by the treatment of GSH, the effect of GSH was lower than other antioxidant treatments. Because it is already added 1 mM L-glutamine into the culture medium, addition of GSH is considered to have a little effect.

An excessively high concentration of antioxidant induced embryo apoptotic effects in an *in vitro* culture system by breaking of redox balance (Kitagawa *et al*., 2004). Thus, the selection of the optimum concentration of antioxidants is considered to be important for the embryo development. In this experiment, we determined the optimal concentration of antioxidants for oocytes through the parthenogenesis. Continuously, we selected the 50 μM Vit. C for the SCNT experiment by direct comparison among the antioxidants based on the H_2O_2 level of parthenogenetic embryos.

You *et al*. (2010) reported that anthocyanin treatment during IVM improved developmental competence of SCNT embryos. In this study, the treatment of antioxidant during IVM or both IVM and SCNT procedures were found to inhibit ROS generation. However, most significant inhibition of H_2O_2 levels was found when antioxidant was added during only SCNT procedures. Based on this result, the supplementation of antioxidant, 50 μM Vit. C, during the SCNT procedures reduced the micromanipulation-induced physical stress and reduced the H_2O_2 generation level up to similar level of IVF embryos.

In conclusion, the results of this study shows that the treatment of 50 μM Vit. C during SCNT procedures can reduce the ROS generation level of SCNT bovine embryos. Thus, appropriate antioxidant treatment during SCNT procedures can enhance the reprogramming of the SCNT bovine embryos and improve the efficiency of SCNT.

REFERENCES

1. Ali AA, Bilodeau JF, Sirard MA (2003): Antioxidant requirements for bovine oocytes varies during *in vitro* maturation, fertilization and development. Theriogenology 59:939-949.
2. Almeida PA, Bolton VN (1998): Cytogenetic analysis of human preimplantation embryos following developmental arrest *in vitro*. Reprod Fertil Dev 10: 505-513.
3. Brackett BG, Oliphant G (1975): Capacitation of rabbit spermatozoa *in vitro*. Biol Reprod 12:260-274.
4. Choi JY, Kim CI, Park CK, Yang BK, Cheong HT (2004): Effect of activation time on the nuclear remodeling and *in vitro* development of nuclear transfer embryos derived from bovine somatic cells. Mol Reprod Dev 69:289-295.
5. Cordova B, Morato R, Izquierdo D, Paramio T, Mogas T (2010): Effect of the addition of insulin-transferrin-selenium and/or L-ascorbic acid to the *in vitro* maturation of prepubertal bovine oocytes on cytoplasmic maturation and embryo development. Theriogenology 74:1341-1348.
6. Crosby IM, Gandolfi F, Moor RM (1988): Control of protein synthesis during early cleavage of sheep embryos. J Reprod Fertil 82:769-775.
7. Dalvit G, Llanes SP, Descalzo A, Insani M, Beconi M, Cetica P (2005): Effect of alpha-tocopherol and ascorbic acid on bovine oocyte *in vitro* maturation. Reprod Domest Anim 40:93-97.
8. Eppig JJ, Hosoe M, O'Brien MJ, Pendola FM, Requena A, Watanabe S (2000): Conditions that affect acquisition of developmental competence by mouse oocytes *in vitro*: FSH, insulin, glucose and ascorbic acid. Mol Cell Endocrinol 163:109-116.
9. Fridovich I (1999): Fundamental aspects of reactive oxygen species, or what's the matter with oxygen?. Ann NY Acad Sci 893:13-18.
10. Garry FB, Adams R, McCann JP, Odde KG (1996): Postnatal characteristics of calves produced by nuclear transfer cloning. Theriogenology 45:141-152.
11. Gonçalves FS, Barretto LS, Arruda RP, Perri SH, Mingoti GZ (2010): Effect of antioxidants during bovine *in vitro* fertilization procedures on spermatozoa and embryo development. Reprod Domest Anim 45:129-135.
12. Halliwell B, Aruoma OI (1991): DNA damage by oxygen derived species. Its mechanism and measurement in mammalian systems. FEBS Lett 281:9-19.
13. Hwang IS, Bae HK, Park CK, Yang BK, Cheong HT (2012): Generation of reactive oxygen species in bo-

vine somatic cells nuclear transfer embryos during micromanipulation procedures. Reprod Dev Biol 36: 49-53.
14. Inoue K, Kohda T, Lee J, Ogonuki N, Mochida K, Noguchi Y, Tanemura K, Kaneko-Ishino T, Ishino F, Ogura A (2002): Faithful expression of imprinted genes in cloned mice. Science 295:297.
15. Jeong YW, Park SW, Hossein MS, Kim S, Kim JH, Lee SH, Kang SK, Lee BC, Hwang WS (2006): Antiapoptotic and embryotrophic effects of alpha-tocopherol and L-ascorbic acid on porcine embryos derived from *in vitro* fertilization and somatic cell nuclear transfer. Theriogenology 66:2104-2112.
16. Kang YK, Koo DB, Park JS, Choi YH, Chung AS, Lee KK, Han YM (2001): Aberrant methylation of donor genome in cloned bovine embryos. Nat Genet 28: 173-177.
17. Kitagawa Y, Suzuki K, Yoneda A, Watanabe T (2004): Effects of oxygen concentration and antioxidants on the *in vitro* developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. Theriogenology 62:1186-1197.
18. Lafleur MV, Hoorweg JJ, Joenje H, Westmijze EJ, Retel J (1994): The ambivalent role of glutathione in the protection of DNA against singlet oxygen. Free Radic Res 21:9-17.
19. Luvoni GC, Keskintepe L, Brackett BG (1996): Improvement in bovine embryo production *in vitro* by glutathione-containing culture media. Mol Reprod Dev 43:437-443.
20. Nedambale TL, Du F, Yang X, Tian XC (2006): Higher survival rate of vitrified and thawed *in vitro* produced bovine blastocysts following culture in defined medium supplemented with beta-mercaptoethanol. Anim Reprod Sci 93:61-75.
21. Nonogaki T, Noda Y, Narimoto K, Umaoka Y, Mori T (1991): Protection from oxidative stress by thioredoxin and superoxide dismutase of mouse embryos fertilized *in vitro*. Hum Reprod 6:1305-1310.
22. Olson SE, Seidel GE Jr (2000): Culture of *in vitro*-produced bovine embryos with vitamin E improves development *in vitro* and after transfer to recipients. Biol Reprod 62:248-252.
23. Orsi NM, Leese HJ (2001): Protection against reactive oxygen species during mouse preimplantation embryo development: role of EDTA, oxygen tension, catalase, superoxide dismutase and pyruvate. Mol Reprod Dev 59:44-53.
24. Padayatty SJ, Katz A, Wang Y, Eck P, Kwon O, Lee JH, Chen S, Corpe C, Dutta A, Dutta SK, Levine M (2003): Vitamin C as an antioxidant: evaluation of its role in disease prevention. J Am Coll Nutr 22:18-35.
25. Raha S, Robinson BH (2000): Mitochondria, oxygen free radicals, disease and ageing. Trends Biochem Sci 25:502-508.
26. Rosenkrans CF Jr, First NL (1994): Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes *in vitro*. J Anim Sci 72:434-437.
27. Silva PF, Gadella BM, Colenbrander B, Roelen BA (2007): Exposure of bovine sperm to pro-oxidants impairs the developmental competence of the embryo after the first cleavage. Theriogenology 67:609-619.
28. Tatemoto H, Muto N, Sunagawa I, Shinjo A, Nakada T (2004): Protection of porcine oocytes against cell damage caused by oxidative stress during *in vitro* maturation: role of superoxide dismutase activity in porcine follicular fluid. Biol Reprod 71:1150-1157.
29. Wongsrikeao P, Nagai T, Agung B, Taniguchi M, Kunishi M, Suto S, Otoi T (2007): Improvement of transgenic cloning efficiencies by culturing recipient oocytes and donor cells with antioxidant vitamins in cattle. Mol Reprod Dev 74:694-702.
30. You JY, Kim JY, Lim JM, Lee ES (2010): Anthocyanin stimulates *in vitro* development of cloned pig embryos by increasing the intracellular glutathione level and inhibiting reactive oxygen species. Theriogenology 74:777-785.

(Received: 8 June 2012/ Accepted: 26 June 2012)