Developmental Ability of Bovine Embryos Nuclear Transferred
with Frozen-thawed or Cooled Donor Cells*

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ABSTRACT : This study was designed to investigate the *in vitro* developmental ability and apoptosis of bovine embryos nuclear-transferred (NT) with frozen-thawed or cooled donor cells. Cultured adult bovine ear cells were used as donor cells after sub-culturing to confluence (CC), cooling to 4°C for 48 h, or freezing-thawing (FT). Apoptotic cells in blastocysts were evaluated for apoptosis by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) method. Fusion, cleavage and blastocyst rates were 69.0 (167/242), 68.8 (115/167), and 29.9 (50/167) with CC cells, 70.4 (88/125), 69.3 (61/88), and 29.6 (26/88) with cooled cells and 66.1 (117/177), 70.1 (82/117), and 13.7 (16/117) with FT cells, respectively. Blastocyst rates of NT embryos derived from FT cells were significantly lower than those from CC or cooled cells ($p<0.05$). In addition, NT blastocysts produced by using FT cells showed significantly higher apoptosis rates (6.4±4.0%) than those produced by CC (2.8±1.7%) or cooled (2.3±1.3%) cells. However, cooling of donor cells had no significant adverse effect on blastocyst rate as well as apoptosis rate. Therefore, our results suggest that cooled cells may be used as an alternative to freshly cultured confluent culture cells, as donor cells, for the production of Somatic nuclear cloned cattle. *(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 9 : 1242-1248)*

Key Words : Somatic Cell Nuclear Transfer, Frozen-thawed, Cooled, Apoptosis, Bovine

INTRODUCTION

Somatic cell nuclear Transfer (SCNT) technology is a powerful tool for production of cloned animals and has become routine in many laboratories. However, the success rate of SCNT is highly variable and is influenced by number of factors including preparation of donor cells. Nearly all cell types undergo some form of tissue culture and/or cryopreservation prior to their use. G0/G1 phase of cell cycle which is reported to be optimal phase of donor cells for use in SCNT (Campbell et al., 1996; Kato et al., 1998; Wells et al., 1999) is generally achieved either by several days of serum starvation or culturing them till confluence which synchronizes the random cell cycle phase of somatic cells under culture to G0/G1 phase. Similarly, frozen-thawed donor cells have been used for SCNT without additional *in vitro* culture after thawing (Tani et al., 2000; Lai et al., 2001). Dong et al. (2004) used frozen-thawed fetal skin derived somatic cells for SCNT and reported the production of cloned calves. Cooling of confluence-cultured somatic cells in refrigerated condition has also been reported for preparation of donor cells for bovine somatic cell nuclear transfer (Liu et al., 2001; Adams et al., 2004; Arat et al., 2004). These cell culture/

cryopreservation techniques, which are very similar across published reports, are considered to be relatively benign but may have profound effects on final outcome of the cloning attempts.

Apoptosis is a normal feature of *in vitro* as well as *in vivo* embryos at early developmental stages (Hardy, 1997) and plays a role in regulating the cell number of embryos and maintaining the cellular quality in the inner cell mass lineage by eliminating damaged cells or those expressing inappropriate phenotypes or developmental potential (Handyside and Hunter, 1986; Parchment, 1993; Brison, 2000). However, though the elimination of unwanted cells is essential in embryonic development, apoptosis also has the potential to eliminate normal cells leading to the death of embryo. Therefore, the number of cells and the amount of apoptosis in embryos are important parameters that are emerging as useful indicators of embryonic development and quality (Brison et al., 1997, 1998). Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining is a useful technique to visualize the apoptotic cells and has been used efficiently to confirm cell death in bovine blastocysts.

The present study was designed to examine the cloning efficiency of bovine ear cell derived donor cells that are cultured to confluence and used either fresh or after cooling to 4°C for 48 h or after freezing-thawing at the quiescent state. Processed donor cells were used for NT immediately after thawing without additional *in vitro* culture and *in vitro* produced bovine blastocysts were evaluated based on their cell number and DNA fragmentation.

MATERIALS AND METHODS

All chemicals were purchased from Sigma, St. Louis, MO, USA unless otherwise specified and were embryo tested.

In vitro maturation of bovine oocytes

Bovine ovaries were collected from a slaughterhouse and transported to the laboratory in saline at 37°C. Cumulus-oocyte complexes (COCs) were recovered from antral follicles of 2 to 8 mm in diameter using 10 ml-syringe fitted with an 18-gauge hypodermic needle. The COCs with evenly granulated cytoplasm and compact cumulus cells of more than three layers were selected and washed thrice in HEPES buffered Tyrode's Lactate medium (TL-HEPES; Parrish et al., 1985) supplemented with 1 mg/ml bovine serum albumin (BSA- Fraction V). Selected COCs were matured in groups of ten in 50 μl droplets of maturation medium: TCM-199 (Gibco BRL, Grand Island, NY, USA) supplemented with 25 mM sodium bicarbonate, 10% (v/v) fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), 0.2 mM sodium pyruvate, 1 μg/ml follicle stimulating hormone (Folltropin V; Veterpharm, Belleville, Ontario, Canada), 25 μg/ml gentamicin sulfate and 1 μg/ml estradiol-17β under warm paraffin oil at 39°C and humidified atmosphere of 5% CO_2 in air.

Parthenogenetic activation of bovine oocytes

Matured COCs were treated with 0.1% hyaluronidase to remove cumulus cells by repeated pipetting for 2 to 3 min and washed in TL-HEPES medium supplemented with 0.3% BSA (w/v). Activation of oocytes was then achieved by exposing them to 5 μM ionomycin in CR1aa medium for five minutes and subsequently to 2 mM 6-dimethyl aminopurine (6-DMAP) in CR1aa medium for 3 h. These activated oocytes were washed thrice in CR1aa medium supplemented with 0.3% BSA (CR1-BSA) and cultured in 50 μl droplets of CR1-BSA medium under paraffin oil at 39°C and humidified atmosphere of 5% CO_2 in air.

In vitro fertilization (IVF) of bovine oocytes

Matured COCs were washed thrice each with TL-HEPES medium and fertilization-Tyrode's Albumin Lactate Pyruvate medium (Fert-TALP; Rosenkrans et al., 1993) and transferred into 44 μl droplets of Fert-TALP preincubated under paraffin oil. Motile spermatozoa were collected from frozen-thawed semen by discontinuous percoll density gradient and adjusted to get a final concentration 2 million sperms/ml. Heparin (2 μg/ml) and PHE stock solution (2 mM Penicillamine, 20 μM hypotaurine and 1 μM epinephrine) were then added, each 2 μl, to each Fert-TALP droplets as capacitating agent and sperm motility enhancer respectively and sperm-oocytes co-incubation was done at

39°C and humidified atmosphere of 5% CO_2 in air for 48 h.

Preparation of donor cells

Primary bovine ear cells were isolated from adult bovine ear by digestion with 0.05% trypsin Ethylene diamine tetra acetic acid (trypsin-EDTA; Gibco BRL, Grand Island, NY, USA) for 2 h at 37°C in air and cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% (v/v) FBS till confluence at 37°C and humidified atmosphere of 5% CO_2 in air. After attainment of confluence, cells were sub-passaged and passage four ear cells were trypsinized and washed with fresh Ca^{++}/Mg^{++} free phosphate buffered saline (PBS; Gibco BRL, Grand Island, NY, USA) for use as frozen-thawed (FT) and cooled cells. To use as FT donor cells, the cells were pelleted and resuspended in DMEM supplemented with 20% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen as frozen aliquots. At the time of use, frozen aliquots were thawed and used immediately as donor cells for SCNT without additional *in vitro* culture. To use as cooled donor cell, pelleted cells were resuspended in DMEM supplemented with 10% (v/v) FBS and stored at 4°C for 48 h before use. Cultured to confluent cells (CC) were used for nuclear transfer between passages two and eight of culture.

Oocyte enucleation and donor cell microinjection

Enucleation of recipient oocytes and microinjection of donor cells were performed as described by Collas and Barnes (1994) with minor modifications. Briefly, oocytes were stripped off cumulus cells by treatment with 0.1% (w/v) hyaluronidase and enucleated by aspirating first polar body and MII plate in a small volume of surrounding cytoplasm using beveled glass pipette (25 μm in diameter) in CR1aa medium supplemented with 0.3% (w/v) BSA and 7.5 μg/ml cytochalasin B (CB). Enucleation was confirmed by staining the aspirated portion of cytoplasm with 5 μg/ml Hoechst 33342. Enucleated oocytes were then incubated in modified CR1aa medium supplemented with 0.3% (w/v) BSA till injection of donor cell. One hour after enucleation, a separate 10 to 15 μm pipette (inner diameter) containing the donor cell was introduced through the slit on the zona pellucida formed during enucleation and the donor cell was expelled into perivitelline space. Round shaped, translucent, small sized cells with no detectable abnormality in plasma membrane, when viewed under inverted microscope, were only used for microinjection. Close contact of the donor cell membrane with the plasma membrane of oocyte was visually confirmed prior to fusion.

Fusion and activation of nuclear transferred couplets

Karyoplast-cytoplast couplets were washed in fusion solution, composed of 0.3 mM mannitol, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$ and manually aligned between two

Table 1. Development competence of bovine embryos produced by parthenogenetic activation, *in vitro* fertilization (IVF) and nuclear transfer (NT)

Treatments	No. of oocytes	No. (%) of oocytes fused	No. (%) of embryos		Total cell number (Mean±SD)
			Cleaved	Blastocysts	
Activation	270	-	197 (73.0)	65 (33.0)	101.4±6.8[b]
IVF	220	-	177 (80.5)	62 (35.0)	126.7±20.4[a]
NT	250	161 (64.4)	123 (76.4)	49 (30.4)	111.8±6.3[b]

[a, b] Values within column with different superscripts differ significantly ($p < 0.05$).

Table 2. Apoptosis rates of bovine embryos produced by parthenogenetic activation, *in vitro* fertilization (IVF) and nuclear transfer (NT)

Treatments	No. of blastocysts examined	Total cell number (Mean±SD)	Apoptotic cell /cell ratio
Activation	10	97.0±4.5[b]	3.4±2.8
IVF	15	123.9±14.7[ab]	2.9±3.9
NT	8	110.8±8.1[a]	3.2±1.2

[a, b] Values within column with different superscripts differ significantly ($p < 0.05$).

stainless steel electrodes (1 mm apart) of fusion chamber filled with fusion solution. Fusion was induced by a double DC pulse (2.1 KV/cm for 30 µsec) delivered by BTX Electro cell Manipulator 200 (BTX, Gentronics, San Diego, CA, USA). Fusion of the couplets was evaluated after 30 minutes of incubation in CR1aa medium supplemented with 0.3% (w/v) BSA and 10 µg/ml lectin and then they were activated by 5 minutes exposure to 5 µM ionomycin and 3 h incubation in 2 mM 6-DMAP in CR1aa medium supplemented with 0.3% (w/v) BSA. Activated embryos were cultured in 50 µl droplets of CR1aa medium supplemented with 0.3% (w/v) BSA for first two days followed by subsequent 6-day culture in CR1aa medium supplemented with 10% (v/v) FBS in experiment 1 and 2, and with estrus cow serum (ECS) in experiment 3.

TUNEL assay

TUNEL assay kit (*In situ* Cell Death Detection Kit, Roche Diagnostic Corp., Indianapolis, IN, USA) was used to visualize the apoptotic cells in embryos. Blastocysts were fixed in 4% (w/v) paraformaldehyde in PBS, pH 7.4, for 30 minutes at room temperature and permeabilized in PBS with 1.0% (v/v) Triton X-100 and 0.1% sodium citrate solution for 30 minutes. Broken ends of DNA in dead cells were then labeled with TdT and fluorescein-dUTP for 60 minutes at 39°C and counter-staining of blastocyst was done with 5 µg/ml Hoechst 33342 to visualize the total cells. Stained embryos were then mounted in universal mount (2130 Memorial Pkwy SW, Huntsville, AL, USA) and stored in the dark at 4°C until analysis.

Experimental design

In experiment 1, the development competence and apoptosis rate of bovine embryos produced by *in vitro*

fertilization (IVF), parthenogenetic activation and SCNT was examined and compared. In experiment 2, influence of BSA and estrous cow serum (ECS) supplementation to *in vitro* culture (IVC) medium on developmental competence and apoptosis rate NT embryos was investigated to determine the optimal IVC media for NT embryos. In experiment 3, effect of donor cell processing on developmental competence and apoptosis of NT blastocyst was analyzed. Confluence cultured bovine ear cells were used as donor cells either fresh or after cooling to 4°C for 48 h or after freeze-thawing. Based on the result of experiment 2, NT blastocysts were cultured in IVC media supplemented with ECS from third day onwards.

Statistical analysis

Data from at least three replications were pooled and significant difference among treatment groups in each experiment was determined by the ANOVA.

RESULTS

Development of bovine embryos produced by activation, IVF and NT

As shown in Table 1, fusion rate of reconstructed embryos was 64.4% while the cleavage rate of embryos derived from activation, IVF and NT were 73.0, 80.5 and 76.4% respectively. The rates of development to blastocyst stage were 33.0 (activation), 35.0 (IVF) and 30.4% (NT). The total cell number of blastocysts were 101.4±6.8 (activation), 126.7±20.4 (IVF) and 111.8±6.3 (NT). There was no significant difference between cleavage rate and development rate to blastocyst stage among the three groups. However, total cell number of blastocyst in IVF group was significantly higher than those in other two groups ($p < 0.05$), but did not differ significantly between activation and NT groups.

Apoptosis in bovine embryos produced by activation, IVF and NT

Day-8 blastocysts produced by activation, IVF and NT were analyzed for DNA fragmentation by TUNEL assay, and total number of cells was compared with apoptotic cells (Figure 1). As shown in Table 2, total cell number of blastocysts were 97.0±4.5 (activation), 123.9±14.7 (IVF) and 110.8±8.1 (NT), while the apoptotic cells per embryo
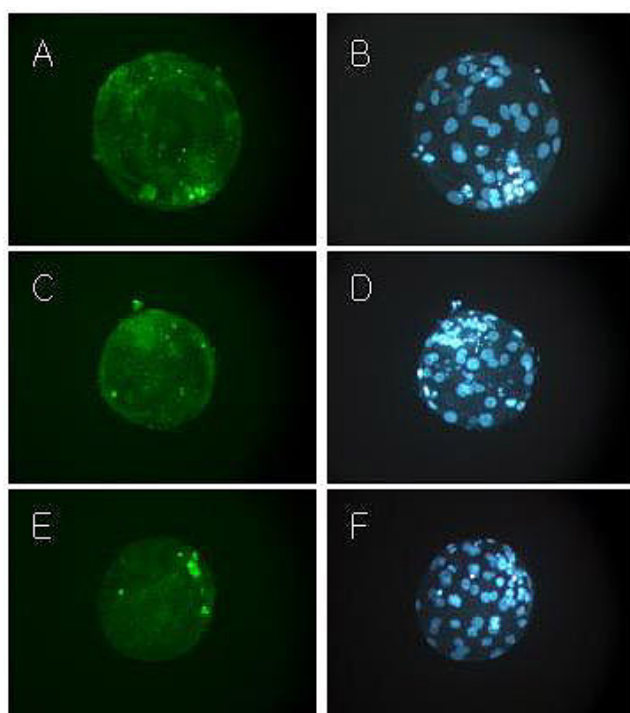
Figure 1. TUNEL (green; A, C and E) and Hoechst 33342 (blue; B, D and F) staining for detection of apoptosis and cell nuclei, respectively, of bovine blastocysts produced by activation (A and B), IVF (C and D) and NT (E and F). Magnification×200.

were 3.4±2.8 (activation), 2.9±3.9 (IVF) and 3.2±1.2 (NT). There was no significant difference in apoptosis rate among three groups, although there was a difference in their total cell number. DNA fragmentation, a hallmark of apoptosis, was noticed mainly in presumptive ICM cells and only to a lesser degree in trophectoderm in all the groups.

Effect of BSA or ECS supplementation to IVC medium on developmental of bovine NT embryos

NT embryos were cultured in CR1aa medium supplemented either with BSA or ECS and analyzed for their development rate and apoptosis. As shown in Table 3, cleavage rates were 75.5 and 73.8 while blastocyst rate were 13.7 and 31.1% in BSA and ECS groups, respectively. Total cell numbers of blastocysts were 101.9±11.8 (BSA) and 121.2±13 (ECS). There was no significant difference between the two groups for cleavage rate. However, blastocyst rate and total cell number were significantly higher in ECS group than BSA group ($p<0.05$).

Table 3. Development competence of bovine nuclear transferred (NT) embryos cultured in the presence of BSA or estrous cow serum (ECS)

Treatments*	No. of oocytes fused	No. (%) of embryos cleaved**	No. (%) of blastocysts	Total cell number (Mean±SD)
BSA	139	105 (75.5)	19 (13.7)[b]	101.9±11.8[b]
ECS	183	135 (73.8)	57 (31.1)[a]	121.2±13.4[a]

* Embryos treated at 3-day after NT.
** Cleavage rates at 3-day after NT.
[a, b] Values within column with different superscripts differ significantly ($p<0.05$).

Table 4. Apoptosis rate of bovine nuclear transferred (NT) embryos cultured in the presence of BSA or estrous cow serum (ECS)

Treatment	No. of blastocysts examined	Total cell number (Mean±SD)	Apoptotic cell/ cell ratio
BSA	11	100.8±10.1[b]	3.5±1.3[a]
ECS	16	117.2±11.5[a]	2.6±1.5[a]

[a, b] Values within column with different superscripts differ significantly ($p<0.05$).

TUNEL assay of NT blastocysts showed that the number of apoptotic cells per blastocyst and total cell number of blastocysts were 3.5±1.3 and 100.8±10.1 in BSA group and 2.6±1.5 and 117.2±11.5 in ECS group, respectively (Table 4). The total cell number of blastocysts was significantly higher in ECS group ($p<0.05$) but there was no significant difference between the two groups for apoptosis rate.

In vitro development of bovine NT embryos using confluent cultured (CC), frozen-thawed (FT) or cooled ear cells

As shown in Table 5, fusion rate of oocytes fused with CC, FT or cooled ear cells were 69.0, 66.1 and 70.4%, respectively, while the cleavage rates were 68.8, 70.1 and 69.3%, respectively. The developmental rates of embryos to blastocyst stage were 29.9 (CC), 13.7 (FT) and 29.6% (cooled). The total cell number of NT blastocysts were 120.9±16.7 (CC), 115.3±13.2 (FT) and 114.5±9.2 (cooled). The fusion and cleaved rate did not differ significantly among the three groups. However, development of embryos to 8- to 16- cell stage in CC group was significantly higher than other two groups. Similarly development to the blastocyst stage was significantly different among all three

Table 5. *In vitro* development of bovine embryos nuclear transferred with confluence culture (CC), frozen-thawed (FT) or cooled ear cells

Treatments	No. of oocytes	No. (%) of oocytes fused	No. (%) of embryos			Total cell number (Mean±SD)
			Cleaved	8- to 16-cell	Blastocysts	
CC cells	242	167 (69.0)	115 (68.8)	75 (44.9)[a]	50 (29.9)[a]	120.9±16.7
FT cells	177	117 (66.1)	82 (70.1)	36 (30.7)[b]	16 (13.7)[b]	115.3±13.2
Cooled cells	125	88 (70.4)	61 (69.3)	39 (44.3)[a]	26 (29.6)[ab]	114.5±9.2

[a, b] Values within column with different superscripts differ significantly ($p<0.05$).

Table 6. Apoptosis in bovine embryos nuclear transferred with confluence culture (CC), frozen-thawed (FT) or cooled ear cells

Treatments	No. of blastocysts examined	Total cell number (Mean±SD)	Apoptotic cell /cell ratio
CC	18	112.9±14.3	2.8±1.7[b]
FT	8	116.5±6.6	6.4±1.9[a]
Cooled	12	114.4±9.7	2.3±1.3[b]

[a, b] Values within column with different superscripts differ significantly (p<0.05).

groups (p<0.05) with CC cells group showing highest blastocyst rate and FT cells group showing the lowest. Total cell number of NT blastocysts did not differ significantly among the three groups.

Apoptosis in NT blastocysts produced using CC, FT or cooled ear cells

NT blastocysts obtained by using CC, FT or cooled ear cells as donor cells were analyzed for apoptosis rate by TUNEL staining (Table 6). These blastocysts showed total cell number of 112.9±14.3 (CC), 116.5±6.6 (FT) and 114.4±9.7 (cooled) and apoptosis rate of 2.8±1.7 (CC), 6.4±1.9 (FT) and 2.3±1.3 (cooled). There was no significant difference among the three groups in total cell number. However, blastocysts produced by using FT cells showed the significantly higher apoptosis rate than others (p<0.05), whereas blastocysts from other two groups did not differ in their apoptosis rate. Figure 2 showed the frequency of total number of cells compared with apoptotic cells in NT embryos produced with CC, FT or cooled cells as donor cells.

DISCUSSION

The present study was designed to evaluate the development potential, blastocyst cell number and apoptosis ratio in NT embryos produced by using donor cells prepared by three different processing viz. freeze-thawing at -196°C in LN_2 (FT), cooled at 4°C for a period of 48 h or confluence cultured (CC). Our results suggest that there is no significant adverse effect of donor cell processing on fusion rate, cleavage rate and embryo quality in terms of cell number. However, cryopreservation of the donor cells in LN_2 has significant detrimental effect on the development potential to blastocyst stage as well as apoptosis ratio while somatic cells cooled at 4°C for 48 h has same developmental efficiency as that of confluent cultured cells.

The cause of the disproportion in development rate to blastocyst observed in the present study may be ascribed to cryo-injury (Kato and Tsunoda, 1996) and/or to their effect on the cell cycle stage of the donor cell (Tani et al., 2000). Low temperature preservation of donor cells at 4°C caused
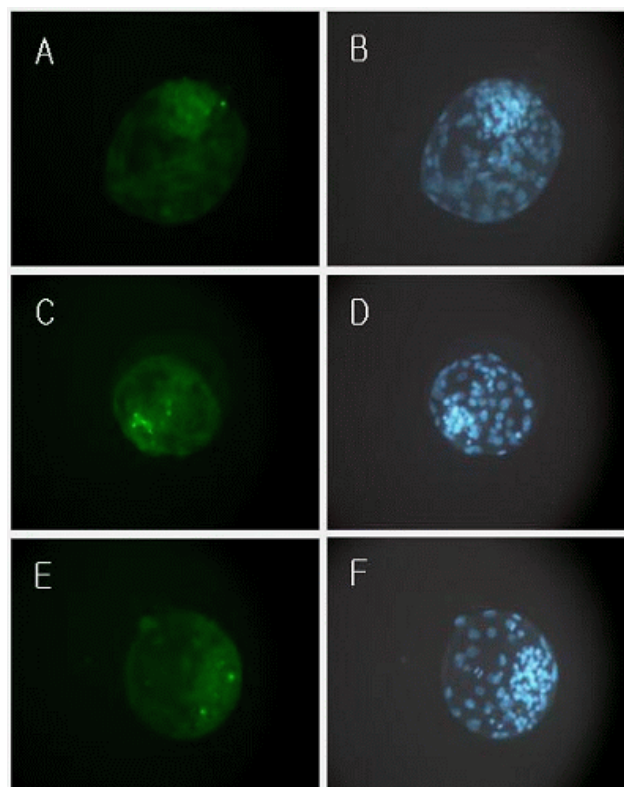

Figure 2. TUNEL (green; A, C and E) and Hoechst 33342 (blue; B, D and F) staining for detection of apoptosis and cell nuclei, respectively, of bovine NT blastocysts produced by using confluent cultured (A and B), frozen-thawed (C and D) or cooled (E and F) bovine ear cells (Magnification×200).

the cell membrane damage to the extent of 60% but the blastocyst rate did not differ from that of controls when stored donor cells were used within two days (Kato and Tsunoda, 1996). However, the significantly lower rate of blastocyst formation was obtained due to the degeneration of plasma membrane during micromanipulation when donor cells were stored for longer duration. On the contrary, Liu et al. (2001) reported the beneficial effect of refrigeration of donor cells at 4°C for 1 to 2 weeks in eliminating the need of confluence culturing or serum starvation for inducing quiescence. This is in concurrence with our findings in Table 5. However, cooled cell derived NT embryos showed the higher rate of developmental block at 8- to 16- cell stage, the stage at which the maternal zygotic transition (MZT) occur in cattle (Kopecny et al., 1989). Thus, in the light of report from Memili and First (1998) that transcription of embryonic genes during the first 4-cell cycles is essential for embryo development beyond 9- to 16- cell stage, it is plausible to hypothesize that cooling may not only have caused damage to plasma membrane of the donor cells but also to other cytoplasmic/nuclear structure. When such cells with intact plasma membrane but damaged nuclear status is selected by chance, owing to morphology and cell size criteria of donor cell selection used in the present study,

such reconstructed embryos probably failed to cross the MZT stage but showed normal early cleavage which was under the control of ooplasmic factors (Dominko et al., 1999). No previous evidences, however, have documented the developmental potential, blastocyst cell number and occurrence of apoptosis in NT embryos produced by thawed somatic cells frozen in LN_2 or cooled at 4°C for a period of 48 h.

Apoptosis is a widespread feature in embryos both *in vivo* and *in vitro* (Nurber et al., 2002), but is detected in standard *in vitro* culture only in advanced stage embryos and not during early cleavages indicating that it is dependent on the developmental stage of the embryo (Matwee et al., 2000). This process ensures the regulation of cell populations and cell lineages in all major mammalian systems, and has many specialized functions during development (Byrne et al., 1999). A negative correlation between embryo cell number and apoptosis rate has been reported (Fahrudin et al., 2002). Therefore, in the present study, analysis of cell number by Hoechst 33342 staining and apoptosis by TUNEL assay was performed at the blastocyst stage. No difference in the cell number was observed among all the groups. However, apoptosis rate was significantly higher in FT group suggesting that relatively more number of cells in FT derived embryos were damaged or had inappropriate phenotypes or developmental potential (Handyside and Hunter, 1986; Parchment, 1993; Hardy, 1997; Brison, 2000).

Furthermore, apoptosis was seen predominantly within the ICM in all experimental groups. This observation may suggest for the aberrant trophectoderm: ICM ratio that has been implicated as a contributory factor in the etiology of the large offspring syndrome (LOS) observed in cloned animals (Thompson et al., 1995; Han et al., 2003). Similar observation of predominant apoptosis in ICM has been reported in preimplantation bovine embryos (Byrne et al., 1999) but not in mouse (Jurisicova et al., 1998) and human (Hardy, 1997). This coupled with the observation of the present study that no difference in cell number noticed in all the three group utilizing three different donor cell processing imply that *in vitro* culture conditions (Brison and Schultz, 1997; Devreker and Hardy, 1997; Kamjoo et al., 2002) or SCNT protocol and not the donor cell processing is contributory factor for higher apoptosis in ICM.

Apoptosis is often viewed as an adaptive mechanism of embryos to allow embryonic survival and development following stress (Paula-Lopes and Hansen, 2002). Analysis of cell number and apoptosis ratio is thus also related to the embryo quality, as embryos with large number of cells and less apoptosis ratio are more likely to implant and give rise to live offspring (Van Soom et al., 1997). In the present study, since the cell number and apoptosis ratio was similar in cooled cell and CC cells groups, it suggests that cooling of donor cells to 4°C for 48 h do not affect the embryo quality, and those embryos which reached to the blastocyst stage could possibly develop to offspring successfully.

Therefore, results of this study suggest that bovine ear cells cooled at 4°C for 48 h could be used successfully as donor cell for production of cloned animals by SCNT without compromising the viability, developmental ability and quality of embryos. However, in instances wherein use of CC or cooled cells seems not feasible, such as long distance transport of donor cells from valuable animals, FT cells could be used with some degree of compromise with development ability.

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