Introduction

Success has now been achieved with somatic cell cloning in various species using both adult and fetal cells (Wilmut et al., 1997; Wakayama et al., 1998; Baguisi et al., 1999; Shiga et al., 1999; Wells et al., 1999; Ogura et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000). Cloning by using the somatic cells offers the advantage of easy accessibility and non-invasiveness without animal sex or age limitations. However, the development rates are still low and less than 5% of all reconstructed embryos develop to live full-term offspring. Much of this inefficiency results from low initial pregnancy rates and early pregnancy losses. First trimester losses of more than 50% are common for somatic cell nuclear transfer (NT) pregnancies in cattle (Wells et al., 1999; Hill et al., 2000; Kubota et al., 2000), whereas 5% of natural pregnancies by artificial insemination and 11% of pregnancies from transfer of in vitro produced embryos would be expected to be lost by day 60 of gestation (Alexander et al., 1995; Hasler et al., 1995; Forar et al., 1996). The exact mechanisms for high rates of early embryonic loss in NT embryo transfer may relate to one deficiency or a combination of deficiencies in the nuclear transfer manipulation, nuclear reprogramming, the cultured donor cells, or the in vitro culture systems used (Yong and Yuqiang, 1998; Zakharchenko et al., 1999; Kubota et al., 2000). These deficiencies, either collectively or singularly, may lead to inappropriate patterns of gene expression at specific key stages during embryonic development and induce chromosomal abnormalities in NT embryos, contributing to embryonic loss (Campbell et al., 1993; Kielbassa et al., 1997; Niemann and Wrenzycki, 2000; Wrenzycki et al., 2001; Boiani et al., 2002).

Recently, assessment of embryonic cells with fragmented DNA has been carried out using the terminal deoxynucleotidyl transferase (TdT) nick-end labelling (TUNEL). This method facilitates the analysis of apoptosis as nuclear DNA fragmentation in fragmented mammalian embryos. Apoptosis occurs as a normal feature of preimplantation development: probably in response to environmental stressors and gross chromosome abnormalities (Hardy, 1997; Matwee et al., 2000). Apoptosis is characterized by the loss of phospholipid symmetry in the plasma membrane, chromatin condensation, internucleosomal DNA fragmentation, separation of the nucleus into discrete masses, and blebbing of the plasma membrane to form apoptotic bodies that are

Analysis of DNA fragmentation in bovine somatic nuclear transfer embryos using TUNEL

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The production of cloned animals is an inefficient process because of early or late embryonic losses. This study focused on the DNA fragmentation that occurs during embryonic development. The occurrence of DNA fragmentation was examined in bovine embryos produced by in vitro fertilization (IVF) and somatic cell nuclear transfer (NT) using the terminal deoxynucleotidyl transferase (TdT) nick-end labelling (TUNEL). IVF and NT embryos at the two-cell to blastocyst stage were stained by TUNEL for the analysis of DNA-fragmented nuclei and with propidium iodide for determination of the total number of cells. DNA fragmentation was first detected in NT embryos at the four-cell stage, but in IVF embryos at the six- to eight-cell stage. The percentage of embryos with at least one DNA-fragmented nucleus increased with the advance of the developmental stage of embryos in both IVF and NT groups. The DNA-fragmented nucleus index in NT embryos that developed beyond the four-cell stage was significantly higher (P < 0.01) than that of IVF embryos at the same stage. In the both IVF and NT groups, TUNEL-labelled cells were detected in almost all blastocysts and were mainly observed in presumptive inner cell mass (ICM) cells of embryos. The DNA-fragmented nucleus index was negatively correlated with the total number of cells in NT blastocysts, but this relationship was not observed in IVF blastocysts. These results suggest that the high occurrence of DNA fragmentation observed in NT embryos may be related to early embryonic loss after transfer.
phagocytosed or extruded without causing damage to surrounding cells (Wyllie et al., 1980). It has been suggested that apoptosis may maintain cellular quality in the inner cell mass (ICM) of the blastocyst by eliminating damaged cells or those expressing inappropriate phenotype or developmental incompetence (Hardy, 1997). The elimination of unwanted cells is essential in development, but conversely, apoptosis has the potential to eliminate viable cells, leading to the death of the organism. Therefore, the number of cells and the amount of apoptosis in the embryos are important parameters that are emerging as useful indicators of embryonic development and health (Brison and Schultz, 1997, 1998).

This study was conducted to investigate the timing of the onset of DNA fragmentation, deemed to indicate apoptosis, in bovine embryos produced by somatic cell NT. In addition, the incidence of DNA fragmentation in embryos produced by in vitro fertilization (IVF) and NT were compared.

Materials and Methods

Donor cell lines

Primary culture of fetal fibroblast cells was established from skin tissues of a fetus obtained at an abattoir as described by Fahrudin et al. (2001). Briefly, the tissues were cut into small pieces and dispersed by exposure to 0.25% (w/v) trypsin-EDTA. The cell suspension was then transferred into 35 mm culture dishes containing Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Grand Island, NY) supplemented with 5% (v/v) fetal bovine serum (FBS) and 50 μg gentamicin ml−1 (Sigma, St Louis, MO), then cultured at 38.5°C in an atmosphere of 5% CO2 and 95% air with high humidity. When confluence was achieved, cells were trypsinized for 5 min and the recovered cells were centrifuged at 500 g for 5 min, washed, and then either frozen in Dulbecco’s phosphate-buffered saline (PBS, Gibco) containing 5% (v/v) dimethyl sulphoxide (Wako Purechemical Co., Osaka) before storage at −80°C, or transferred into a new 35 mm culture dish containing DMEM supplemented with 5% FBS. Cells for nuclear transfer were cultured for four to six passages, and then starved by culture in DMEM containing 0.5% FBS for 4–5 days as described by Campbell et al. (1996a).

Nuclear transfer and embryo culture

The nuclear transfer procedure has been described by Fahrudin et al. (2001). Briefly, recipient oocytes were matured for 21 h in tissue culture medium 199 (TCM199 medium; Gibco) supplemented with 0.02 mg FSH ml−1 (Denka Pharmaceutical Co., Kawasaki), 100 ng somatotrophin ml−1 (Sigma), 5% FBS and 50 μg gentamicin ml−1. After maturation culture, the oocytes were denuded by gently pipetting in PBS containing 330 IU hyaluronidase ml−1 (Sigma) and then placed in manipulation medium (PBS supplemented with 5 μg cytochalasin B ml−1 and 3 mg bovine serum albumin ml−1). A small amount of cytoplasm directly below the polar body was removed in the manipulation medium. The enucleated oocytes were incubated in CR1aa medium (Rosenkrans et al., 1993) containing 10 μg Hoechst 33342 ml−1 (Calbiochem, San Diego, CA) for 10–20 min and then examined under epifluorescent illumination for the presence of a metaphase chromosome. Only successfully enucleated oocytes were used for the cloning procedure. Individual starved fetal cells were transferred into the perivitelline space of enucleated oocytes at 21–22 h after maturation, and the oocyte–fibroblast couplets were fused with a single pulse of 1 kV cm−1 for 50 μs delivered by BTX2001 (BTX, San Diego, CA). After fusion, the couplets were incubated in a CR1aa medium supplemented with 5% FBS for 20 min before chemical activation. The successfully fused couplets were activated by an incubation of 5 min in CR1aa medium supplemented with 10 μg calcium ionophore ml−1 (Sigma) followed by 5–6 h of culture in CR1aa medium with 10 μg cycloheximide ml−1 and 5% FBS. The couplets were transferred into 100 μl drops (five to ten couplets per drop) of fresh CR1aa medium with 5% FBS and subsequently cultured for 7 days at 38.5°C in an atmosphere of 5% CO2 and 95% air with high humidity.

In vitro fertilization

The incidence of DNA fragmentation of embryos fertilized in vitro was examined by fertilizing some oocytes matured for 21 h with frozen-thawed semen according to the method described by Otoi et al. (1993) with minor modifications. Briefly, after maturation, the cumulus–oocyte complexes were fertilized in vitro with frozen semen and BO medium (Brackett and Oliphant, 1975). Two straws of frozen semen were thawed in a water bath at 37°C for 1 min, and the spermatozoa were processed to induce capacitation in vitro. An aliquot of semen was transferred to BO medium without bovine serum albumin (BSA), but supplemented with 5 mmol caffeine l−1 (Sigma). The spermatozoa were washed twice by centrifugation at 500 g for 5 min. The pellet of spermatozoa was resuspended in BO medium that contained 3 mg BSA ml−1, 10 μg heparin ml−1 (Novo-Heparin 1000; Novo Industry A/S, Osaka), and 2.5 mmol caffeine l−1 to give a concentration of 1.5 × 106 spermatozoa ml−1. The cumulus–oocyte complexes were transferred to microdroplets of spermatozoa for insemination. After 5 h of co-incubation with spermatozoa, each oocyte was transferred to a culture medium (CR1aa medium supplemented with 5% FBS and 50 μg gentamicin ml−1) and was incubated at 38.5°C in an atmosphere of 5% CO2 and 95% air with high humidity. Only cleaved embryos were transferred into a fresh culture medium 48 h after co-incubation with spermatozoa and subsequently cultured for an additional 5 days.
Detection of DNA fragmentation by TUNEL

The status of chromatin in fragmented embryos produced by IVF and NT was analysed by using a combined technique for simultaneous nuclear staining and TUNEL by a modification of the procedures of Brison and Schultz (1997). Embryo stages from the two-cell to blastocyst stages were selected at the following times after fertilization and fusion: two-cell, 30 h; four-cell, 38 h; six- to eight-cell, 49 h; 9–16-cell, 100 h; morula, 128 h; and blastocyst, day 7. The embryos were washed four times in PBS containing 3 mg polyvinylalcohol ml\(^{-1}\) (PBS–PVA), and fixed overnight at 4°C in 3.7% (w/v) paraformaldehyde diluted in PBS. After fixation, they were washed four times in PBS–PVA, permeabilized in PBS containing 0.1% (v/v) Triton-X100 for 40 min, and incubated in a blocking solution (PBS containing 10 mg BSA ml\(^{-1}\)) overnight at 4°C. They were washed four times in PBS–PVA and incubated in fluorescein-conjugated dUTP and TdT (TUNEL reagents; Roche Diagnostics, Tokyo) for 1 h in an incubator at 38.5°C and 5% CO\(_2\) in air. Positive controls (one or two embryos per TUNEL analysis) were incubated in 1000 IU deoxyribonuclease I ml\(^{-1}\) (DNase I; Sigma) for 20 min at 38.5°C and 5% CO\(_2\) in air, and washed twice in PBS–PVA before TUNEL. Negative controls (one or two embryos per TUNEL analysis) were incubated in fluorescein–dUTP in the absence of TdT. After TUNEL, the embryos were washed three times in PBS–PVA and counterstained with 50 μg propidium iodide ml\(^{-1}\) after RNase treatment (50 μg RNase ml\(^{-1}\) for 60 min at room temperature) to label all nuclei. Embryos were then washed in the blocking solution, treated with an anti-bleaching solution (Slow-Fade; Molecular Probes, Eugene, OR), and mounted on a glass slide. Labelled nuclei were examined using a Nikon Diaphot microscope fitted with epifluorescent illumination. Two standard filter sets were used for detection of fluorescein isothiocyanate (FITC) alone (emission wavelength: 525 nm), and for detection of propidium iodide alone (emission wavelength: 560 nm). The total number of cells and the number of cells with DNA-fragmented nuclei were counted, and the DNA fragmentation index was calculated by dividing the number of cells with DNA-fragmented nuclei by the total number of cells (which included the DNA-fragmented nuclei). Analysis was carried out blindly on individual experimental groups of embryos.

Statistical analysis

The proportion of cells with DNA-fragmented nuclei out of the total number of cells (DNA-fragmented nucleus index) was arcsine-transformed and analysed by one-way analysis of variance. The significance of difference between means was compared by a post hoc, Fisher’s protected least-significant-difference test. The percentage of embryos with DNA-fragmented nuclei was analysed by chi-squared analysis or, when some expected values were ≤ 5, Fisher’s exact probability test was used. The total number of cells of embryos was analysed by Student’s \(t\) test. Relationships between the total number of cells and the DNA-fragmented nucleus index were examined by a single linear regression analysis, and the correlation coefficients were determined. Differences at a probability value (\(P\)) of 0.05 or less were considered to be significant.

Results

All stages of development from the two-cell to the blastocyst stage were examined by TUNEL for measurement of DNA-fragmented nuclei and propidium iodide for determining the total cell number of embryos (Fig. 1). There was no evidence of DNA fragmentation at the two-cell stage in either group. However, DNA fragmentation was detected in NT embryos at the four-cell stage (1/30, 3.3%), but not in IVF embryos at the same stage. The percentage of embryos with at least one DNA-fragmented nucleus increased with the advance of the developmental stage of embryos in both groups. In the NT group, all blastocysts contained at least...
Fig. 2. Detection of DNA fragmentation and all nuclei in bovine embryos by terminal deoxynucleotidyl transferase (TdT) nick-end labelling (TUNEL) (green channel) and propidium iodide (red channel), respectively (original magnification: × 200). (a) Nuclear transfer (NT) and (c,e) IVF blastocysts showing labelling with both channels. (b) NT and (d,f) IVF blastocysts showing propidium iodide staining of all nuclei. Most TUNEL labelling indicative of DNA fragmentation is in the presumptive inner cell mass (ICM) of both NT and IVF blastocysts (arrows).
one cell with DNA-fragmented nuclei. In the six- to eight-cell and morula stages, moreover, the percentages of NT embryos with a DNA-fragmented nucleus were higher ($P < 0.05$) than those of IVF embryos. The DNA-fragmented nucleus index in NT embryos that developed beyond the four-cell stage was significantly higher ($P < 0.01$), than that of IVF embryos at the same stage. The total number of cells ($\pm$ SEM) of NT blastocysts was lower ($P < 0.01$) than that of IVF blastocysts (97.2 $\pm$ 7.2 versus 120.9 $\pm$ 5.1). When the correlation between the total number of cells and the DNA-fragmented nucleus index of blastocysts developed 7 days after IVF and NT were analysed, the regression line of NT blastocysts was $y = 16.0 – 0.08x$ ($r = –0.49$, $P < 0.01$), indicating a negative correlation between the total number of cells and the DNA-fragmented nucleus index. However, no correlations were seen in IVF blastocysts ($y = 5.56 – 0.01x$, $r = –0.1$, $P > 0.05$).

A total of 61 embryos served as a positive control on an individual experimental group of NT and IVF embryos for the TUNEL assay and a total of 60 embryos served as a negative control. The embryos incubated with DNase showed uniform TUNEL labelling in all nuclei, ensuring appropriate detection of DNA fragmentation by this method (positive control). In contrast, none of the nuclei was labelled in the embryos incubated without TdT (negative control). Representative images of labelled blastocysts produced by IVF and NT for total cell counts and measurement of DNA-fragmented nuclei are shown (Fig. 2). A morphological characteristic of fragmented nuclei was observed by TUNEL staining. Although DNA fragmentation was observed in the trophoderm, most DNA fragmentation was located in presumptive ICM cells of blastocysts in both groups.

**Discussion**

It has been suggested that DNA fragmentation indicating cell death or apoptosis is a common feature of the development of mammalian embryos (Hardy, 1997). Jurisicova et al. (1996) presented evidence of apoptotic changes in human fragmented embryos by using the 4,6-diamidino-2-phenylindole–TUNEL method. They found that about 75% of embryos fertilized in vitro with morphological fragmentation had apoptotic changes. Matwee et al. (2000) showed that all bovine blastocysts produced in vitro have apoptotic cells. Similarly, in the present study, TUNEL-labelled cells, which reflect the integrity of the DNA, were detected in almost all IVF and NT blastocysts (> 94%). Brison and Schultz (1997) showed that, in mouse blastocysts, the amount of apoptosis in vitro was higher than it was in vivo, and that the degree of apoptosis depended on the environment of the culture, such as the medium and the culture method. Moreover, it has been suggested that suboptimal conditions in the culture system can induce apoptosis in embryos produced in vitro (Deveker and Hardy, 1997; Kamjoo et al., 2002). A high oxygen tension in the in vitro culture system can cause embryonic arrest at a specific stage of development (Ho et al., 1994), in which oxygen free radicals can cause the DNA strand breaks that result in cell cycle arrest or cell death. In fact, the present results showed that the percentage of embryos with at least one DNA-fragmented nucleus increased with the advance of the developmental stage of the embryo, irrespective of the embryonic source. This observation is consistent with previous studies (Byrne et al., 1999; Matwee et al., 2000), which demonstrated that cell death or apoptosis in bovine IVF embryos was dependent on the developmental stage. Therefore, DNA damage observed in IVF and NT embryos may, in part, be induced by suboptimal conditions in the in vitro culture system.

It has been suggested that the onset of apoptosis is related to the activation of the embryonic genome, in which the major events take place at the two-cell stage in mice (Jurisicova et al., 1998) and the four-cell stage in humans (Jurisicova et al., 1996). In cows, the embryonic genome is activated relatively late in development at the 9–16-cell stage (Frei et al., 1989). Matwee et al. (2000) demonstrated that DNA fragmentation detected by the TUNEL method was first observed in bovine IVF embryos at the 8–16-cell stages. In the present study, similarly, the occurrence of DNA fragmentation was first observed in IVF embryos at the six- to eight-cell stage and increased in embryos that developed beyond 9–16-cell stage. In NT embryos, however, DNA fragmentation was first detected at the four-cell stage, and a marked increase in the extent of DNA-fragmented nuclei was apparent at the six- to eight-cell stage. These observations indicate that the onset of apoptosis may correlate with the major burst of embryonic genome activation, but the timing of onset of DNA fragmentation during embryonic development may differ between IVF and NT embryos.

In the present study, the incidence of DNA-fragmented nuclei was apparently higher in NT embryos than in IVF embryos, when the embryos developed beyond the four-cell stage. The percentage of embryos with DNA-fragmented nuclei also tended to be higher in NT embryos than in IVF embryos. The NT techniques involve exploiting the cytoplasm of the metaphase-arrested oocyte that may facilitate the remodelling and reprogramming of somatic cell nuclei. However, nuclear reprogramming depends on many factors, which include the source and the degree of differentiation of donor nuclei, and the cell cycle synchrony between donor nucleus and recipient cytoplast (Campbell et al., 1996b). Previous reports indicated that the incidence of chromosomal abnormalities in NT embryos relates to synchronization of donor and recipient cell cycle stage (Barnes et al., 1993; Campbell et al., 1993). Inappropriate choice of cell cycle stage during nuclear transplantation may lead to chromosome damage or aneuploidy, because of inappropriate regulation of DNA replication during the first cell cycle (Campbell et al., 1993). As one possible role for apoptosis is the elimination of cells with abnormal chromosomes (Hardy, 1997), one factor that contributes to the higher incidence of DNA fragmentation in NT embryos.
may be an increase of abnormal chromosomes induced by inappropriate synchronization in the cell cycle of the donor nucleus and recipient cytoplasm through reconstruction into enucleated oocytes.

The number of cells and the amount of apoptosis in the embryos are important parameters of embryo development and health (Brison and Schultz, 1997, 1998). It is thought that embryos with a large number of cells are more likely to implant and give rise to live offspring (Van Soom et al., 1997). In addition, DNA fragmentation may play a role in the regulation of the total ICM cell number, which is likely to be important, as an oversized ICM could result in an abnormal development of the fetus, such as an enlarged offspring (Thompson et al., 1995). Several reports have demonstrated that cell death or apoptosis in blastocysts are most predominant in ICM cells and are occasionally seen in the trophectodermal lineage (Brison and Schultz, 1997; Byrne et al., 1999). Similarly, in the present study, most DNA fragmentation appeared to be present in presumptive ICM cells of both IVF and NT embryos. Moreover, the total number of cells of NT blastocysts was lower than that in IVF blastocysts, and the DNA-fragmented nucleus index was negatively correlated with the total number of cells in NT blastocysts, but not in IVF blastocysts. These findings indicate that NT blastocysts consisting of a small number of cells have a high incidence of DNA fragmentation or apoptosis, which may relate to low pregnancy rates and embryonic losses after transfer.

In conclusion, the present results indicate that the DNA-fragmented nucleus index and the percentage of embryos with DNA-fragmented nuclei increase in NT embryos, compared with IVF embryos, in which an increase of DNA fragmentation in NT embryos may contribute to early pregnancy losses.

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