

Serum-Starved Apoptotic Fibroblasts Reduce Blastocyst Production but Enable Development to Term after SCNT in Cattle

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Abstract

Cell cycle synchronization by serum starvation (SS) induces apoptosis in somatic cells. This side effect of SS is hypothesized to negatively affect the outcome of somatic cell nuclear transfer (SCNT). We determined whether apoptotic fibroblasts affect SCNT yields. Serum-starved, adult, bovine fibroblasts were stained with annexin V-FITC/propidium iodide to allow apoptosis detection by flow cytometry. Positive and negative cells sorted by fluorescence activated cell sorting (FACS) and an unsorted control group were used as nuclear donors for SCNT. Reconstructed embryos were cultured *in vitro* and transferred to synchronized recipients. Apoptosis had no effect on fusion and cleavage rates; however, it resulted in reductions in blastocyst production and quality measured by apoptotic index. However, reconstructed embryos with apoptotic cells resulted in pregnancy rates similar to that of the control on day 30, and generated one live female calf. In conclusion, we showed that apoptotic cells present in serum-starved cultures negatively affect embryo production after SCNT without compromising full-term development. Further studies will evaluate the ability of the oocyte to reprogram cells in specific phases of apoptosis.

Introduction

THE SUCCESSFUL PRODUCTION OF ANIMALS by somatic cell nuclear transfer (SCNT) has many potential applications in basic research, medicine, and agriculture. Cloned animals including sheep (Schinieke et al., 1998; Wilmut et al., 1997), goats (Baguisi et al., 1999), mice (Wakayama et al., 1998), pigs (Polejaeva et al., 2000), cattle (Cibelli et al., 1998; Kato et al., 1998; Wells et al., 1999), cats (Shin et al., 2002), rabbits (Chesne et al., 2002), horses (Galli et al., 2003), dogs (Lee et al., 2005), and buffaloes (Shi et al., 2007) have been successfully produced by nuclear transfer using SCNT. Nonetheless, the low efficiency of SCNT is a major obstacle to the widespread use of this technology (Heyman, 2005).

The inefficiency is manifested by a low rate of embryonic development and a high pregnancy loss throughout

gestation, resulting in poor survival of live-born young. It has been generally accepted that inadequacy of the SCNT procedure (Galli et al., 2002; Machaty et al., 1999), asynchrony between donor karyoplasts and recipient oocytes (Campbell et al., 1996), chromosomal and epigenetic abnormalities of the donor cell (Giraldo et al., 2006, 2007; Mastro Monaco et al., 2006), altered embryonic gene expression patterns (Wrenzycki et al., 2001), and higher embryonic apoptosis (Park et al., 2004) are among the factors responsible for the abnormal development of cloned embryos and fetuses.

Cell cycle coordination between donated cell nuclei and recipient oocyte cytoplasm has been widely recognized as a key factor for adequate maintenance of integrity and ploidy in SCNT reconstructed embryos (Campbell et al., 1996). Serum starvation is a commonly used method to synchronize cells for cloning (Boquest et al., 1999; Cho et al., 2005; Kues et al.,

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2000; Wilmut et al., 1997). However, it has been shown that, besides its very efficient cell cycle synchronization capacity, serum restriction induces apoptosis in murine, human, and rodent cell lines (Lindenboim et al., 1995; Mills et al., 1997; Rawson et al., 1991; Rühl et al., 1999), as well in cells of livestock species such as pigs and cattle (Boquest et al., 1999; Cho et al., 2005; Kues et al., 2002; Peng et al., 1998).

Apoptosis, a type of programmed cell death, is a well-conserved phenomenon across mammalian species that is characterized by modifications in the plasma membrane (phosphatidylserine exposure), chromatin condensation, internucleosomal DNA fragmentation, and blebbing of the plasma membrane to form apoptotic bodies that are phagocytosed without causing damage to surrounding cells (Taylor et al., 2008; Wyllie et al., 1980). The detection of phosphatidylserine by the annexin-V assay (Brumatti et al., 2008; Martin et al., 1995) and DNA double-strand breaks by terminal deoxynucleotidyl transferase (TdT) nick-end labeling (TUNEL) are among most useful techniques for detection of apoptotic cells at early and late phases, respectively.

Park et al. (2004) proposed that the survival and physiology of donor somatic cells before SCNT directly influence clone embryo development; they showed, for the first time, that the treatment of bovine donor cells with putative apoptosis inhibitors prior to SCNT improved posterior preimplantation development. However, it remains unclear if the apoptotic cells from regular cultures result in embryos and fetuses in nuclear transfer experiments. Therefore, it would be of great interest to elucidate whether cells undergoing apoptosis could be a source for suitable nucleus donor cells for somatic cloning.

The aim of this study was to further investigate the role of the physiological status of donor cells in embryo production and quality with respect to SCNT. Specifically, we aimed to determine if putative annexin-positive bovine fibroblasts are suitable as nuclei donors for SCNT by verifying if the use of these cells could alter preimplantation development, apoptotic index, and pregnancy rates of developed embryos. To accomplish this, apoptosis was detected in cultures of bovine serum-starved fibroblasts by an annexin V assay (Brumatti et al., 2008; Martin et al., 1995). Annexin V-positive cells were isolated by fluorescence-activated cell sorting (FACS) and used for nuclear transfer. Embryos were subsequently monitored for developmental competence, total cell number, and apoptotic index (TUNEL-positive nuclei among total cell number). Moreover, some embryos were transferred to synchronized recipients, and pregnancies were recorded and monitored until termination of the pregnancy or birth. We have shown that the use of annexin-positive cells as nuclear donors for SCNT affects embryonic development and blastocyst total cell number without increasing the apoptosis rate in developed embryos. Despite affecting blastocyst production and quality, one live-born calf has been obtained from an annexin V-positive cell after embryo transfer to recipients.

Materials and Methods

Unless stated otherwise, chemicals and growth media were purchased from Sigma (St. Louis, MO). Incubator conditions for oocytes and embryos, as well as for fibroblast cultures, were maintained at 38.5°C in air with 5% CO₂ and maximum humidity.

Primary cell cultures and isolation of cells

Bovine skin fibroblasts were obtained from adult Nelore female cattle by ear-punch biopsy. After removal of hair and rinsing in 70% ethanol, the tissue was washed in Dulbecco's PBS (DPBS), minced thoroughly with small scissors, and placed on plastic Petri dishes, allowed to partially dry to increase attachment to the surface of the plastic, and then carefully filled with Dulbecco's modified Eagle's medium (DMEM; Gibco BRL®, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Gibco BRL®), and 50 µg/mL gentamicin. In this experiment, cells were utilized between passages 3 to 6.

Cell cycle synchronization and analysis

Cell cycle comparisons were made among cells that had been serum starved for 24 or 72 h and cycling cells (non-starved). For the experiment, cells were seeded on 75-cm² plastic flasks (TPP, Trasadingen, Switzerland) at a concentration of 4 × 10⁴ cells/mL in DMEM with 10% FBS, leading to greater than 60% confluency. After 24 h, the medium was changed to DMEM with 0.5% FBS (serum starvation) and distributed in the following experimental groups: (1) serum starvation for 1 day, and (2) serum starvation for 3 days. Nonstarved cycling cells kept in DMEM with 10% FBS served as controls.

DNA amount analysis was performed by flow cytometry (FACS Calibur, Becton Dickinson, San José, CA) following DNA staining with propidium iodide. Cell cycle analysis was performed using the multicycle DNA content and cell cycle analysis software (Phoenix Flow Systems Inc., San Diego, CA). Briefly, 3 × 10⁶ cells were pelleted and fixed in 1% ice-cold formaldehyde for 20 min at 4°C. Ice-cold methanol was added, after which the cells were placed at -20°C for 10 min. The cells were washed twice in PBS, resuspended in 20 µg/mL of propidium iodide for 30 min at 37°C in darkness, and then submitted to flow cytometry analysis.

Detection of apoptosis by flow cytometry and FACS

After cell cycle synchronization by serum starvation, cells were then trypsinized and incubated with annexin V (Anx) that had been conjugated to FITC (Apoptest-FITC; Dako, Hoeven, The Netherlands) at a dilution of 1:100 in the calcium-containing binding buffer provided with the kit. After 10 min of incubation, propidium iodide (PI) was added at a final concentration of 5 µg/mL to detect necrotic cells, and the data were analyzed using a FACS Aria flow cytometer (Becton Dickinson) with 488 nm of laser light excitation. Data were analyzed using the FACSDiva software application (Becton Dickinson). The cell populations were observed in a "dot plot" graphic presenting annexin-negative cells considered as population of annexin V-negative/PI-negative cells, apoptotic cells (annexin V-positive/PI-negative cells), necrotic cells (annexin V-negative/PI-positive cells), and late apoptotic/necrotic cells (annexin V-positive/PI-positive cells). Annexin-negative and apoptotic cells were sorted by FACS, into room-temperature DMEM with 10% FBS containing a physiological concentration of calcium. Cells were then centrifuged and used for nuclear transfer. A group of the unstained and unsorted cells, which were cultured and synchronized as described above, were used to calibrate for fluorescence background and for SCNT (control group).

Nuclear transfer

Bovine oocytes were obtained by aspiration of small antral follicles. Immature cumulus–oocyte complexes were cultured in Tissue Culture Medium 199 (TCM-199) supplemented with 10% FBS, 0.2 mM pyruvate, 50 μ g/mL gentamicin, 0.5 mg/mL FSH and 150 mg/mL LH. Eighteen hours after the start of maturation, cumulus cells were completely removed from the oocyte by manual pipetting in the presence of 0.2% hyaluronidase, and oocytes with an extruded first polar body were selected for enucleation. The oocytes were labeled with 10 μ g/mL DNA fluorochrome (Hoechst 33342) for 10 min at room temperature in SOF containing 0.2 mM pyruvate and 3 mg/mL bovine serum albumin (BSA), and washed and transferred in a manipulation drop of SOF–HEPES (Wells et al., 1999) supplemented with 7.5 μ g/mL cytochalasin B. All manipulations were performed on an inverted microscope (Leica, Solms, Germany) equipped with Hoffman optics and micromanipulators (Narishige, Tokio, Japan). The first polar body and metaphase II (MII) plates were removed by aspiration with a 15- μ m inner diameter enucleation pipette (ES transferTip, Eppendorf, USA). To ensure that oocyte chromatin was removed, aspirated cytoplasm was exposed to UV light and examined for the presence of the removed polar body and metaphase plate.

After enucleation of all oocytes, one single donor cell from sorted and control cell populations was placed into the perivitelline space of each enucleated oocyte. SCNT couplets were placed into a fusion chamber filled with 0.3 M mannitol. One pulse of alternating current (5 sec, 50 V/cm), followed by two continuous current electric pulses (45 μ sec each, 1.75 kV/cm), were applied to promote fusion in a Multiporator (Eppendorf, Hamburg, Germany). Successfully fused SCNT couplets were activated by using ionomycin and 2 mM of 6-dimethylaminopurine (6-DMAP), as described by Susko-Parrish et al. (1994). Twenty-six hours after the start of maturation, SCNT units were placed in 5 μ M ionomycin in TCM199-HEPES medium (supplemented with 1 mg/mL fatty acid-free BSA) for 5 min. SCNT units were then moved into TCM199-HEPES medium (supplemented with 30 mg/mL BSA) for 5 min. This was followed by incubation of SCNT units in SOF medium (supplemented with 6-DMAP for 3 h. At the end of incubation, SCNT units were washed in SOF medium (Vajta et al., 1999) and placed into *in vitro* culture medium drops. Cleavage and blastocyst rates were evaluated at second and seventh day, respectively. Non-manipulated, control metaphase II arrested oocytes were activated by using the same protocol (parthenogenetic embryos), *in vitro* cultivated, and used as a control for oocyte quality, activation procedure, and *in vitro* culture.

Detection of DNA fragmentation by TUNEL

The status of chromatin in embryos produced from annexin-negative, annexin-positive, and control cells was analyzed by using a TUNEL assay. Blastocysts were washed in PBS containing 1 mg polyvinylalcohol/ml (PBS–PVA), and fixed for 1 h at room temperature with 3.7% (w/v) paraformaldehyde diluted in PBS. After fixation, they were washed three times in PBS–PVA and permeabilized in PBS containing 0.5% (v/v) Triton X-100 and 0.1% (w/v) sodium citrate for 1 h at 4°C. They were washed three times in PBS–PVA and incubated in fluorescein-conjugated dUTP and TdT

(In situ Cell Apoptosis Detection Kit; Roche Diagnostics, Mannheim, Germany) for 1 h in a humidified chamber at 37°C. Positive controls (one or two embryos per TUNEL analysis) were incubated in 1000 IU deoxyribonuclease I/mL (DNase I; Sigma, St. Louis, MO) for 1 h at 37°C, 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 10 μ g/mL Hoechst 33342 to label all nuclei. Blastocysts were mounted on glass slides with antifade (Vectashield, Vector Laboratories, Burlingame, CA). Labeled nuclei were examined using a Zeiss Axioplan microscope (Carl Zeiss, NY) fitted with epifluorescent illumination. Two standard filter sets were used for detection of fluorescein isothiocyanate (FITC) (emission wavelength: 525 nm) and Hoechst 33342. Both 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.

Embryo transfer and pregnancy monitoring

Day 7 SCNT blastocysts derived from annexin-negative, annexin-positive, and control cells were transferred non-surgically into the uterine horn ipsilateral to the ovary containing a palpable corpus luteum of previously synchronized recipient cows (one good-quality embryo or one intermediate, together with one poor-quality were transferred per cow). Pregnancy status was determined by rectal palpation on day 30 and by ultrasound examination on day 60 of gestation. Pregnancies were then allowed to develop to term. Abortion rate was evaluated on a monthly basis.

Statistical analysis

The percentages of cells in each stage of the cell cycle for the experimental groups, as well as apoptotic index, are given as the mean value of replicates \pm standard error mean (SEM) and were analyzed by analysis of variance (ANOVA), and Bonferroni posttests (cell cycle values were arcsin-transformed previously). Fusion rate, embryonic development, and pregnancy rates were analyzed by a chi-square test. The chosen significance level was 5%. A significance level of 10% was considered only where stated.

Results

Cell cycle analysis

To evaluate the effect of serum starvation on the cell cycle, the relative percentages of the proportions of cells in the G0/G1 (2C DNA content), S (2C–4C) and G2/M (4C DNA content) stages are calculated (Table 1). Serum starvation was an effective means to synchronize adult fibroblasts cell cycle yielding an increase of \sim 20% in G1/G0 cell population, and a reduction of \sim 50% in S-phase cells of the cell cycle compared to cycling cells ($p < 0.05$). There was no effect of serum starvation treatment length (24 or 72 h) with regard to mean number of synchronized fibroblasts in G1/G0 phases of cell cycle ($p > 0.05$).

TABLE 1. CELL CYCLE SYNCHRONIZATION OF BOVINE ADULT FIBROBLASTS CULTURED FOR DIFFERENT DURATIONS OF SERUM STARVATION

Culture conditions	% Cell cycle (mean \pm SEM)		
	G0/G1	S	G2/M
Cycling cells ¹	60.9 \pm 9.6 ^a	9.3 \pm 2.3 ^a	29.8 \pm 8.9
24-h starvation ²	82.6 \pm 3.3 ^b	5.1 \pm 0.6 ^b	12.3 \pm 0.8
72-h starvation ²	84.1 \pm 5.9 ^b	2.8 \pm 0.2 ^b	13.1 \pm 1.1

¹Nonconfluent cycling fibroblasts cultured in DMEM supplemented with 10% FBS for 48 h.

²Starved, nonconfluent fibroblasts cultured in DMEM supplemented with 0.5% FBS.

^{a,b}Different superscripts within same column indicate significant difference ($p < 0.05$). Passages 4–8, three replicates.

Detection of cell apoptosis by flow cytometry

Bovine adult fibroblasts were serum starved for 24 h, stained with an annexin V assay, and analyzed for apoptosis by flow cytometry. Five thousand cells were recorded per replicate. The experiment was repeated three times. Results are presented in Table 2.

The results showed that approximately ~15% of the cells were classified as apoptotic (annexin V-positive), ~3.6% as necrotic (PI-positive), ~1.5% as late apoptotic (annexin V/PI-positive) and ~79.7% as annexin-negative cells.

FACS and SCNT embryo development

Cell population was visualized in dot plot graphics generated by the flow cytometer (Fig. 1), and divided into four subpopulations (four quadrants) according to the annexin V and PI fluorescence signals from non stained samples (background). Apoptotic cells, shown in right-bottom quadrant, and annexin-negative cells, shown in left-bottom quadrant, were sorted in DMEM 10% FBS medium and used as nuclear donors for cloning.

A total of 765 oocytes were micromanipulated in this experiment. Results are shown in Table 3.

In comparison to the control cells, there was no effect on fusion rate through the use of annexin-positive cells as nuclear donors ($p > 0.05$). Meanwhile, sorted annexin-negative cells were able to fuse more efficiently to enucleated oocytes ($p < 0.05$). Cleavage of embryos reconstructed with annexin-positive cells (72.7%) and annexin-negative cells (83.4%) were similar to cleavage of embryos reconstructed with control cells (77.3%; $p > 0.05$). The use of annexin-positive cells as nuclear donors for cloning significantly reduced blastocyst production in comparison to the use of annexin-

negative cells (18.8 vs. 34.7, respectively; $p < 0.05$) and control cells (18.8 vs. 27.3%, respectively; $p = 0.06$) as nuclear donors.

Blastocyst cell number and TUNEL

Results for total cell number, TUNEL-positive nuclei, and apoptotic indexes for embryos cloned from annexin-negative, positive cells, and control cells are shown in Table 4. Cloned embryos reconstructed from annexin-positive cells had the same amount of blastomeres (81.5 \pm 9.3) than embryos reconstructed from annexin-negative (97.6 \pm 8.7) and control cells (104.7 \pm 8.6; $p > 0.05$). However, it was observed that apoptotic index tended to be higher in embryos cloned from annexin-positive cells compared with annexin-negative and control cells ($p = 0.07$).

Embryo transfer and pregnancy results

A total of 81 embryo transfers were made. Surrogate bovine females receiving one (good-quality), or two (one intermediate and one poor-quality) embryos were monitored for pregnancy and abortion rates. Results are shown in the Table 5.

At the first ultrasound exam, 30 days after embryo transfer, there was no difference ($p > 0.05$) in the pregnancy rates between annexin-positive (18.1%), annexin-negative (13.3%), and control (27.5%) groups. At 60 days after embryo transfer it was observed that all pregnancies from annexin-negative group were lost, whereas annexin-positive and control groups showed a reduction in pregnancy rates (13.6 and 10.3%, respectively).

Between 60 days and term, abortion was monitored on a monthly basis, and it was observed that two pregnancies were lost in annexin-positive group, whereas all pregnancies were lost in control group. Considering the number of pregnancies on day 30 as 100%, a 66.6% loss was observed (two losses of three) and 100% loss was observed (three losses of three) in the apoptotic and control groups, respectively.

The overall live birth outcome of SCNT embryos in this study was 1.2%; however, for the annexin-positive group the birth rate was 4.5%. The live calf obtained from the annexin-positive group died ~12 h after birth with symptoms of respiratory distress syndrome. Although we produced live animals in our laboratory (Miglino et al., 2007), for an unknown reason this cell line resulted in lower rates of calving and survival.

Discussion

In this study we have shown that adult bovine fibroblasts that were cultured under conditions of serum starvation for

TABLE 2. APOPTOSIS, NECROSIS, AND LATE APOPTOSIS OF BOVINE FIBROBLASTS SERUM STARVED (0.5% FBS) FOR 24 H^a

	% (Mean \pm SEM)			
	Annexin-negative	Apoptosis	Necrosis	Late apoptosis/necrosis
24-h starvation	79.7 \pm 4.7	15.1 \pm 2.6	3.6 \pm 2.3	1.5 \pm 0.5

^aApoptosis was detected by annexin V/propidium iodide (PI) staining assay. Annexin-negative, cells without annexin and PI signal. Apoptosis, cells with annexin signal only. Necrosis, cells with PI signal only. Late apoptosis/necrosis, cells with annexin and PI signals.

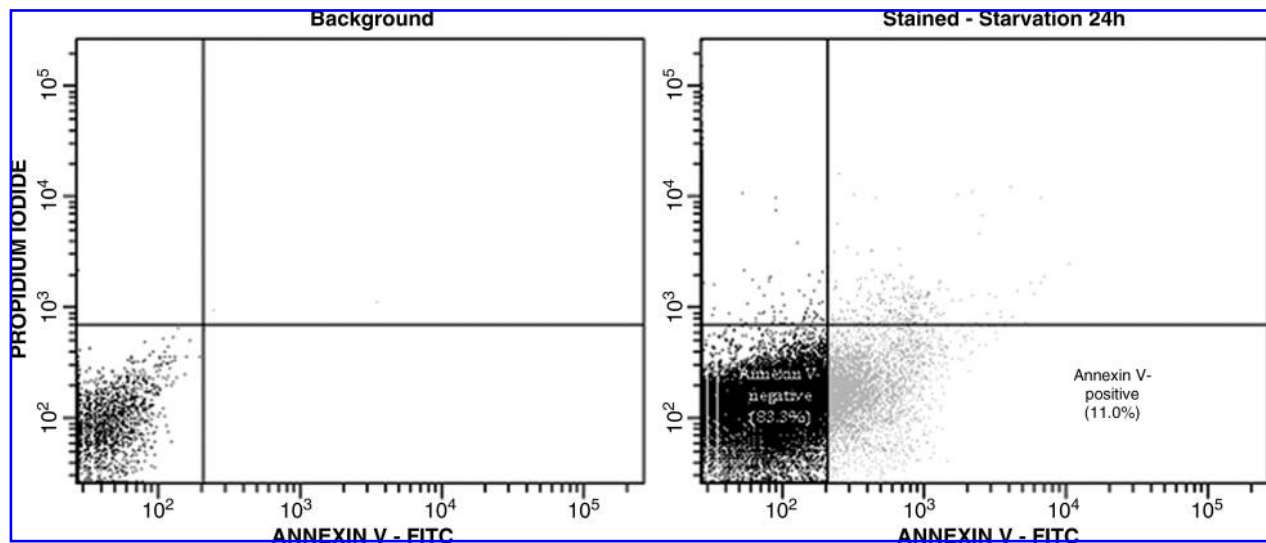


FIG. 1. Apoptosis in bovine adult fibroblasts serum starved for 24 h. An unstained cell sample was utilized to define four quadrants (Background). Apoptotic (Annexin V-positive cells; lower right quadrant), and annexin-negative cells (lower left quadrant) were sorted by FACS into sterile tubes with DMEM 10% FBS and utilized for SCNT.

24 h, subjected to an apoptosis assay with annexin V assay and sorted by FACS can be used as nuclear donors for SCNT. Despite the negative effect on embryo development and tendency to increase apoptosis in blastocysts these cells could be reprogrammed after SCNT and live offspring could be produced.

Cell cycle coordination between donated cell nuclei and recipient oocyte cytoplasm has been widely recognized as a key factor to the adequate maintenance of integrity and ploidy in SCNT reconstructed embryos. It has been shown, independent of species (Campbell et al., 1996), that cytoplasm of metaphase II enucleated oocytes requires the use of somatic cell nuclei in G1/G0 phase of cell cycle to achieve adequate embryo. Several approaches can be used to arrest cells at G1/G0 in the cell cycle for SCNT, and serum starvation is among the most commonly used methods. The use of serum starvation for cell cycle synchronization in SCNT was first suggested in the original "Dolly's paper" (Wilmut et al., 1997). Besides inducing arrest of cells in G1 phase of cell cycle by dramatically reducing proliferating stimuli such as growth factors, serum starvation for several days induces the cell to enter into a quiescent, reversible state of the cell cycle: the G0 phase (reviewed by Collier, 2007).

As reviewed by Oback and Wells (2003), cell cycle synchronization of bovine fibroblasts at G1/G0 phase for cloning can be achieved by the use of serum starvation during 3–7 days. In this study serum starvation was evaluated for synchronization and the results showed that incubation in DMEM with a reduced amount of serum (0.5% FBS) for only 24 h was enough to synchronize more than 80% of the fibroblasts at G1/G0 phase of cell cycle. In support of our findings, Sun et al. (2008) showed that serum starvation of bovine transgenic recombined fibroblasts for 24 h was efficient to synchronize ~86.5% of the cells at G1/G0. Nonetheless, according to the latter, the proportion of G1/G0 cells was significantly increased when serum starvation lasted for 3 (~91.5%), 4 and 5 days rather than 1 day only. In our study, additional 48 h of serum starvation (72 h) showed a slight decrease in cells at S phase but did not increase the amount of synchronized fibroblasts at G1/G0 phase. This difference could be explained by differences in the protocol of cell preparation, because we used cells at 60–70% confluence, whereas Sun et al. (2008) used cells that were closer to confluence.

The cell cycle synchronization protocol of using serum starvation for 24 h was utilized in combination with enucleated

TABLE 3. *IN VITRO* DEVELOPMENT TO BLASTOCYST STAGE OF CLONED EMBRYOS RECONSTRUCTED USING ANNEXIN-POSITIVE, ANNEXIN-NEGATIVE, AND CONTROL FIBROBLASTS¹

	No. reconstructed oocytes	No. fused (%)	Cleaved (%) ²	Blastocysts (%) ²
Annexin-negative	259	193 (74.5) ^a	161 (83.4) ^a	67 (34.7) ^a
Annexin-positive	243	165 (67.9) ^{a,b}	120 (72.7) ^b	31 (18.8) ^{b,*}
Control	263	172 (65.4) ^b	133 (77.3) ^{a,b}	47 (27.3) ^{ab}

¹Bovine fibroblasts were stained by an annexin V/PI assay and sorted by FACS according to their fluorescence pattern. Annexin-negative cells (without annexin V and PI signals). Annexin-positive cells (annexin V signal only). Control: unstained, and unsorted cells.

²Percentage of number of oocytes successfully fused.

*Trend was observed for the comparison with control ($p = 0.06$).

^{a,b}Different superscripts within same column differed significantly ($p < 0.05$; chi-square test).

TABLE 4. MEAN NUMBER OF TOTAL¹ AND APOPTOTIC BLASTOMERES² IN BOVINE EMBRYOS CLONED FROM ANNEXIN-POSITIVE AND ANNEXIN-NEGATIVE CELLS

	Total embryos tested (n)	Total cells per embryo (mean ± SEM)	Apoptotic cells per embryo (mean ± SEM)	Apoptotic index (mean ± SEM)
Annexin-negative	21	97.6 ± 8.7	4.8 ± 0.6	0.051 ± 0.009 ^a
Annexin-positive	14	81.5 ± 9.3	6.4 ± 1.0	0.087 ± 0.013 ^b
Control	16	104.7 ± 8.6	5.4 ± 0.8	0.054 ± 0.009 ^a

¹Total cell number was detected with Hoechst 33342 staining (10 µg/mL).

²Apoptosis was detected with TUNEL assay.

^{a,b}Different superscripts within the same column differed statistically ($p = 0.07$).

metaphase II oocytes as recipients for subsequent SCNT procedures. Besides the efficient capacity for cell cycle synchronization, it has been shown that serum starvation can increase the occurrence of apoptosis, along with DNA damage resulting from inadequate replication. Gibbons et al. (2002) suggested that serum starvation for cell cycle synchronization of the fibroblasts may be related to the late-term fetal loss observed in bovine clones. Cho et al. (2005) showed that serum starvation for 5 days increased apoptosis in bovine fibroblasts in comparison to cell confluency. Moreover, serum deprivation for at least 48 h induces apoptosis in murine and human cell lines (Lindenboim et al., 1995; Mills et al., 1997; Rawson et al., 1991; Ruhl et al., 1999), as well as in pig fetal fibroblasts (Kues et al., 2000, 2002).

In this study, our objective was not to investigate if serum starvation induces apoptosis; however we have shown, using the annexin V assay, that ~15% of adult nonconfluent bovine fibroblasts cultured under serum starvation for only 24 h were apoptotic. The annexin V-binding assay provides a very specific, rapid, and reliable technique to detect apoptosis by flow cytometry, or by fluorescence microscopy (Martin et al., 1995, 1996). Annexin V binds efficiently to the negatively charged phospholipid phosphatidylserine, which is rapidly externalized on the outer leaflet of the plasma membrane in response to increases in intracellular Ca²⁺, cell injury or apoptosis-inducing agents (Chang et al., 1993; Comfurius et al., 1994). Phosphatidylserine externalization has been considered as an early event in apoptosis occurring before plasma membrane permeability, persisting until late stages of the process and serving as a trigger for the apoptotic cells clearance via recognition by macrophages. Therefore, annexin V-positive cells detected in this study

may represent a heterogeneous cell population concerning apoptosis progression.

Because the annexin V-binding assay is a very simple, rapid, and reliable assay that does not require fixation or processing of cells (reviewed by Brumatti et al., 2008), we tested the hypothesis that annexin V-positive (apoptotic) bovine fibroblasts sorted by FACS are suitable nucleus donor cells for somatic cloning. Our results indicate that the use of these annexin-positive cells for SCNT did not affect fusion and cleavage in developing embryos in comparison to the use of control cells ($p > 0.05$). On the other hand, the use of annexin-negative cells for SCNT significantly increased fusion ($p < 0.05$). During electrofusion, application of a voltage pulse occurs within liquid medium contained in a fusion chamber with two opposing electrodes. Applying a voltage induces a high-strength electric field. This electric field is further amplified in the cell membrane owing to the low molecular rearrangement of the phospholipids in the form of nanoscale pores (Clow et al., 2009). As stated previously, annexin V binds to phospholipid phosphatidylserine, which is rapidly externalized on the outer leaflet of the plasma membrane in apoptotic cells. Nonetheless, fusion rates were similar in both annexin-negative and positive cells, which may be indicative of the presence of some agent in the detection kit that might improve fusion.

Concerning blastocyst development and quality, the use of annexin-positive cells as nuclear donors for SCNT negatively affected both production ($p < 0.05$) and apoptotic index in blastocysts ($p = 0.07$) in comparison to the use of annexin-negative and control cells as nuclear donors. Embryo total cell number was not affected by the use of either annexin-positive or annexin-negative cells as nuclear donors ($p > 0.05$).

TABLE 5. PREGNANCY RESULTS AFTER TRANSFER OF BLASTOCYSTS CLONED FROM ANNEXIN-POSITIVE, ANNEXIN-NEGATIVE, AND CONTROL CELLS¹

	No. Transf.	Pregnant D30 (%)	Pregnant D60 (%)	Abortion (%) ²	Birth (%) ³
Annexin-negative	30	4 (13.3) ^a	0 (0) ^a	—	—
Annexin-positive	22	4 (18.1) ^a	3 (13.6) ^b	2 (66.6)	1 (4.5)
Control	29	8 (27.5) ^a	3 (10.3) ^b	3 (100)	0 (0)
Total	81	16 (19.7)	6 (7.4)	5 (83.3)	1 (1.2)

¹Cells were stained for annexin V assay, sorted by FACS in flow cytometer, and used for NT.

²Related to pregnancy on day 60.

³Related to number of transferred embryos.

^{a,b}Different letters within the same column differed significantly (chi-square test, $p < 0.05$).

Studies demonstrating the production of healthy offspring derived from freeze-dried sperm by intracytoplasmic spermatozoa injection (ICSI) in mice (Wakayama et al., 1998), the production of lambs cloned from denatured (heated to 55°C or 75°C) somatic cells (Loi et al., 2002) and the rescue of H₂O₂-treated pronuclei after their transfer to cytoplasm of untreated enucleated zygotes highlight that even harsh treatments imposed on donor cells can be overcome once these cells are into the oocyte cytoplasm, resulting in quite normal embryonic and even fetal development. Here we have shown, for the first time, that apoptotic bovine fibroblasts, detected by an annexin V assay and sorted by FACS, are developmentally competent nuclear donors for SCNT. Embryos cloned from annexin-positive cells were able to establish pregnancies in a similar way to embryos cloned from control and annexin-negative cells, as diagnosed on day 30 after embryo transfer. All pregnancies established after the transfer of annexin-negative cells and control cells were lost between days 30–60 and 60–birth, respectively. Surprisingly, despite a 75% pregnancy loss, one live Nelore calf was delivered by cesarean section on day 285 of gestation from a recipient cow receiving one embryo cloned from an annexin-positive (annexin V-positive) cell.

Apoptosis or programmed cell death is a genetically regulated, multistep cascade of events for the elimination of cells in response to specific cell signals. There is a point in the apoptotic process when an irreversible commitment occurs and cell death cannot be prevented. This so-called point of no return has, as yet, not been well characterized (Kroemer et al., 2009). Despite being able to trigger apoptosis in somatic cell culture, as has already been shown, serum starvation cannot be considered as a potent apoptosis inducer when compared to pharmacological apoptosis inducers such as staurosporine. Moreover, because serum starvation is not a strong “death signal,” one can speculate that cells enter apoptosis at different times after initiation of serum starvation. The Annexin V assay has been shown to detect early apoptotic cells as well as cells at later phases of the apoptotic process as proposed by Clodi et al. (2000) and Span et al. (2002). Therefore, considering that embryos were cloned from a population of annexin V-positive cells sorted by flow cytometry, the reduction in blastocyst production suggests that the apoptotic reversal did not occur efficiently in all somatic cell transfers because of heterogeneity in the apoptotic process in the annexin V-positive cells. We speculate that there was a limit for the reversal of the apoptotic process after somatic cell transfer into the oocyte’s cytoplasm. If the apoptotic point of no return and this limit of cloning apoptotic cells are the same, we do not know, but our results suggest that, in the apoptotic process, positive expression of annexin V overlaps with the point in the cell death cascade where reversibility and the point of no return occur. Therefore, the use of cells selected for annexin V staining following SCNT can represent a model for the further molecular characterization of the point of no return/reversibility of apoptotic process.

It has become clear that apoptosis is also characterized by a variety of biochemical changes that occur in several organelles, including the nucleus. Studies have shown that striking modifications occur at nuclear matrix level, which suggests that early proteolysis of key nuclear matrix proteins during apoptosis may open sites of nuclease hypersensitiv-

ity similar to those observed in the transcriptionally active regions of chromatin. However, these modifications are difficult to identify because apoptosis often occurs in an asynchronous way. In addition, recent evidence supports the notion that, depending on the inducing agent, the hierarchical sequence and kinetics of degradation events (both proteolysis and DNA fragmentation) contributing to nuclear disassembly during apoptosis are variable (reviewed by Martelli et al., 2001). Kikyo and Wolffe (2000) proposed that the repressive nucleoprotein structures established during somatic cell commitment (differentiation) must be completely reversed by the oocyte cytoplasm, and the efficiency of this reversal determines the developmental success of nuclear transfer embryo. The treatment of ovine granulosa cells with supraphysiological temperatures (65°C) has been shown to induce destabilization of repressive nucleoprotein complexes and to improve embryo and animal production when these cells were utilized as nuclear donors for SCNT (Loi et al., 2002). Therefore, one hypothesis is that the only animal cloned in this study originated from an apoptotic cell that was harboring a nucleus with “relaxed chromatin structures,” thereby facilitating reprogramming by the host cytoplasm.

In conclusion, we confirmed through a novel approach that apoptosis in cell culture negatively affects SCNT, which is in agreement with previously proposed ideas from Park et al. (2004). However, based on the observations that embryos, and one live animal, were obtained through the use of annexin-positive cells, we speculate that serum starvation triggers an asynchronous apoptotic stimulus that the oocyte is able to reverse, depending on the stage of apoptotic process in which the cells are at the time of fusion. Nuclear transfer experiments using cells that have undergone induced apoptosis (synchronous apoptotic stimulus) at different times after induction could be applied to evaluate this hypothesis.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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