Sperm Cytosolic Factor Activation for Bovine Somatic Cell Nuclear Transfer

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ABSTRACT

In this study I report that *in vitro* development rates of bovine nuclear transfer embryos activated either with boar sperm cytosolic factor (SCF) or with ionomycin followed by cycloheximide (CHX) and subsequent *in vivo* developmental rates after embryo transfer are related to blastocyst quality as evaluated by apoptosis analysis. SCF was extracted from porcine semen then purified for post-activation injection after nuclear transfer. The optimal timing for SCF injection was determined to be at least 22 h post-IVM for parthenogenetic activation of bovine oocytes. A total of 364 oocytes were successfully enucleated and 268 (73.6%) fused and were injected with SCF. The survival rate of fused and injected embryos was 109/113 (96.5%) after 2 h. The cleavage rates of nuclear transfer embryos after 3 d of culture in the ionomycin/CHX treated group were significantly higher than those of the SCF-activated group (93.3% *vs.* 81.7%, $p<0.01$, respectively). However, at 7 d and 9 d there was no significant difference between the total developmental rates to blastocyst for either treatment group. Total blastocyst cell numbers were also not significantly different between the two activation treatments (ionomycin/CHX: 149.5 ± 7.7 *vs.* SCF: 139.3 ± 4.4 cells). In contrast, the apoptotic levels in the SCF blastocysts were higher than those produced after the chemical treatment (28.2 ± 5.1% *vs.* 8.8 ± 0.6%, respectively). A total of 18 expanded or hatching blastocysts was transferred to nine synchronized recipients in each activation group; 5/9 (55.5%) and 2/9 (22.2%) were pregnant at 40 d in the ionomycin/CHX treatment and SCF activated group, respectively. However, only one went to term in the ionomycin/CHX treatment while none of the pregnancies from the SCF group were maintained by 90 d.

In conclusion, these results suggest that SCF derived from different species is a limited activator to be used for activation after bovine nuclear transfer in lieu of a chemical activation protocol.

(Key words : porcine sperm cytosolic factor, bovine nuclear transfer, activation)

INTRODUCTION

Offspring from somatic cell cloning have been reported in sheep (Wilmut et al., 1997), cattle (Kato et al., 1998), mice (Wakayama et al., 1998), goat (Baguisi et al., 1999), pig (Polejaeva et al., 2000), cat (Shin et al., 2002), rabbit (Chesne et al., 2003), rat (Zhou et al., 2003), mule (Wood et al., 2003), horse (Galli et al., 2003), dog (Lee et al., 2005) and wolf (Kim et al., 2007). Also, this technology has been combined with transgenic technology and resulted transgenic animal production (Schnike et al., 1997; Cibelli et al., 1998; Baguisi et al., 1999; McCreath et al., 2000; Yin et al., 2008). Since the nuclear transfer procedure itself does not induce adequate activation of oocytes, artificial activation is necessary before or after electrofusion to mimic sperm-induced oocyte cytoplasmic calcium oscillations (Cuthbertson et al., 1985; Fissore et al., 1992; Liu et al., 1998). The efficiency of the nuclear transfer technique and support to term of cloned offspring depends on the activation regimen chosen (Nagai, 1987; Ware et al., 1989; Presicce and Yang 1994; Susko-Parrish et al., 1994; Hill et al., 2000).

During normal physiological fertilization in the eggs of all mammals studied to date, fertilization induces a large intracellular calcium rise (Jaffe, 1980; Whitaker and Patel, 1990) which is followed by multiple oscillations in $[Ca^{2+}]_i$ that last for several hours (Miyazaki et al., 1986; Miyazaki et al., 1993). These $[Ca^{2+}]_i$ spikes are thought to be required for egg activation, which consists of a sequence of events that includes cortical granule exocytosis, resumption of meiosis and extrusion of the second polar body, pronuclear formation, DNA synthesis and first mitotic cleavage (Kline and Kline, 1992; Schultz and Kopf, 1995). In addition, it has been shown that injection of sperm cytosolic factor (SCF), obtained from sperm of several

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species, is able to induce $[Ca^{2+}]_i$ responses similar to those observed during normal fertilization (Swann and Lai, 1997) and completely support normal activation, including *in vitro* development to the blastocyst stage (Stice and Robl, 1990; Wu et al., 1997; Fissore et al., 1998; Wu et al., 1998). In studies involving animal cloning, there is one report that porcine sperm cytosolic extract from crude sperm induced intracellular calcium oscillation in bovine MII oocytes, which were then used as activation treatment and support for full term development in bovine somatic cell nuclear transfer (Knott et al., 2001). Thus, SCF derived from semen of different species may be a useful tool as a "natural" activator to further investigate the function and molecular targets of $[Ca^{2+}]_i$ oscillations not only in the initiation of development but also to observe its effects on *in vivo* developmental survival rates after nuclear transfer.

Mammalian preimplantation embryos cultured *in vitro* are characterized by variable morphology and developmental potential. Cell death has been observed during preimplantation embryogenesis both *in vivo* and *in vitro* in a variety of mammalian species (Brison and Schultz, 1997; Hardy, 1997; Brison and Schultz, 1998; Long et al., 1998; Matwee et al., 2000; Spanos et al., 2000). It has been suggested that apoptosis may be involved in early embryonic arrest and that cytoplasmic fragments are equivalent to apoptotic bodies, i.e., the end product of apoptosis (Jurisicova et al., 1996). Thus, total embryo cell number and apoptosis levels are important parameters that are emerging as useful indicators of normal embryo development and survival to predict the production of live offspring (Watson et al., 2000).

Therefore, this study was conducted to compare the effects of SCF derived from different species and chemical activation on oocytes used for bovine nuclear transfer. *In vitro* developmental rates and fetal survival rates after embryo transfer to recipients were evaluated. Furthermore, the pattern of cell death in the cloned blastocysts was examined by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) method.

MATERIALS AND METHODS

1. All Chemicals were Purchased from Sigma Unless Specified Individually

1) *In Vitro* Maturation

Immature bovine oocytes collected from abattoirs derived ovaries were matured for 20 hr under 38.5℃, 5% CO_2 incubator in TCM 199 (Gibco Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Gibco), 0.1 units/ml FSH(Sioux Biochem, Sioux City, IA), 0.1 units/ml LH (Sioux Biochem), 1 μg/ml estradiol (Sigma, St Louis, MO), 28 μg/ml pyruvate (Sigma), 0.05 μg/ml epidermal growth factor (EGF; Sigma) and 1% Penicillin/Streptomycin (Gibco). The expanded cumulus-oocyte complexes were denuded by vortexing for 3 min in 0.1% hyaluronidase (Sigma) in TL-Hepes after which the oocytes were washed and placed in TCM 199 with 10% FCS.

2) Cell Culture and Preparation of Donor Cells

Adult fibroblast cells were isolated from an ear punch obtained from the bull and cultured in Dulbecco Modified Eagle medium (DMEM; Gibco) supplemented with 10% FCS, and then frozen in LN_2 for storage. These cell lines were thawed and maintained in 4-well multidishes (Nunc, Denmark) in DMEM/F-12 supplemented with 10% FCS + 1% penicillin/ streptomycin (10,000 U/ml penicillin G, 10,000 μg/ml streptomycin) for 3～5 d under 38℃, 5% CO_2 incubator. Prior to nuclear trasnfer the cultured donor cells were trypsinized with trypsin-EDTA solution (Sigma, T-4174 X10) for less than 1 min with gentle pipetting. After the addition of 3 ml of Hepes-buffered TCM 199 (Gibco) supplemented with 10% FCS, the donor cells were washed by centrifugation (3 min, 200 g), then resuspended in medium.

3) Sperm Cytosolic Factor Preparation

Sperm cytosolic factor (SCF) was prepared from boar semen as previously described (Gordo et al., 2000). Briefly, semen samples were washed twice with TL-Hepes medium, and the sperm pellet was resuspended in a solution containing 75 mM KCl, 20 mM Hepes, 1 mM EDTA, 10 mM glycerophosphate, 1 mM dithiothreitol, 200 μM PMSF, 10 μg/ml leupeptin, pH 7.0. The resulting suspension was lysed by sonication for 30～35 min at 4℃ (XL2020; Heat Systems Inc., Farmingdale, NY). The lysate was then centrifuged twice at 10,000 g, and the supernatants were collected and ultracentrifuged at 100,000 g for 1 h at 4℃. Ultrafiltration membranes (Centricon 30; Amicon, Beverly, MA) were used to wash and concentrate the extracts to 60 mg/ml of protein. The crude sperm extracts were then precipitated by exposure to a saturated solution of ammonium sulfate (50% final ammonium sulfate concentration) and centrifuged at 10,000 g for 15 min at 4℃, and the precipitates

were collected and stored at −80℃ until use. The stock SCF was stored at a protein concentration of 15 mg/ml and diluted to 5 mg/ml in buffer (75 mM KCl, 20 mM Hepes, pH 7.0) for injection.

4) Enucleation, Recombination and Electrofusion

Oocytes were enucleated at 21 hr post-maturation (hpm). Prior to enucleation, oocytes were placed for 10 minutes in Hepes buffered TCM 199 supplemented with 10% FCS containing 5.0 μg/ml cytochalasin B and 5 μg/ml Hoechst 33342. All oocytes were carefully selected for presence of the first polar body and a homogeneous cytoplasm. Enucleation was achieved with a beveled 18~20 μm outer diameter glass pipette mounted on Narishige micromanipulators (Medical Systems Corp., Great Neck, NY) on a Zeiss Microscope (Axiovert 135; Zeiss, Germany). Only oocytes in which the removal of both the polar body and metaphase chromosomes was confirmed by observation under UV light were included in the experiment. After trypsin/EDTA treatment of cultured donor cell lines, fibroblasts of median (18~20 μm) size and morphologically round smooth shape were combined with enucleated oocytes. Following recombination, the oocyte-fibroblast couplets were returned to TCM199 with 10% FCS before electrofusion. Electrofusion was performed in 0.3 M mannitol solution (0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$) with two 25 μsec 2.3 kV/cm DC pulses delivered by a BTX Electrocell Manipulator 200 (BTX, San Diego, CA) within 24 hpm. All couplets were moved to TCM 199 supplemented with 10% FCS containing 5.0 μg/ml cytochalasin B and cultured for 1 hr, prior to being transferred to cytochalasin B-free medium for an additional hour. Fusion was then evaluated and only successfully fused embryos were selected and subjected to an activation treatment.

5) SCF Microinjection Technique

Microinjection procedures were modified from the method of Wu et al. (1997). Oocytes or nuclear transfer embryos were placed in a 100 μl drop of TL-Hepes supplemented with 2.5% sucrose (w:v). A 2 μl drop of SCF (5 mg/ml protein concentration) was placed on the same dish. All drops were overlaid with mineral oil. SCF injections were performed on a Zeiss microscope with Narishige manipulators coupled with a Piezo-electric actuator (Primetech, Japan). The amount of injected solution was determined to be about 5~10 pl, resulting in a final intracellular SCF concentration of approximately 2% of the concentration in the injection pipette.

6) Post Fusion Activation of Electrofused Nuclear Transfer Embryos

Chemical oocyte activation was performed 2 hr after fusion by a 4 min incubation in 5 μM ionomycin (Calbiochem, San Diego, CA) followed by 4 min in TL-Hepes with 30 mg/ml BSA. Oocytes were then washed twice in TL-Hepes with 3 mg/ml BSA. Successfully fused embryos were then placed in a well with 500 μl of M199 supplemented with 10% FBS containing 10 μg/ml CHX (Sigma) and 5 μg/ml cytochalasin B for 5 hr (Presicce and Yang, 1994), while the porcine SCF injection was performed 2 hr after fusion with only fused embryos.

7) Evaluation of Activation and Pronucleus Formation with Hoechst 33342 Staining

To evaluate DNA morphology after injection of SCF in bovine MII oocytes, all oocytes were fixed in 3.7% paraformaldehyde in PBS supplemented with 0.05% Triton-X for 4 min. This was followed by washing with PBS supplemented with 3 mg/ml PVP for 4 min, after which the oocytes were mounted in 90% glycerol in PBS supplemented with 1.9 mM Hoechst 33342 to visualize their chromatin. Slides were examined using a Nikon inverted microscope (Nikon, Japan) and images recorded.

8) *In Vitro* Culture after Fusion

Embryos were cultured in G1.2/G2.2 media (Colorado Center for Reproductive Medicine, Englewood, Co) for 7 d as described by Gardner et al. (1994). Embryo development was assessed 7 d after cell fusion by determining the cleavage stage and embryo cell number (Fig. 1).

9) TUNEL Assay and Propidium Iodide (PI) Labeling of Embryonic Nuclei

TUNEL and PI labeling was carried out as described pre-

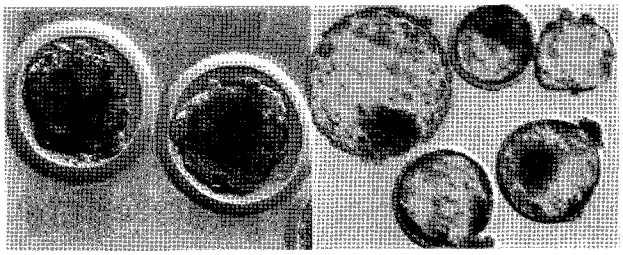

Fig. 1. Sperm factor activated NT embryos developed to compacted morulae (left) and hatching, hatched blastocysts (right) cultured in G1G2 for 7 days.

viously (Watson et al., 2000). Briefly, zona pellucida-intact 7 d nuclear transfer blastocysts were fixed in 1% paraformaldehyde in PBS, pH 7.4, for 1 hr at room temperature. The blastocysts were washed twice in 60 mM PIPES/25 mM Hepes buffer, pH 7.4, and stored in the second wash in a sealed 4-well plate at 4℃ until ready for labeling. An *in situ* cell death detection kit using fluorescein-conjugated dUTP (Roche Molecular Biochemicals, Laval, PQ, Canada) was used for labeling apoptotic cells. Blastocysts were washed with PBS and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 15 min. They were then washed twice with PBS before labeling. Positive control embryos were treated with 50 U/ml RQ1 RNase-free Dnase for 20 min at 37℃ and then washed twice with PBS prior to labeling. The TUNEL reagent was prepared immediately before use and kept on ice. Blastocysts were placed in 50-μl drops of TUNEL reagent, covered with filtered paraffin oil in 35-mm plastic dishes and incubated in the dark at 37℃ for 60 min in a humidified chamber. The blastocysts were washed three times in PBS with 1% BSA and incubated with 50 μg/ml Rnase A in the dark at room temperature for 60 min. Total cell nuclei were labeled with 40 μg/ ml PI (Sigma-Aldrich) in sterile H_2O in the dark at room temperature for 60 min (Fig. 2). The embryos were washed twice in PBS with 1% BS and mounted in Fluoroguard antifade mounting reagent (Bio-Rad, Mississauga, ON, Canada).

10) Imaging Microscopy and Analysis

Initially, the blastocysts were viewed with confocal microscopy, however, this proved to be inefficient for accurate cell counting of total nuclei in 7 d nuclear transfer blastocysts. To circumvent this, standard epifluorescence microscopy methods were adapted and applied to flattened blastocysts to generate digitized images that were analyzed with Northern Exposure image analysis software (Empix Imaging, Mississauga, ON, Canada) to quantify cell counts. Digital still images were captured through an integrating Sony (Park Ridge, NJ) XC-75 CCD video camera module. The camera was controlled through a personal computer (PII-400, 128 MB RAM, 8.4 GB HD, Hewlett-Packard 800 series CD-writer; Palo Alto, CA) running Empix Imaging software. Images of the fluorescein-labeled apoptotic cells and the PI-labeled nuclei were recorded using a Leica Orthoplan microscope (Milton Keynes, Bucks, UK) equipped with a Ploempak epifluorescence module and a full turret of high numerical aperture Plan-APO fluorite lenses.

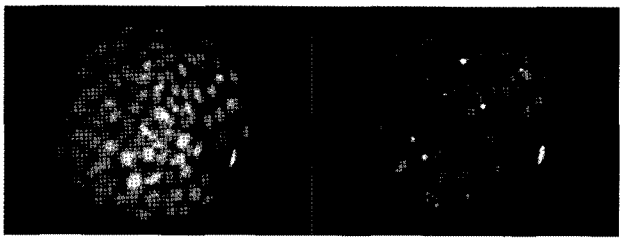

Fig. 2. TUNEL images of PI stained blastocyst blastocyst (left) and fluorescence image for apoptotic nuclei (right) produced from porcine sperm cytosolic factor activated NT embryo.

11) Embryo Transfer and Pregnancy Diagnosis

Nuclear transfer blastocysts were classified by their morphology on 7 d. Two hatching or expanded blastocysts were non-surgically transferred to each recipient when available. Pregnancy status was assessed by transrectal ultrasonography (Aloka 500, 5-MHz transducer; Aloka Co., Tokyo, Japan) at 40 d after nuclear transfer and rechecked regularly.

12) Statistics

Statistical comparisons between treatment groups were carried out using the chi-square test for activation rates of bovine oocytes after SCF injection and *in vitro* developmental rates of bovine nuclear transfer embryos cultured in G1.2/G2.2 medium, ANOVA with Fisher's exact value was used for comparison of total cell numbers and apoptotic levels of d 7 nuclear transfer embryos. All analyses were performed using the Statview® software (SAS Institute, Cary, NC). Experiments were repeated at least three times, and the number of oocytes used per experiment is indicated in the Results section and in the tables.

13) Experimental Design

The objective of each experiment was as follows: Experiment 1: Evaluation of bovine oocyte activation after porcine SCF injection. Experiment 2: Comparison of *in vitro* development rates of bovine nuclear transfer embryos after chemical *vs.* SCF activation. Experiment 3: Comparison of total blastocyst cell number and nuclear apoptotic levels in nuclear transfer embryos between the same two activation treatment groups as in Experiment 2 using the TUNEL assay. Experiment 4: Comparison of *in vivo* survival rates of nuclear transfer blastocysts from the two different activation treatments after embryo transfer to recipients.

RESULTS

Experiment 1: Effect of Porcine SCF on Parthenogenetic Activation of Bovine Oocytes and Post Survival Rates after SCF Injection with Nuclear Tranfer Embryos

To determine the optimal timing for SCF activation of bovine oocytes, SCF was injected into oocytes at different hour post maturation (hpm). A total of 91 bovine oocytes were injected and survived without lysis or rupture of the oolemma after injection. Intact oocytes were cultured for 18~20 h after injection before being evaluated for activation. Activation rates (as determined by formation of one or more pronuclei) were 14.8%, 65.0% and 79.5% when oocytes were injected at 19~21, 22~26 and 26~28 hpm, respectively (Table 1). As a result of these experiments, it was determined that for the nuclear transfer experiments, the oocytes would be activated at least 26 hpm.

In addition, the electrofusion and survival rates after SCF injection for post-fusion activation treatment were evaluated. A total of 364 oocytes were successfully enucleated and 268 (73.6%) of those fused with the cytoplast and were subsequently injected with porcine SCF. The survival rates of fused and injected embryos were 109/113 (96.5%) after 2 h after injection (data not shown).

Experiment 2: *In Vitro* Development Rates of Bovine Nuclear Transfer Embryos Produced from Chemical vs. SCF Activation

Table 1. Activation rates of bovine oocytes after sperm cytosolic factor injection at different hour post maturation (hpm)

Treatment group	No. of injected MII oocytes	No. activated	
		PN (%)	MII
hpm 19~21	27	4 (14.8)[a]	23
hpm 22~26	20	13 (65.0)[b]	7
hpm 26~28	44	35 (79.5)[b]	9

As determined by the chi-square test, treatments with different superscripts were significantly different ($p<0.001$).

To compare the *in vitro* developmental rates of nuclear transfer embryos activated by SCF injection with those of activated with a standard chemical treatment, nuclear transfer experiments were conducted with either ionomycin activation followed by CHX and cytochalasin B treatment *vs.* SCF activation. The cleavage rates of nuclear transfer embryos after 3 d of culture in the ionomycin/CHX treatment group were significantly higher than those obtained after SCF activation (93.3% v. 81.7%, $p<0.01$, respectively; Table 2). However, there were no significant differences between the two activation treatments at 7 d or 9 d in terms of total developmental rates to blastocyst.

Experiment 3: Comparison of Total Blastocyst Cell Number and Nuclear Apoptotic Levels of 7 d Nuclear Transfer Embryos Activated by SCF Injection or Chemical Treatment

To investigate the total cell number and apoptotic levels of nuclear transfer embryos, blastocysts were fixed and processed for TUNEL labeling. There were no significant difference in total cell numbers of blastocysts between the two activation groups (ionomycin/CHX: 149.5 ± 7.7 *vs.* SCF: 139.3 ± 4.4 cells; Table 3). In contrast, the apoptotic levels of SCF blastocysts were higher than those resulting from chemical activation (28.2 ± 5.1% *vs.* 8.8 ± 0.6% apoptotic nuclei, respectively).

Experiment 4: *In Vivo* Survival Rates of Nuclear Transfer Embryos after Transfer to Recipients

To evaluate the *in vivo* survival rates of embryos produced

Table 3. Comparison of total cell numbers and apoptotic levels of d 7 nuclear transfer embryos activated either by SCF injection or chemical activation

Treatment	No. total nuclei	% apoptotic nuclei
Ionomycin/CHX/CB	149.5 ± 7.7	8.8 ± 0.6[a]
SCF	139.3 ± 4.4	28.2 ± 5.1[b]

Values are given as mean ± SEM. As determined by the ANOVA (Fisher's exact value) with different superscripts were significantly different ($p<0.001$).

Table 2. *In vitro* developmental rates of bovine nuclear transfer embryos following activation by either SCF or Ionomycin+CHX

Treatment	No. cultured	No. cleaved (%)	No. 7 d BL (%)	No. of total BL (9 d, %)
Ionomycin+CHX(CB)	119	111 (93.3)[a]	44 (39.6)	49 (44.1)
Sperm cytosolic factor	109	89 (81.7)[b]	26 (29.2)	40 (44.9)

As determined by the chi-square test, treatments with different superscripts were significantly different ($p<0.01$).

Table 4. Pregnancy rates at 40 d and 90 d after embryo transfer of nuclear transfer produced embryos

	No. embryos transferred	No. recipient	No. pregnant (%)	
			At day 40	At day 90
Ionomycin/CHX	18	9	5 (55.6)	1 (11.1)
SCF	18	9	2 (22.2)	0

by nuclear transfer activated by either SCF injection or by ionomycin/CHX treatment. A total of 18 expanded or hatching blastocysts were transferred to nine synchronized recipients for each activation group. Of the ionomycin/CHX activated embryos, 5/9 recipients were pregnant at 40 d while 2/9 of the recipients receiving SCF treated embryos were pregnant at the same time. By 90 d, only one of the recipients of the ionomycin/CHX activated embryos was still pregnant, while none of the recipients which had received a SCF-activated embryo was pregnant (Table 4).

DISCUSSION

During normal fertilization, a series of $[Ca^{2+}]_i$ oscillations in the oocyte's cytoplasm results in calcium being released from the oocyte's endoplasmic reticulum (Miyazaki et al., 1986). It is thought that these $[Ca^{2+}]_i$ spikes trigger oocyte activation (Kline and Kline, 1992). Different theories exist on sperm-mediated oocyte activation. In the receptor theory, the initiation of the activation reaction begins as the sperm binds to an oolemmal receptor, which leads to receptor-mediated production of inositol-1,4,5-triphosphate (IP3). The IP3 produced then triggers the release of $[Ca^{2+}]_i$ from intracellular stores. Components of pathways involved in receptor-mediated IP3 production have been observed in mammalian oocytes, including G-protein and tyrosine kinase-coupled receptors (Williams et al., 1992; de Nadai et al., 1996; Dupont et al., 1996; Miyazaki, 1988). However, to date there has been no conclusive proof for this theory (Wu et al., 1998). An alternative hypothesis involves a soluble "sperm cytosolic factor" which is released from the sperm when the gametes fuse. This SCF might induce $[Ca^{2+}]_i$ oscillations by interacting with some component in the oocyte's cytoplasm. Support for this theory comes in part from experiments with sperm injected directly into the cytoplasm of oocytes, which has shown that the injected sperm can induce $[Ca^{2+}]_i$ spikes and activate the oocyte despite a lack of any gamete membrane interactions (Tesarik et al., 1994; Dozortsev et al., 1995). In addition, the injection of cytosolic sperm extracts into oocytes of different species has been demonstrated to induce $[Ca^{2+}]_i$ signals similar to those induced at fertilization, and which are capable of oocyte activation (Stice and Robl, 1990; Swann 1990; Swann 1992; Wu et al., 1998). However these theories remain to be fully substantiated.

Based on the second theory, in this study SCF extracts from porcine sperm were used for application in the bovine nuclear transfer procedure. Before using the SCF for nuclear transfer, SCF was tested by injection into mature bovine oocytes and confirmed that $[Ca^{2+}]_i$ oscillations were elicited as reported previously (data not shown) (Wu et al., 1997; Fissore et al., 1998). The results from the experiments presented here indicate that bovine oocytes can successfully be acated by injection with porcine SCF although there were significant differences in the activation efficiency depending on the timing of injection relative to the *in vitro* maturation times of the oocytes. In addition, these results support the conclusion that porcine SCF can also be used for activation of oocytes during bovine nuclear transfer procedures. Since the SCF is derived from sperm, the physiological activators of oocytes, SCF could be considered to be a more "natural" activator than standard chemical activation protocols. A previous report showed patterns of oscillation with porcine SCF (Knott et al., 2001) in which the appropriate amplitudes, frequencies and durations of calcium rise, similar to fertilization. However, it remains unclear because those were not exactly the same phenomena of fertilization in homologous species, even if the SCF is not related to the species' specificity.

The overall survival rates of oocytes microinjected with SCF after nuclear transfer and electrofusion were 96.5% 2 hr post-injection. This indicates that further microinjection after electrofusion did not significantly affect the total nuclear transfer efficiency. When comparing the *in vitro* development rates of nuclear transfer embryos activated with two different activation protocols, the total blastocyst developmental rates in this study were very similar to those of previous report (Wells et al., 1999; Zakhartchenko et al., 1999; Kubota et al., 2000) but better than Knott et al. (2001) who reported 13% of blastocyst

developmental rates with porcine SCF activation in bovine nuclear transfer. It was noted that the cleavage rates of nuclear transfer embryos after 3 d of culture in the ionomycin/CHX treatment group were significantly higher than those of SCF-activated embryos (93.3% vs. 81.7%, respectively). It is possible that the variation of *in vitro* developmental rates may be altered by activation protocols for nuclear transfer as well as parthenogenetic activation. However, by 7 d and 9 d of culture the differences between the two treatments were no longer apparent, at least in terms of numbers of blastocysts produced. These results suggest that SCF activation alone might not be as efficient as ionomycin treatment followed by the protein synthesis inhibitor CHX for promoting *in vitro* cleavage. However, since there was no significant difference in blastocyst development rates between the chemical and SCF treatments it does not appear that the treatments differ in their ability to promote the formation of later embryos.

Indicators of embryo viability such as metabolism, embryo cell number, cryopreservation survival, and especially pregnancy and calving results are critical for the embryo transfer industry (Krisher et al., 1999). Therefore, it was examined by total blastocyst cell number and apoptosis levels of *in vitro* produced nuclear transfer 7 d blastocysts. Apoptosis is a physiological process of cell death that occurs in eukaryotic multicellular organisms during cell division, cell differentiation and programmed cell death. It can also occur at the embryonic stage in response to abnormalities or impaired development of embryonic blastomeres (Matwee et al., 2000).

I confirmed that normal cell death in bovine nuclear transfer embryos occurs by apoptosis, as defined by the TUNEL assay. The formation of the mammalian blastocyst involves processes required for tissue homeostasis and morphogenesis, e.g., cell proliferation and differentiation, as well as cell death. During the first four rounds of cell division, normal cell death does not occur in blastomeres. It was shown during blastocyst formation in a variety of species including mouse, rat, and human and occurs predominantly in the ICM (Brison and Schultz, 1997; Brison and Schultz, 1998) and bovine *in vitro* matured, fertilized, cultured blastocysts (Watson et al., 2000). Although the role of cell death in the early embryo is unknown, it may involve the removal of cells with abnormal properties or inappropriate potential.

It has been reported that 9.5～13% of average apoptotic levels were evaluated blastocyst from bovine IVM/IVF/IVC system (Watson et al, 2000). These were similar to those of 7d nuclear transfer embryos activated with ionomycin followed by CHX+CB treatment, however the high apoptosis levels in SCF activated nuclear transfer embryos were observed in this study. Gordo et al. (2000) have shown that $[Ca^{2+}]_i$ oscillations are required to activate mouse oocytes and critical for the initiation of development, but if induced at significantly higher frequency than normal, they may trigger abnormal activation and developmental arrest. Although it is not exactly the same as normal fertilization, Ca^{2+} transients related to duration, frequency, and intensity may affect DNA synthesis, protein translation, and initiation of transcription (Fissore et al., 1999a).; however, the impact of such effects on development is unknown (Fissore et al., 1999b). In addition, the different pregnancy rates achieved in this study for the ionomycin/CHX and SCF activation treatments after nuclear transfer may have been caused by factors such as those mentioned above. The high rates of fetal loss following early pregnancy diagnosis seen in the present study have been observed in cloned pregnancies in cattle, sheep and mice (Schnieke et al., 1997; Wilmut et al., 1997; Cibelli et al., 1998; Wakayama et al., 1998; Wells et al, 1999) including abnormal placentation for the cloned fetus (Hill et al., 2000).

In summary, the results presented here indicate that SCF derived from different species is a limited activator to be used for activation treatment during bovine nuclear transfer. SCF may be thought of as a more natural activator than standard chemical treatments since it is derived from the sperm, which is thought to be the physiological initiator of activation at fertilization. SCF causes multiple, fertilization-like $[Ca^{2+}]_i$ oscillations at injection, whereas chemical treatments usually only stimulate a single $[Ca^{2+}]_i$ release. Furthermore, transferred donor cell nuclei were successfully reprogrammed after SCF activation and resulted in embryos which sustained pregnancies at least to 40 d in recipients. Further research is needed to optimize the concentration of SCF injected, the timing of activation, as well as the gene expression patterns that result after this activation to clarify the role of SCF in activation. This would also likely enhance the full term developmental capability of the embryos produced and could increase the total efficiency rates for cloning cattle by nuclear transfer.

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