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***In vitro* development of cloned bovine embryos produced by handmade cloning using somatic cells from distinct levels of cell culture confluence**

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ABSTRACT. The relationship between the level of cell confluence near the plateau phase of growth and blastocyst yield following somatic cell cloning is not well understood. We examined the effect of distinct cell culture confluence levels on *in vitro* development of cloned bovine embryos. *In vitro*-matured bovine oocytes were manually bisected and selected by DNA staining. One or two enucleated hemi-cytoplasts were paired and fused with an adult skin somatic cell. Cultured skin cells from an adult Nelore cow harvested at three distinct culture confluence levels (70-80, 80-90, and >95%) were used for construction of embryos

and hemi-embryos. After activation, structures were cultured *in vitro* as one embryo (1 x 100%) or as aggregates of two hemi-embryos (2 x 50%) per microwell. Fusion, cleavage and blastocyst rates were compared using the χ^2 test. The fusion rate for hemi-embryos (51.4%) was lower than for embryos (67.6%), with no influence of degree of cell confluence. However, blastocyst rates improved linearly (7.0, 17.5, and 29.4%) with increases in cell confluence. We conclude that degree of cell culture confluence significantly influences subsequent embryo development; use of a cell population in high confluence (>90%) for nuclear transfer significantly improved blastocyst yield after cloning.

Key words: Somatic cell nuclear transfer; Cell culture confluence; Cell cycle; Embryo aggregation; Cattle

INTRODUCTION

In vitro embryo production by somatic cell nuclear transfer (SCNT) has evolved significantly in the past decade. However, the procedure *per se* has been implicated as an important cause of incomplete genomic remodeling (Humpherys et al., 2002), which in turn is linked to increased rates of fetal and placental abnormalities and pregnancy losses (Constant et al., 2006; Farin et al., 2006; Bertolini et al., 2007).

The synchrony between the cell cycle of the donor nucleus and the recipient cytoplasm is considered to be one of the key factors needed to increase nuclear reprogramming capacity and, thereby, cloning efficiency (Campbell et al., 1996). The use of quiescent donor cells in the G0 or arrested in the G1 phases of the cell cycle has become a rule in cloning (Gibbons et al., 2002), since such cell cycle phases are considered by many investigators as more suitable for proper reprogramming (Campbell et al., 1996; Wells et al., 1999; Kubota et al., 2000; Kues et al., 2000; Liu et al., 2004). In addition, the use of donor cells at other cell cycle phases usually leads to poor embryo development after cloning due to either chromosome pulverization induced by premature chromosome condensation when occurring in S phase, or aneuploidy in G2/M phases (Campbell et al., 1996).

Several protocols have been proposed over the years for the cell cycle synchronization of donor cells in G0/G1 phase. Some protocols require the manipulation of the culture conditions, such as serum starvation (Wilmut et al., 1997) or cell contact inhibition by high culture confluence (Boquest et al., 1999). However, wide variations in time for proper cell cycle synchronization or culture confluence, with more unpredictable results, make such procedures less practical for scheduled SCNT routines, requiring a certain level of expertise and knowledge of the cell lineage or type in use (Choresca et al., 2009). Alternatively, certain protocols make use of drugs that directly or indirectly halt the cell cycle, such as treatments with roscovitine (Gibbons et al., 2002), dimethyl sulfoxide (Hashem et al., 2006) or cycloheximide (Goissis et al., 2007). The use of such cell cycle-synchronizing agents may be effective, but the rather broad spectrum of interference with other cellular processes, along with potential toxic side effects, may induce cell damage or death, which may be consistent with DNA damage (Koo et al., 2009), along with unintended consequences of concern for further development (Gibbons et al., 2002).

As observed, all procedures have advantages and limitations. Among them, cell confluence by contact inhibition appears to be one of the most widely used methods nowadays (Campbell et al., 2007; Sun et al., 2008). With such method, by attempting to induce growth inhibition by cell contact, changes in cell growth pattern or culture conditions will occasionally delay or accelerate cell growth, resulting in suboptimal cell populations for cloning, especially when cultures may be sub-confluent (Campbell et al., 1996; Cho et al., 2005; Choresca et al., 2009). Recently, Sun et al. (2008) demonstrated that a high proportion of cells in G0/G1 (91.4%) were present in highly confluent cell cultures at the plateau phase (>90% confluence), with that proportion significantly falling (59.3%) when cultures were in log phase (50-60% confluence). However, the relationship between the level of cell confluence in a culture dish at or near the plateau phase of cell growth and blastocyst yield following cloning by SCNT still needs to be better characterized. Therefore, this study aimed to examine the effect of three distinct somatic cell culture confluence intervals prior to nuclear transfer on *in vitro* development of cloned bovine embryos.

MATERIAL AND METHODS

All media and reagents were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), unless otherwise stated.

Isolation and culture of bovine ear skin cells

Primary skin cell cultures were established from ear biopsies aseptically collected from an adult Nellore female donor, following our standard procedures (Ribeiro et al., 2009). Briefly, upon biopsy collection, the ear tissue was diced into 3-mm pieces and placed in 35-mm tissue culture dishes (Corning Incorporated, NY, USA) containing Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 0.22 mM sodium pyruvate, 26.2 mM sodium bicarbonate, 100 IU/mL penicillin G, 100 µg/mL streptomycin sulfate, 0.25 µg amphotericin B/mL, and 10% fetal bovine serum (FBS; Gibco-BRL). Dermal fibroblast-like cell cultures were established, expanded and maintained at 38.5°C, 5% CO₂, and 95% humidity, until needed for cloning.

Bovine embryo production

Prior to the cloning procedure, cell cultures were assessed by morphological evaluation and segregated into three distinct culture confluence intervals at or near the plateau phase of growth: lower confluence (L), 70-80%; medium confluence (M), 80-90%, and higher confluence (H), >95%. The cells were then used for the reconstruction of hemi-embryos (50% volume) and embryos (100% volume). Bovine embryos were produced by handmade cloning or by parthenogenesis, based on our established procedures (Ribeiro et al., 2009), as described below.

In vitro maturation and cytoplasm selection

After *in vitro* maturation (17 h) of selected cumulus-oocyte complexes from bovine ovaries obtained from a local slaughterhouse, cumulus-oocyte complexes were denuded by

pipetting in HEPES-buffered M-199 + 10% FBS (HM), followed by polar body selection and zona pellucida removal in 0.5% protease in HEPES-buffered M-199 + 0.01% PVA (HP). Zona-free oocytes were rinsed several times in HM, incubated for 5 to 10 min in 5 µg/mL cytochalasin B in HM in groups of up to 3 oocytes in 5-µL drops under oil, and hand-bisected (Ultra-Sharp Splitting Blade, Bioniche, USA). Hemi-oocytes were selected after screening for nuclear material under UV light in 10 µg/mL bisbenzimidazole in HM (Hoechst 33342).

Embryo reconstruction and activation

For reconstruction and nuclear transfer, one or two enucleated hemi-oocytes (hemi-cytoplasts) were quickly exposed to 500 µg/mL phytohemagglutinin in HP, and then attached to one donor cell, achieving approximately either 50 or 100% of the final cytoplasmic volume, respectively. Only small, round and intact cells, with visibly soft membranes, were used for embryo reconstruction, in an attempt to increase the proportion of cells in G0/G1 phase of the cell cycle (Boquest et al., 1999). Following attachment, reconstructed hemi-embryos (50% volume) or embryos (100% volume) were electrofused by two 1.2-kV/cm DC pulses for 10 µs, after a brief exposure to a 30-V pre-fusion AC pulse, in electrofusion medium (300 mM mannitol, 0.1 mM MgSO₄·7H₂O, 0.05 mM CaCl₂·2H₂O, 0.5 mM HEPES, 0.01% PVA), in a BTX 453 chamber (BTX Instruments, Genetronics, San Diego, CA, USA) coupled to an electrofusion apparatus (Voltain™ EP-1 Cell Fusion System, Cryologic, Australia). Fusion outcome was assessed within 60 to 90 min, followed by chemical activation of reconstructed clone embryos, hemi-embryos, and zona-intact oocytes (control group by parthenogenesis) in 5 µM ionomycin in HM for 5 min, and incubation in 2 mM 6-DMAP in mSOFaa + 0.4% BSA for 4 h. Activation was initiated 24.8 ± 1.0 h after the onset of maturation, with a fusion-activation interval of 2.5 ± 0.8 h.

In vitro culture conditions

Cloned embryos were cultured *in vitro* individually in microwells using the well-of-the-well (WOW) system, based on Vajta et al. (2000), and modified by Feltrin et al. (2006). Activated cloned embryos (1 x 100%) or two cloned hemi-embryos (2 x 50%, embryo aggregation) were placed in each microwell (WOW), comprising approximately 100% of the final normal embryo volume per microwell. All structures were cultured *in vitro* in 400 µL mSOFaa + 5% mare estrous serum, supplemented with 5 µg/mL insulin, 5 µg/mL transferrin and 5 ng/mL sodium selenite (I-1884), under mineral oil, at 39°C and 5% CO₂, 5% O₂ and 90% N₂ for 7 days, to the blastocyst stage. Cleavage rate was assessed 48 h after activation. Control groups containing zona-intact parthenote embryos were cultured *in vitro* under the same conditions as above, but in 4-well dishes with no microwells, and served in this study as controls for egg quality, manipulation and culture conditions.

Statistical analysis

Fusion, cleavage and blastocyst rates were compared using the χ^2 test (Minitab, State College, PA, USA), for $P < 0.05$. The dependence of embryo developmental rate on cell confluence was analyzed by linear regression analysis.

RESULTS

A maturation rate of 65.6% (2506/3822) was observed after 11 replications. A total of 1897 hemi-cytoplasts were used to reconstruct 455 hemi-embryos (50%) and 695 embryos (100%). The fusion rate for hemi-embryos (234/455, 51.4%) was lower than for embryos (470/695, 67.6%), with cell confluence having no effect on fusion.

Results for embryo development are summarized in Table 1. Cleavage rates were similar between all groups, but blastocyst rates increased linearly ($R = 0.999$, $P = 0.023$) with increase in cell confluence. The mean blastocyst rates for both embryo types combined (2 x 50% and 1 x 100%) were 7.0, 17.5, and 29.4% for L, M, and H confluence intervals, respectively, with cell confluence being a strong predictor of development to the blastocyst stage (regression equation $y = 11.2x - 4.4$, where y is blastocyst rate and x is cell confluence interval). The aggregation scheme did not appear to have influenced, positively or negatively, the developmental outcome to the blastocyst stage.

Table 1. *In vitro* development of cloned bovine embryos from distinct cell culture confluences and aggregation scheme.

Embryo type	Cell culture confluence	Aggregation scheme	N [†]	Cleavage rate		Blastocyst rate	
				N	%	N	%
Clones	70-80% (L)	2 x 50%	8	6	75.0	0	0.0 ^a
		1 x 100%	134	104	77.6	10	7.5 ^b
	80-90% (M)	2 x 50%	34	22	64.7	7	20.6 ^{c,d}
		1 x 100%	149	115	77.2	25	16.8 ^c
	>95% (H)	2 x 50%	50	42	84.0	17	34.0 ^d
		1 x 100%	164	130	79.3	46	28.0 ^d
Parthenotes	n/a	1 x 100%	144	103	71.5	31	21.5 ^{c,d}

^{a,b,c,d}P < 0.05 (columns). L = lower confluence; M = medium confluence; H = higher confluence. [†]11 replications; n/a = not applicable. Cleavage and blastocyst rates were analyzed by the well-of-the-well (WOW) system.

DISCUSSION

Since the first successful live birth of a cloned mammal from adult origin (Wilmut et al., 1997), SCNT procedures have evolved considerably. However, the cloning process as a whole still remains inefficient, with crucial steps yet to be better refined and the associated biological events to be fully characterized. For the time being, pre- and postnatal deviations in development will continue as unpredictable consequences of cloning by SCNT, limiting the transfer of such technology to commercial applications (Farin et al., 2006; Bertolini et al., 2007).

Successful development is dependent on numerous factors, including nuclear-transfer procedures, laboratory and technician proficiencies, type, source and quality of recipient cytoplast, donor cell type and lineage, method of reconstruction, activation process, embryo culture system, and donor and recipient cell cycle stages (Campbell et al., 2007). The influence of cell confluence at or near the plateau phase of the cell growth curve on *in vitro* embryo development was clearly demonstrated in this study. A significant linear increase in blastocyst rate (7.0, 17.5, and 29.4%) followed an increase in cell confluence and, by inference, an increase in cell contact inhibition. Such differences in development are likely to be due to a greater proportion of cells

in G0/G1 in highly confluent cell cultures (H, at the plateau) when compared with cells in the transition between the log phase and the plateau phase (L and M). We did not evaluate the cell cycle distribution by flow cytometry in each cell confluence interval in this study, but our results agree, at least in part, with findings of a previous study in which more than 85% of cells were in G0/G1 when from highly confluent cultures, whereas cell cultures in log phase (50-60% confluence) had only 74.1% of cells in G0/G1 (Boquest et al., 1999). The high proportion of cells at the G0/G1 stage in highly confluent cell populations was also recently demonstrated in cattle (Sun et al., 2008), goldfish (Choresca et al., 2009) and dogs (Koo et al., 2009), which compared different methods for synchronization of somatic cells in this specific phase, analyzed by flow cytometry.

Gibbons et al. (2002) compared serum starvation and roscovitine treatment as cell synchronizing protocols, observing a similar *in vitro* embryo developmental rate with both treatments, but higher *in vivo* development using roscovitine in the cell culture prior to cloning. Also, Choresca et al. (2009), after analyzing serum starvation, culture to confluence, and roscovitine treatment in goldfish cells in culture, and Koo et al. (2009), comparing the use of serum starvation, culture to confluence, and cycloheximide, DMSO, and roscovitine treatments in cultured dog cells, recommended the use of roscovitine due to a slightly higher proportion of cells in G0/G1, or due to a faster synchronizing response after the onset of the treatment compared with the other protocols. However, the latter studies only evaluated the cell cycle proportion in cells in culture, not using such cells for embryo cloning.

Using bovine cells arrested in G0/G1, Sun et al. (2008) observed that cell contact inhibition by high culture confluence was more effective in supporting development to the blastocyst stage than by serum starvation (58 vs 44%, respectively), whereas Hayes et al. (2005) did not observe any differences in *in vitro* development of bovine embryos between treatments. Nonetheless, the authors only used cells in high confluence for cloning, at the plateau phase, not evaluating cloning efficiency at lower confluence intervals. All-in-all, most authors above describe conflicting evidence suggesting beneficial effects by the use of roscovitine treatment for cell cycle synchronization. However, providing the necessary time for a cell culture to attain high confluence, the proportion of cells in G0/G1 in those studies appeared to be higher than any other protocol. Therefore, serum starvation or the use of chemical agents for cell cycle synchronization prior to somatic cell cloning not only impose a potential stressful or toxic condition on cells, but also do not appear to promote any improvement in embryo development compared with cell contact inhibition by high confluence.

The evaluation of the potential benefit of aggregating embryos was based on previous studies, including ours (Ribeiro et al., 2009), in which increased blastocyst rates and/or better embryo quality was observed following the aggregation of cloned mouse (Boiani et al., 2003) or bovine (Misica-Turner et al., 2007; Ribeiro et al., 2009) embryos during *in vitro* culture. Within each confluence interval, the aggregation scheme had no influence on blastocyst development. However, the 2 x 50% group from the intermediate confluence interval (M) demonstrated a similar pattern of development, unlike the two groups of embryos (2 x 50% and 1 x 100%) produced using cells at the highest confluence. Such data suggest that the aggregation of embryos may promote a paracrine positive effect on embryo development, perhaps becoming more evident when cell donor conditions are sub-optimal, corroborating observations by others (Boiani et al., 2003; Misica-Turner et al., 2007). However, the benefit of embryo aggregation on development of cloned embryos varying donor cell culture conditions still needs to be further elucidated.

In summary, we demonstrated the existence of a positive linear effect of cell confluence level at or near the plateau phase of the growth curve on subsequent *in vitro* embryo development after cloning by SCNT. Cell contact inhibition is an efficient method for cell donor synchronization for cloning, providing that the cell population is harvested at a high culture confluence condition.

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