CLONING AND STEM CELLS
Volume 11, Number 3, 2009

DOI: 10.1089/clo.2009.0022

Original Article

Developmental Potential of Bovine Hand-Made Clone Embryos Reconstructed by Aggregation or Fusion with Distinct Cytoplasmic Volumes

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Abstract

Animal cloning has been associated with developmental abnormalities, with the level of heteroplasmy caused by the procedure being one of its potential limiting factors. The aim of this study was to determine the effect of the fusion of hemicytoplasts or aggregation of hemiembryos, varying the final cytoplasmic volume, on development and cell density of embryos produced by hand-made cloning (HMC), parthenogenesis or by *in vitro* fertilization (IVF). One or two enucleated hemicytoplasts were paired and fused with one skin somatic cell. Activated clone and zona-free parthenote embryos and hemiembryos were *in vitro* cultured in the well-of-the-well (WOW) system, being allocated to one of six experimental groups, on a per WOW basis: single clone or parthenote hemiembryos (1×50%); aggregation of two (2×50%), three (3×50%), or four (4×50%) clone or parthenote hemiembryos; single clone or parthenote embryos (1×100%); or aggregation of two clone or parthenote embryos (2×100%). Control zona-intact parthenote or IVF embryos were *in vitro* cultured in four-well dishes. Results indicated that the increase in the number of aggregated structures within each WOW was followed by a linear increase in cleavage, blastocyst rate, and cell density. The increase in cytoplasmic volume, either by fusion or by aggregation, had a positive effect on embryo development, supporting the establishment of pregnancies and the birth of a viable clone calf after transfer to recipients. However, embryo aggregation did not improve development on a hemicytoplast basis, except for the aggregation of two clone embryos.

Introduction

Somatic cell nuclear transfer (SCNT) technology is an excellent model for studies in developmental biology and mammalian embryology. However, somatic cell cloning usually leads to incomplete genomic remodeling, which is linked to increased rates of fetal and placental abnormalities and pregnancy losses (Bertolini et al., 2007; Bourc'his et al., 2001; Hill et al., 2000; Reik et al., 2003; Xue et al., 2002). Distinct epigenetic features, manifested by increased levels of DNA methylation (Kang et al., 2001), along with suggested changes in DNA methyltransferase mRNA relative abundance in SCNT-derived bovine embryos (Wrenzycki et al., 2001), are thought to affect the pattern of expression of a variety of imprinted and nonimprinted genes after *in vitro* embryo manipulations (Bertolini et al., 2002, 2004; Wrenzycki et al., 1998, 1999, 2001; Yang et al., 2007). Remarkably, a certain level of variation in gene expression after cloning is fully tolerable and compatible with development (Wells et al., 2003; Yang et al., 2007). Yet, the mechanisms involved in reprogramming after nuclear transfer (NT) are still widely unknown. A better understanding of such mechanisms is essential for NT to become more reliable and efficient for practical uses.

In early embryos, genomic reprogramming occurs rapidly after fertilization, with the chromatin reconfiguration being established before and during pronuclear formation (Reik et al., 2001). Comparatively, a somatic nucleus in NT procedures is introduced into an enucleated oocyte at a point in development compatible with the pronuclear stage (Reik et al., 2001). Consequently, chromatin configuration does not seem to occur properly in time and space after cloning, creating a functional and molecular asynchrony between the

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donor nucleus and the ooplasm. In this process, the ooplasmic components seem to be insufficient to modify or erase marks of differentiation in the donor genome, leading to faulty or incomplete epigenetic reprogramming (Bird, 2002).

One of the least investigated aspects associated with faulty reprogramming after cloning by SCNT is the heteroplasmic nature of the process per se. In conventional cloning, the fusion of a somatic cell to an enucleated oocyte causes a merge of two distinct cytoplasms, along with their components, which represents an unpredictable factor in development. With the establishment of hand-made cloning (HMC) procedures (Vajta et al., 2000, 2001, 2003), the level of heteroplasmy is further increased, because two enucleated hemioocytes are usually needed for embryo reconstruction. Consequently, the process of heteroplasmy by fusion of distinct oocytes can, on one hand, promote or increase developmental capacity (Liu and Keefe, 2000), also increasing cell density (Tecirlioglu et al., 2005). Alternatively, the fusion of incompatible oocytes or between oocytes with distinctive maturation and developmental capacities can create a detrimental level of heteroplasmy to cloning efficiency (Vajta et al., 2005). An alternative to circumvent the degree of heteroplasmy caused by fusion during cloning by HMC, at least partially, is embryo aggregation (Misica-Turner et al., 2007; Vajta et al., 2005), which could minimize any unfavorable effects caused by fusion. Due to the importance of such events on genomic reprogramming and developmental normality after somatic cell cloning, systematic studies comparing the developmental potential of bovine hand-made clone embryos reconstructed by aggregation or fusion with distinct cytoplasmic volumes are still a need. Thus, the aim of this study was to determine the effect of the level of heteroplasmy after embryo reconstruction by fusing hemioocytes or by aggregating hemiembryos, varying the final cytoplasmic volume (50, 100, 150, and 200%), on *in vitro* development and cell density of day −7 blastocysts.

Materials and Methods

All chemicals were from Sigma Chemical Co. (St. Louis, MO), unless stated otherwise. Procedures involving animals in this study were approved by the Animal Ethics Committee of the University of São Paulo, Brazil.

Generation of primary somatic cell cultures

For the establishment of adult skin cell primary cultures, ear biopsies aseptically collected from an adult donor Nelore cow were diced into 3-mm pieces and placed in 60-mm tissue culture dishes (Corning Incorporated, Corning, NY) containing Dulbecco's modified Eagle's Medium (DMEM, Gibco-BRL, Grand Island, NY) supplemented with 0.22 mM sodium pyruvate, 26.2 mM sodium bicarbonate, 10,000 UI/mL penicillin G, 10 mg/mL streptomycin sulfate, and 10% fetal calf serum (FCS; Gibco-BRL). Cell cultures were established, expanded, and maintained at 38.5°C, 5% CO_2, and 95% humidity. When >90% confluent, cells from passages 1 to 3 were harvested using trypsin–EDTA solution for 5 to 7 min, spun at $300\times g$ for 5 min, resuspended in culture medium, and cultured in four-well dishes (Nunc, Denmark) at 25 to 50×10^3 cells/mL for use in cloning by SCNT or stored frozen at −196°C in cryovials containing 10% DMSO in culture medium.

Bovine embryo production

Bovine embryos were produced by *in vitro* fertilization (IVF), cloning, or parthenogenetic procedures, as described below.

In vitro maturation (IVM). Selected cumulus–oocyte complexes (COCs) from bovine ovaries obtained from local slaughterhouses were *in vitro*-matured at a 38.5°C, 5% CO_2 and 95% humidity, according to procedures by Vieira et al. (2002) and Bertolini et al. (2004).

IVF. *In vitro* sperm capacitation and IVF followed procedures modified from Parrish et al. (1986), as established in our laboratory (Vieira et al., 2002; Bertolini et al., 2002). Briefly, frozen–thawed bovine sperm cells were segregated by swim up in Sperm-TALP medium. Sperm cells, after *in vitro* capacitation, and oocytes, after 20 to 24 h of IVM, were coincubated for 18 to 20 h (IVF = day 0) in IVF-TALP medium, when cumulus cells were removed by pipetting (IVF control group).

Nuclear transfer (SCNT-derived) by HMC and parthenote embryos. Skin cell cultures from early passages (P1–P3) were used for the production of bovine SCNT embryos by modified HMC procedures, based on Vajta et al. (2003). In brief, after 17 h of IVM, COCs were denuded by pipetting in HEPES-buffered M-199 + 10% FCS (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 5 oocytes in 5 μL drops under oil and hand-bisected (Ultrasharp Splitting Blade, Bioniche, Athens, GA). Hemioocytes were selected after screening for nuclear material under UV light in 10 μg/mL bisbenzimide in HM (Hoechst 33342) in hemicytoplasts and hemikaryoplasts (enucleated hemioocytes and metaphase II hemioocytes, respectively).

Clone embryos were reconstructed by fusing either one or two hemicytoplasts to one donor cell to attain approximately either 50 or 100% of the final cytoplasmic volume, respectively. Adhesion for reconstruction was accomplished after a quick exposure of hemioocyte(s) to 500 μg/mL phytohaemoagglutinin in HP. For clone hemiembryos, one enucleated half (hemicytoplast) and one donor cell were conjoined, whereas reconstruction of clone embryos was performed by first sticking a somatic cell on a hemicytoplast, followed by the adhesion of a second hemicytoplast to the former, creating a linear arrangement, with the donor cell staying on the edge. Reconstructed hemiembryos (50% volume) or embryos (100% volume) were electrofused by two 1.0-kV/cm DC pulses for 20 μsec, after a brief exposure to a 15-V prefusion AC pulse, in electrofusion medium (300 mM mannitol, 0.1 mM $MgSO_4$ $7H_2O$, 0.05 mM $CaCl_2$ $2H_2O$, 0.5 mM HEPES, 0.01% PVA), into a BTX 453 chamber (BTX Instruments, Genetronics, San Diego, CA) coupled to an electrofusion apparatus (BTX Electro Cell Manipulator 200, Biotechnologies & Experimental Research Inc., San Diego, CA). Fusion rates for reconstructed hemiembryos (one hemicytoplast + one donor cell = 50% volume) and embryos (two hemicytoplasts + one donor cell = 100% volume) were assessed by microscopic examination after 60 min.

Hemiembryo and embryo activation. Reconstructed clone embryos after 2.4 ± 0.7 h from fusion (25.0 ± 2.4 h from the onset of maturation), zona-free and zona-intact oocytes, and hemikaryoplasts (metaphase II hemioocytes) were chemically activated in 5 μM ionomycin in HM for 5 min, followed by incubation in 2 mM 6-DMAP in mSOFaa + 0.4% BSA for 4 h.

Experimental groups and in vitro *culture (IVC) conditions*

Based on the volume of ooplasm used for reconstruction of hemiembryos (after fusion of hemicytoplast + donor cell or by activating MII hemikaryoplasts, producing clone or parthenote hemiembryos with 50% cytoplasmic volumes, respectively) or embryos (by fusing hemicytoplast + hemicytoplast + donor cell reconstituting clone embryos with 100% cytoplasmic volume), activated clone, and parthenote hemiembryos or embryos were *in vitro* cultured in the well-of-the-well (WOW) system, based on Vajta et al. (2000), and modified by Feltrin et al. (2006), randomly allocated to to one of six experimental groups as follows: culture of single clone or parthenote hemiembryos (50% volume; G1, 1×50%) or in aggregates of two (G2, 2×50%), three (G3, 3×50%), or four (G4, 4×50%) clone or parthenote hemiembryos per WOW, composing approximately 50, 100, 150, or 200% of the final normal embryo volume, respectively; or culture of single clone or parthenote embryos (100% volume; G5, 1×100%) or in aggregates of two (G6, 2×100%) clone or parthenote embryos per WOW, composing approximately 100 or 200% of the final normal embryo volume, respectively. All structures were *in vitro* cultured in microwells into four-well dishes, containing 400 μL mSOFaa + 0.4% bovine serum albumin (BSA), under mineral oil, at 39°C and gas mixture (5% CO_2, 5% O_2, 90% N_2) for 7 days, to the blastocyst stage. Control groups containing zona-intact parthenote or IVF embryos (G7, Zona-intact, 100% volume) were *in vitro* cultured under the same conditions as above, but in four-well dishes with no microwells. Parthenote embryos were used in this study as controls for egg quality and manipulation and culture conditions in our laboratory. All experimental treatment groups were carried out concomitantly, in 12 replications.

Assessment of embryo quality, stage of development, and cell density

Embryo quality and stage of development were assessed by morphology according to the guidelines from the International Embryo Transfer Society (IETS). In the case of zona-free embryos, especially for expanded and hatched blastocysts, stages of development were assessed by comparing embryo size and morphology with blastocysts from the zona-intact control groups. Grades 1, 2, and 3 day −7 blastocysts from each group were used for the estimation of total embryo cell number. Following fixation in ice-cold absolute ethanol for up to 12 h, blastocysts were exposed to 10 μg/mL bisbenzimide (Hoechst 33342) in HM, transferred to a drop of glycerol on a glass slide and covered with a cover slip before cell counting under UV light in an epifluorescent microscope. Cell counts were performed by two operators and, when differences exceeded 10%, a third operator reanalyzed the sample, with the disposal of the most divergent count.

In vivo *development*

To verify *in vivo* embryo viability, some Grade 1 and/or 2 fresh day −7 clone blastocysts from groups G1 (2×50%) and G5 (1×100%) were nonsurgically transferred to synchronous female recipients at a commercial embryo transfer facility (one or two per recipient). Pregnancy and fetal gender diagnoses were performed by transrectal ultrasonography on days 30 and 60 of gestation, respectively, with weekly ultrasound examinations to term for the detection of losses and potential abnormalities. Pregnancy rates from clone embryos were compared with embryo transfer results on days 30 and 90 from contemporaneous *in vivo* produced embryos.

Data analyses

Data analyses were done using Minitab software (State College, PA). Fusion, cleavage and blastocyst rates were compared by the χ^2 test, whereas distribution analyses of day −7 blastocysts according to stage of development or quality by treatment group were done by the Kruskal-Wallis test. Data regarding total cell number, based on morphological quality, were analyzed by analysis of variance (ANOVA), for a level of significance of 5%, considering ooplasmic volume (50 or 100%), aggregation status (one, two, three, or four hemiembryos or embryos per WOW), embryo type (clone, parthenote, or IVF embryos), stage of development (early blastocyst, blastocyst, expanded blastocyst, or hatching/hatched blastocyst), and embryo quality (good, fair, or poor) as main effects. Pairwise comparisons between treatment groups were performed using the Tukey test. Simple Pearson's correlation and linear regression tests were used for the analysis of relationships and dependence between traits, and a probability test was carried out for the estimation of embryo viability (developmental potential to the blastocyst stage and embryo quality) in each treatment.

Results

A total of 5025 out of 7487 oocytes (67.1%) were selected for the presence of the first polar body. Following zona removal, manual oocyte bisection, and fluorescence screening, enucleated hemioocytes (3334/6001; 55.6%) were used for clone embryo reconstruction by fusion or aggregation, whereas a portion of the nucleated (MII) hemioocytes was used as matching treatment groups for parthenogenetic activation and embryo aggregation. After embryo reconstruction, fusion rate for clone hemiembryos (one hemicytoplast + one cell; 1250/1833, 68.2%) was lower ($p < 0.05$) than for clone embryos (two hemicytoplasts + one cell; 480/642, 74.8%).

Irrespective of embryo type (clone or parthenote), increasing the number of aggregated structures or ooplasmic volume within each WOW improved cleavage and blastocyst rates (Table 1). A positive correlation was observed between the number of aggregated structures per WOW and cleavage rate ($r = 0.679$, $p = 0.008$), and between cleavage and blastocyst rates ($r = 0.770$, $p = 0.001$), with the number of aggregated structures and cleavage rate being good predictors for blastocyst yield ($r = 0.885$, $p < 0.0001$). Embryos with approximately 100% of final ooplasmic volume (G2 and G5) had rates of development to the blastocyst stage similar to controls (G7). Also, the increase in volumes to 150 or 200% (G3, G4, and G6) of final volume tended to improve blastocyst production

Table 1. Cleavage and Blastocyst Rates for Clone and Parthenote Embryos Reconstructed by Aggregation or Fusion with Distinct Cytoplasmic Volumes

Group	Ooplasmic volume (aggregation)[1]	Embryo type	Fusion/activation	n^2	Cleavage rate[2]		Blastocyst rate[2]	
					n	%	*n*	%
G1	1×50%	Clone	H-Cyt+SC	118	87	73.7^{a}	14	11.9^{a}
		Parthenote	H-Kar	130	89	68.5^{a}	13	10.0^{a}
G2	2×50%	Clone	H-Cyt+SC	116	98	84.5^{bc}	27	23.3^{bc}
		Parthenote	H-Kar	128	114	89.1^{cd}	36	$28.1^{b–d}$
G3	3×50%	Clone	H-Cyt+SC	117	112	$95.7^{d–g}$	39	$33.3^{c–f}$
		Parthenote	H-Kar	131	124	$94.7^{d–g}$	40	$30.5^{b–e}$
G4	4×50%	Clone	H-Cyt+SC	117	114	97.4^{fg}	49	$41.9^{e–g}$
		Parthenote	H-Kar	133	129	97.0^{fg}	48	$36.1^{d–f}$
G5	1×100%	Clone	H-Cyt+H-Cyt+SC	199	153	76.9^{ab}	44	22.1^{b}
		Parthenote	H-Kar+H-Cyt	144	134	$93.1^{d–f}$	65	45.1^{fg}
G6	2×100%	Clone	H-Cyt+H-Cyt+SC	124	120	$96.8^{e–g}$	79	63.7^{h}
		Parthenote	H-Kar+H-Cyt	132	130	98.5^{g}	87	65.9^{h}
Zona-intact	100%	IVF		225	168	74.7^{a}	70	$31.1^{b–e}$
		Parthenote	MII oocytes	110	100	$90.9^{c–e}$	55	50.0^{g}

Note: The sign "+" denotes fusion of distinct structures; H-Cyt: hemicytoplasts in MII; H-Kar, hemikaryoplast in MII; SC, somatic cell.
[1]Aggregation of hemiembryos (50%) or embryos (100%).
[2]On a per WOW basis, except for zona-intact embryos.
[a–h]Data in the same column without common superscripts differ, $p < 0.05$.

compared with groups with 100% volumes (G2 or G5), whereas the use of only half of the normal embryo cytoplasmic volume (G1: 1×50%) negatively affected embryo development to the blastocyst stage (Table 1).

Within each group, development to the blastocyst stage was similar between clone and parthenote embryos, except for G5 (1×100%) and G7 (zona-intact), in which parthenote development was higher than clones and IVF embryos ($p < 0.05$), respectively. Nonetheless, such differences were a reflection of lower cleavage rates in clone embryos in G5 and in IVF embryos in G7, because blastocyst rates did not differ between embryo types when development was based on cleavage. For clone embryos in G5, lower cleavage rates may have been caused by the manipulation process imposed by the cloning procedure per se. Conversely, as only about two-thirds of the oocytes used for embryo production by cloning and parthenogenesis had a visible polar body upon selection, cleavage rates for IVF embryos were accordingly lower, because no polar body selection was performed in this subgroup prior to IVF.

Cleavage rate was also evaluated individually for hemiembryos or embryos allocated to each WOW for groups G2, G3, G4, and G6. The proportion of microwells undergoing cleavage in all aggregated clone or parthenote structures was 72.3, 48.9, 54.0, and 87.0% for G2, G3, G4, and G6, respectively. Cleavage rates were similar between parthenote and clone embryos within each group. In G3, 48.9% of the microwells had two cleavages out of three structures, and in G4, 19.0 and 22.2% of the microwells had two and three out of four structures cleaving. Nevertheless, the partial or complete rate of cleavage for aggregated hemiembryos or embryos per microwell in those groups did not seem to affect development to the blastocyst stage or total cell number in day −7 blastocysts, appearing only to compromise slightly the developmental kinetics within the blastocyst stages or embryo quality in G4 and G6 (data not shown).

No differences in mean total cell number were seen between parthenote, clone, and IVF blastocyst per embryo type (99.7 ± 8.5, 106.0 ± 4.5, and 103.8 ± 3.5, respectively), neither within each group (Table 2) nor within stages of development (Fig. 1a). However, cell density in blastocysts increased linearly as the number of 50% aggregates per WOW also increased, from G1 to G4 (Table 2). Moreover, clone embryos in G1, but not parthenotes, had a significantly lower number of cells than G4, G5, and G6 ($p < 0.05$).

Differences in mean total cell number were mostly due to embryo quality. As expected, embryo quality was correlated with cell density ($r = -0.647$, $p < 0.001$), with numbers of cells being significantly different between morphological grades 1, 2, and 3 (147.6 ± 6.0, 99.9 ± 5.2, and 63.8 ± 6.4, respectively; $p < 0.0001$). Total cell number was similar between groups when considering grade 2 or 3 blastocysts, but differences became more evident for grade 1 embryos (Fig. 1b), especially between clone embryos in G1 and in G5 and G6 (Tables 2 and 3). However, embryo quality did not account alone for differences between groups, as developmental kinetics, measured by the proportion of embryos at distinct levels of development at the blastocyst stage, also were divergent between some groups (Tables 3 and 4).

Excluding parthenote embryos, used more as controls for development, the relative efficiency for the production of day −7 blastocysts based on the total number of oocytes used for the replications was significantly lower for aggregated clone embryos in G2 to G4 groups than for clones in G5 and G6, which in turn, was also lower than IVF-derived embryos (Table 4). Reconstruction of clone embryos in G5 (1×100%) and G6 (2×100%) and IVF embryos were 2-, 3-, and 10-fold more likely to result in blastocysts than hemiembryos in G1 and aggregated embryos from G2 to G4. Moreover, clone blastocysts in G5 and G6 tended to be in more advanced stages of development than any other group, which was associated with increased total cell number and better embryo quality, especially in G6 (2×100%).

Table 2. Total Cell Number (LSM ± SEM), by Treatment Group and by Morphological Quality, for Clone and Parthenote Embryos Reconstructed by Aggregation or Fusion with Distinct Ooplasmic Volumes

Group	Ooplasmic volume (aggregation)[1]	Embryo type	Fusion/activation	Mean cell number (n)	Cell number based on embryo quality: Grade 1 (n)	Grade 2 (n)	Grade 3 (n)
G1	1×50%	Clone	H-Cyt+SC	45.4 ± 16.4^{c} (n = 12)	37.3 ± 35.3^{Ac} (n = 2)	70.9 ± 18.9^{Aa} (n = 7)	28.0 ± 28.8^{Aa} (n = 3)
		Parthenote	H-Kar	70.8 ± 20.4^{abc} (n = 10)	102.3 ± 20.4^{Abc} (n = 6)	67.0 ± 28.8^{Aa} (n = 3)	43.0 ± 49.9^{Aa} (n = 1)
G2	2×50%	Clone	H-Cyt+SC	100.6 ± 11.9^{abc} (n = 18)	140.5 ± 20.4^{Aabc} (n = 6)	102.9 ± 18.9^{Aa} (n = 7)	58.4 ± 22.3^{Aa} (n = 5)
		Parthenote	H-Kar	77.6 ± 10.8^{bc} (n = 23)	93.1 ± 22.3^{Ac} (n = 5)	91.9 ± 16.6^{Aa} (n = 9)	47.8 ± 16.6^{Aa} (n = 9)
G3	3×50%	Clone	H-Cyt+SC	86.7 ± 9.5^{abc} (n = 32)	143.1 ± 18.9^{Aabc} (n = 7)	74.5 ± 12.1^{ABa} (n = 17)	42.6 ± 17.6^{Ba} (n = 8)
		Parthenote	H-Kar	106.8 ± 10.3^{abc} (n = 26)	185.2 ± 20.4^{Aab} (n = 6)	91.3 ± 13.8^{Ba} (n = 13)	43.8 ± 18.9^{Ba} (n = 7)
G4	4×50%	Clone	H-Cyt+SC	108.4 ± 8.5^{ab} (n = 37)	156.5 ± 13.3^{Aabc} (n = 14)	105.2 ± 12.9^{ABa} (n = 15)	63.6 ± 17.6^{Ba} (n = 8)
		Parthenote	H-Kar	116.3 ± 7.7^{ab} (n = 42)	180.4 ± 13.8^{Aab} (n = 13)	94.6 ± 13.8^{Ba} (n = 13)	73.8 ± 12.5^{Ba} (n = 16)
G5	1×100%	Clone	H-Cyt+ H-Cyt+SC	133.2 ± 13.7^{a} (n = 18)	235.0 ± 24.9^{Aa} (n = 4)	94.7 ± 15.0^{Ba} (n = 11)	70.0 ± 28.8^{Ba} (n = 3)
		Parthenote	H-Kar+H-Cyt	110.7 ± 8.7^{ab} (n = 55)	79.2 ± 10.4^{Ac} (n = 23)	109.4 ± 9.1^{Aa} (n = 30)	143.5 ± 22.3^{Aa} (n = 5)
G6	2×100%	Clone	H-Cyt+ H-Cyt+SC	116.8 ± 9.6^{ab} (n = 55)	187.4 ± 9.0^{Aab} (n = 31)	112.1 ± 11.2^{Ba} (n = 20)	51.0 ± 24.9^{Ba} (n = 4)
		Parthenote	H-Kar+H-Cyt	125.3 ± 6.4^{a} (n = 76)	164.7 ± 8.0^{Aabc} (n = 39)	124.8 ± 10.0^{ABa} (n = 25)	86.5 ± 14.4^{Ba} (n = 12)
Zona-intact	100%	IVF		99.7 ± 7.7^{abc} (n = 57)	136.6 ± 14.4^{Aabc} (n = 12)	97.7 ± 12.1^{Aa} (n = 27)	64.8 ± 13.3^{Aa} (n = 18)
		Parthenote	MII oocytes	83.0 ± 17.4^{abc} (n = 41)	126.9 ± 10.4^{Abc} (n = 21)	74.0 ± 12.1^{Aa} (n = 15)	48.0 ± 49.9^{Aa} (n = 5)

Note: Fusion of one hemicytoplast + donor cell (1×50%) or two hemicytoplasts + donor cell (1×100%).
[1]Aggregation of hemiembryos (50%) or embryos (100%) produced either by the fusion of a hemicytoplast + donor cell or by the activation of MII hemikaryoplasts.
[A,B,C]Data in the same row without common superscripts differ, $p < 0.05$.
[a,b,c]Data in the same column without common superscripts differ, $p < 0.05$.

On a per WOW basis, blastocyst rates and cell density did show a linear increase as the number of aggregates increased. However, on a per hemicytoplast basis, when blastocyst rate and cell density data from each group were adjusted for the number of hemicytoplasts (50%) used for reconstruction, either by fusion or by aggregation, including an adjustment for parthenote and IVF embryos, the increase in blastocyst rate and total cell number was no longer obvious, except for clone embryos in group G5 (1×100%) and G6 (2×100%). Results from the test of probability outcome indicated the prospect for development at least to the expanded blastocyst stage for transferable-grade embryos (grades 1 and 2) to be of 25, 44, 33, 47, 51, and 69%, for clone embryos in groups G1 through G6, respectively, and 41% for IVF embryos in G7. If only grade 1 expanded and/or hatched stage clone blastocysts are taken into consideration, developmental outcomes for embryos in groups G1 through G6 were 6, 20, 10, 22, 13, and 42%, respectively, and for IVF embryos in G7 was 13%.

Results from the transfer of clone embryos from groups G2 (2×50%) and G5 (1×100%) to female recipients are presented in Table 5, as evidence for *in vivo* embryo viability. Overall, a total of 82 clone embryos were transferred into 46 synchronized female recipients, resulting in 10 pregnancies (21.7%) carrying single embryos on day 30 of gestation. The number of embryos per recipient did not seem to affect pregnancy rates or losses. As expected, a trend was evident for embryo quality to be a predictor of pregnancy ($R = -0.310$, $p = 0.095$), with pregnancies developing from high-quality embryos (grade 1). Cumulative pregnancy losses up to days 60 and 210 of gestation were 50 and 80%, respectively, with losses occurring between days 30 and 35 ($n = 4$), around days 45 ($n = 1$) and 70 ($n = 1$), and on days 155 ($n = 1$) and 196 ($n = 1$) of gestation. A viable 39-kg female calf was born from the transfer of a G2 clone embryo, whereas another viable pregnancy from the transfer of two G5 clone embryos was terminated on day 225 of gestation for the collection of biological material for a parallel experiment.

Discussion

Many aspects related to the physiological and developmental failures after SCNT in mammals have been associated with genomic reprogramming errors that, in turn, appear to be related to epigenetic failure at the DNA methylation, histone

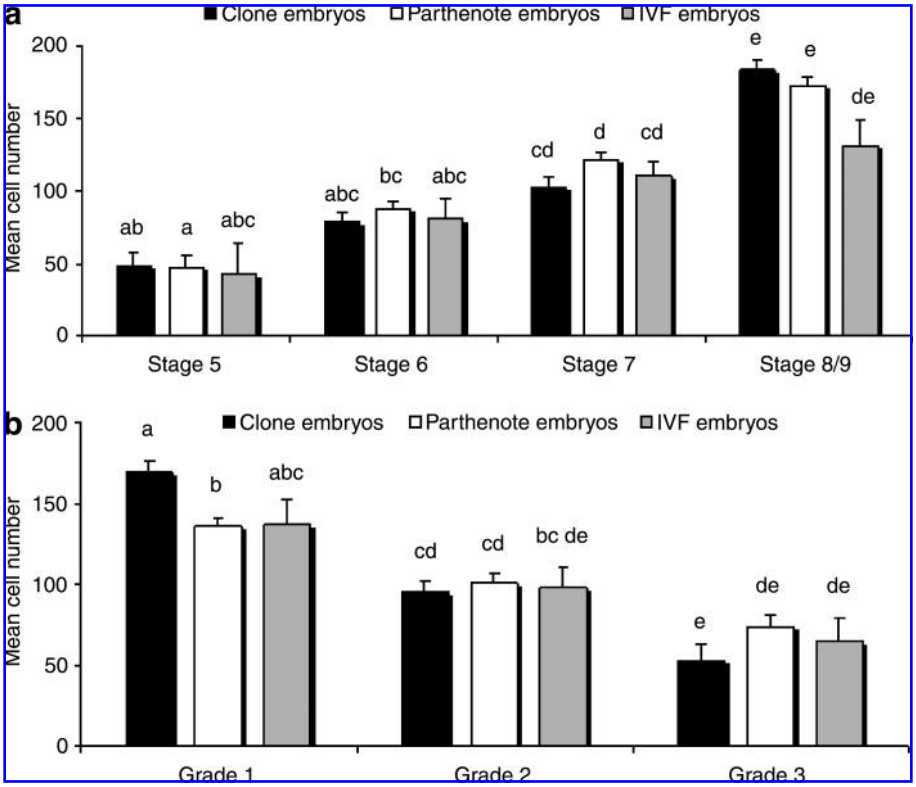

FIG. 1. Total cell number by (**a**) stage of development and (**b**) embryo grade, assessed by morphological quality, for clone, parthenote, and IVF embryos. [a–e]Column bars without common superscripts differ, $p < 0.05$.

acetylation, and/or chromatin remodeling levels. An accurate nuclear reprogramming process after embryo reconstruction is needed for successful SCNT. In this process, the donor nucleus must encompass changes to ascertain that the pattern of gene expression of cloned embryos and concepti becomes similar to that seen in fertilized embryos (Bourc'his et al., 2001). Yet, the intricate mechanisms involved in cell reprogramming are still widely unknown. The use of preimplantation embryos and the establishment of cloning by HMC procedures, along with embryo aggregation methods, are valuable strategies and source of biological material for the study of embryonic development and epigenetic reprogramming that have an impact on developmental biology and mammalian embryology (Misica-Turner et al., 2007; Vajta et al., 2005).

As the recipient oocyte exerts a key role in chromatin remodeling, the effect of the cytoplasmic volume after cloning cannot be neglected. Previous studies have demonstrated that the reduction or increase in cytoplasmic volume may have an important impact on embryo development. The removal of 50 or 25% of the ooplasm during enucleation compromised embryo development, embryo quality, and total cell number in bovine clone blastocysts (Koo et al., 2002; Peura et al., 1998; Westhusin et al., 1996). The use of only 50% of the normal volume for embryo production in this study did affect the developmental potential of cloned embryos to the blastocyst stage, reducing the total cell number per blastocyst, corroborating results from those early reports. Most likely, in conditions when the volume is reduced, such as for hemiembryos, the amount of ooplasmic components may be sufficient to provide support to early cleavage, activation of the embryonic genome (8- to 16-cell stage) and even cavitation, but as cytoplasmic volume does not increase during the first few rounds of embryonic cell division, total cell numbers tend to be limited by total embryo volume (Westhusin et al., 1996).

Increasing the embryonic cytoplasm either by fusing two or three enucleated oocytes to attain 150% of normal volume or by aggregating two or three clone embryos to approximately 200 or 300% of the embryo size improved bovine blastocyst cell density in the inner cell mass and trophectoderm (Misica-Turner et al., 2007; Oback and Wells, 2003; Pedersen et al., 2005; Peura et al., 1998; Tecirlioglu et al., 2005; Vajta et al., 2003). The increase in embryo volume, either by fusion or by aggregation, as seen in this study, also increased the developmental potential and cell density in blastocysts, as reported above. When cytoplasmic volume is increased by fusing cytoplasts, the extra ooplasmic components may boost development beyond the 8- to 16-cell stage, improving embryo development. In addition, the larger cytoplasm will tend to allow or promote more cell divisions to occur, increasing total cell numbers. That seemed to have occurred in this study, as the aggregation of two 100% embryos (G6, 2×100%) was more efficient than the other groups for the production of high-quality (grade 1) blastocysts, which in turn, were more advanced in development and, consequently, contained a higher number of cells (Tables 1–4). However, the *in vivo* developmental potential for aggregated clone embryos for groups G3, G4, and in special for G6, still needs to be further investigated.

The increase in cytoplasmic volume by aggregation may have a distinct explanation than by fusion. In mice, embryo

Table 3. Total Cell Number (LSM ± SEM), by Treatment Group and by Stage of Development, for Clone and Parthenote Embryos Reconstructed by Aggregation or Fusion with Distinct Ooplasmic Volumes

Group	Ooplasmic volume (aggregation)[1]	Embryo type	Fusion/activation	Mean cell number (n)	Cell number based on stage of development*			
					Stage 5 (n)	Stage 6 (n)	Stage 7 (n)	Stages 8/9 (n)
G1	1×50%	Clone	H-Cyt+SC	45.4 ± 16.4^{c} (n = 12)	36.0 ± 23.1^{Aa} (n = 4)	79.1 ± 23.1^{Aa} (n = 4)	48.6 ± 23.1^{Ab} (n = 4)	— (n = 0)
		Parthenote	H-Kar	70.8 ± 20.4^{abc} (n = 10)	43.0 (n = 1)	73.3 ± 20.7^{Aa} (n = 5)	112.1 ± 23.1^{Aa} (n = 4)	— (n = 0)
G2	2×50%	Clone	H-Cyt+SC	100.6 ± 11.9^{abc} (n = 18)	47.3 ± 26.7^{Aa} (n = 3)	65.5 ± 23.1^{Aa} (n = 4)	115.7 ± 17.5^{Aa} (n = 7)	160.4 ± 23.1^{Aa} (n = 4)
		Parthenote	H-Kar	77.6 ± 10.8^{bc} (n = 23)	44.3 ± 18.9^{Aa} (n = 6)	83.2 ± 15.4^{Aa} (n = 9)	88.7 ± 16.3^{Aab} (n = 8)	— (n = 0)
G3	3×50%	Clone	H-Cyt+SC	86.7 ± 9.5^{abc} (n = 32)	45.9 ± 16.3^{Aa} (n = 8)	57.9 ± 14.6^{Aa} (n = 10)	95.0 ± 14.6^{ABab} (n = 10)	178.0 ± 23.1^{Ba} (n = 4)
		Parthenote	H-Kar	106.8 ± 10.3^{abc} (n = 26)	34.3 ± 20.7^{Aa} (n = 5)	72.7 ± 17.5^{Aa} (n = 7)	144.2 ± 18.9^{Aa} (n = 6)	132.4 ± 16.3^{Aa} (n = 8)
G4	4×50%	Clone	H-Cyt+SC	108.4 ± 8.5^{ab} (n = 37)	65.6 ± 23.1^{Aa} (n = 4)	85.4 ± 13.9^{Aa} (n = 11)	109.2 ± 13.9^{ABa} (n = 11)	170.5 ± 13.9^{Ba} (n = 11)
		Parthenote	H-Kar	116.3 ± 7.7^{ab} (n = 42)	34.9 ± 20.7^{Aa} (n = 5)	82.2 ± 11.2^{Aa} (n = 17)	126.7 ± 15.4^{ABa} (n = 9)	185.6 ± 13.9^{Ba} (n = 11)
G5	1×100%	Clone	H-Cyt+H-Cyt+SC	133.2 ± 13.7^{a} (n = 18)	72.3 ± 26.7^{Aa} (n = 5)	63.1 ± 23.1^{Ba} (n = 4)	90.5 ± 26.7^{ABab} (n = 3)	181.4 ± 16.3^{Ba} (n = 8)
		Parthenote	H-Kar+H-Cyt	110.7 ± 8.7^{ab} (n = 55)	48.8 ± 16.3^{Aa} (n = 8)	90.4 ± 10.1^{Aa} (n = 21)	113.1 ± 10.3^{Aa} (n = 20)	141.1 ± 15.4^{Ba} (n = 9)
G6	2×100%	Clone	H-Cyt+H-Cyt+SC	116.8 ± 9.6^{ab} (n = 55)	37.0 ± 20.6^{Aa} (n = 5)	104.1 ± 15.4^{ABa} (n = 9)	118.0 ± 14.6^{ABa} (n = 10)	192.0 ± 8.3^{Ba} (n = 31)
		Parthenote	H-Kar+H-Cyt	125.3 ± 6.4^{a} (n = 76)	57.7 ± 16.3^{Aa} (n = 8)	104.6 ± 9.2^{ABa} (n = 25)	152.2 ± 11.5^{BCa} (n = 16)	187.8 ± 8.9^{Ca} (n = 27)
Zona-intact	100%	IVF		99.7 ± 7.7^{abc} (n = 57)	42.6 ± 20.7^{Aa} (n = 7)	80.4 ± 13.9^{Aa} (n = 16)	109.8 ± 10.3^{Aa} (n = 25)	130.5 ± 17.5^{Aa} (n = 9)
		Parthenote	MII oocytes	83.0 ± 17.4^{abc} (n = 41)	62.3 ± 23.1^{ABa} (n = 4)	75.7 ± 12.8^{Aa} (n = 13)	112.3 ± 10.3^{ABa} (n = 20)	186.3 ± 23.1^{Ba} (n = 4)

Note: Fusion of one hemicytoplast+donor cell (1×50%) or two hemicytoplasts+donor cell (1×100%).
[1]Aggregation of hemiembryos (50%) or embryos (100%) produced either by the fusion of a hemicytoplast+donor cell or by the activation of MII hemikaryoplasts.
[2]Stage 5: early blastocysts; stage 6: blastocysts; stage 7: expanded blastocysts; stages 8/9: hatching and hatched blastocysts.
[A,B,C]Data in the same row without common superscripts differ, $p < 0.05$.
[a,b,c]Data in the same column without common superscripts differ, $p < 0.05$.

Table 4. Probability Outcomes for Development, Distribution of Relative Frequencies and Relative Efficiency per Total Oocytes for Embryo Development to the Blastocyst Stage by Treatment Group Based on embryo Stage, Embryo Quality for Clone Embryos Reconstructed by Aggregation or Fusion with Distinct Ooplasmic Volumes

Group	Ooplasmic volume (aggregation)[1]	Stage of embryo development[2]				Embryo quality			Relative efficiency (blastocysts per total oocytes)	
		Stage 5	Stage 6	Stage 7	Stages 8/9	Grade 1	Grade 2	Grade 3	MII oocytes[3]	Total COC
G1	1×50%	0.333^{Ac}	0.333^{Ab}	0.333^{Ab}	0.000^{Ba}	0.167^{Aa}	0.583^{Ba}	0.250^{ABa}	$1:20^{a}$ (5.0%)	$1:29^{a}$ (3.4%)
G2	2×50%	0.167^{Aabc}	0.222^{Aab}	0.389^{Aab}	0.222^{Abcd}	0.333^{Aa}	0.389^{Aa}	0.278^{Aa}	$1:19^{a}$ (5.3%)	$1:28^{a}$ (3.6%)
G3	3×50%	0.250^{ABbc}	0.312^{Bab}	0.312^{Bab}	0.125^{Aabc}	0.219^{Aa}	0.531^{Ba}	0.250^{Aa}	$1:21^{a}$ (4.7%)	$1:31^{a}$ (3.2%)
G4	4×50%	0.108^{Aabc}	0.297^{Bab}	0.297^{Bab}	0.297^{Bcd}	0.378^{Bab}	0.405^{Bab}	0.216^{Aab}	$1:23^{a}$ (4.4%)	$1:33^{a}$ (3.0%)
G5	1×100%	0.278^{Abc}	0.222^{Aab}	0.167^{Aa}	0.444^{Ade}	0.222^{Aab}	0.611^{Bab}	0.167^{Aab}	$1:10^{ab}$ (10.2%)	$1:14^{ab}$ (6.9%)
G6	2×100%	0.091^{Aa}	0.164^{Aa}	0.182^{Aa}	0.564^{Be}	0.564^{Cab}	0.364^{Bab}	0.073^{Aab}	$1:7b^{b}$ (14.8%)	$1:10^{b}$ (9.9%)
G7	Zona-intact IVF	0.123^{Aabc}	0.281^{Bab}	0.439^{Bb}	0.164^{ABbc}	0.210^{Aab}	0.474^{Aab}	0.316^{Aab}	$1:2^{c}$ (46.4%)	$1:3^{c}$ (31.1%)

[1]Aggregation of hemiembryos (50%) or embryos (100%) produced either by the fusion of a hemicytoplast + donor cell or by the activation of MII hemikaryoplasts.
[2]Stage 5: early blastocysts; stage 6: blastocysts; stage 7: expanded blastocysts; stages 8/9: hatching and hatched blastocysts.
[3]Based on maturation rate for oocytes used for cloning (67.1%).
[A,B,C]Data in the same row, for stage of development or embryo quality, without common superscripts differ, $p < 0.05$.
[a,b,c]Data in the same column without common superscripts differ, $p < 0.05$.
COC, cumulus-oocyte complexes.

aggregation did not improve clone embryo development to the blastocyst stage, but increased cell density in blastocysts and promoted an eightfold higher *in vivo* development than controls (Boiani et al., 2003). In that study, the improvement in *in vivo* development was not considered to be a consequence of an increase in cell numbers, but more a complementation of the genetically identical, but epigenetically different embryos, which exerted an additive effect on the developmental potential of embryos. In our study, an increase in the number of aggregated structures within each WOW was followed by a linear increase in cleavage, blastocyst rate, and cell density. In fact, embryo aggregation may be a means of increasing cloning efficiency, not only by promoting an increase in cell density in developed embryos, but also (1) by influencing cell allocation in the embryonic lineages, which may favor subsequent placental development (Vajta et al., 2005), (2) by eliminating and/or replacing blastomeres of lower developmental capacity (Viuff et al., 2002), or even (3) by compensating, correcting, or regulating epigenetic defects present in some blastomeres through a paracrine fashion (Boiani et al., 2003). However, embryo aggregation in this study did not improve blastocyst yield or cell number, on a hemicytoplast basis, for aggregated clone embryos in groups G2 (2×50%), G3 (3×50%), and G4 (4×50%). In part, the lower fusion rates for the reconstruction of clone hemiembryos for aggregation caused a reduction in the overall cloning efficiency. In addition, variations in total cleavage in each WOW may have diminished or masked any synergistic effect of embryo aggregation. However, such additive effect in embryo development (blastocyst rate, stage of development, embryo quality and, consequently, total cell number), on a per hemicytoplast basis, was apparent for G6 clone embryos (2×100%). Such observation corroborates those of Boiani et al. (2003), Pedersen et al. (2005), Tecirlioglu et al. (2005), and Misica-Turner et al. (2007), in which a nonlinear synergistic increase in blastocyst yield and/or cell density were detected. Possibly, such differences in results may be based on the type of structures used for aggregation in all aforementioned studies and the present one. Most likely, the linear improvement in embryo development and cell density observed by aggregation in this study for 50% aggregates was more a reflection of probability for development than of a paracrine effect or epigenetic complementation, whereas the nonlinear synergistic improvement seen for embryos in G6 (2×100%) and in the other studies may have resulted from the aggregation of structures proner for development.

Table 5. Pregnancy Rate after Transfer of Day 7 Clone Embryos and Contemporaneous Control Embryos to Synchronous Female Recipients

Group	Embryos/recipient	Embryo Transfer		Pregnancy					
				Day 30		Day 60		Day 210	
		Recipients	Embryos	n	%	n	%	n	%
Clones G2 (2×50%)	One	2	2	1	50.0	1	50.0	1[b]	50.0
Clones G5 (1×100%)	One	8	8	1	12.5	0	0.0	—	—
	Two	36	72	8	22.2	4	11.1	1[c]	2.8
In vivo-produced embryos (controls)[a]	One	94	94	50	53.2	50	53.2	—	—

[a]Contemporaneous fresh control bovine embryos, for which day 210 pregnancy was not determined.
[b]Pregnancy resulted in a 39-kg viable calf born after elective C section on day 289 of gestation.
[c]Pregnancy was terminated on day 225 of gestation for the collection of biological materials for a parallel experiment.

Interestingly, embryos produced by aggregation of bovine embryos yielded higher early pregnancy rates than single embryos produced by fusion (Pedersen et al., 2005). Such results opposed those by Tecirlioglu et al. (2005) in which no differences were observed in pregnancy rates between aggregated and nonaggregated clone embryos, despite the significant improvement in embryo development and total cell number in clone blastocysts. In this study, pregnancy rates were lower than contemporaneous controls. Even if numbers were low, pregnancy outcome appeared to be more closely related to embryo quality, which in turn, was correlated with cell density, rather than with embryo type. Nevertheless, further studies are still needed to elucidate this issue.

Differences seen for the fusion of structures with one hemicytoplast + one cell and with two cytoplasts + one cell may be related to two basic factors: alignment of the structures into the electrofusion chamber during the AC pulse and relation between the nature of the DC pulse and cell size. It is known that the efficiency of the AC pulse used for alignment depends on the cell type (Oback and Wells, 2003) and, particularly, on the proper orientation. Vajta et al. (2002) showed that a proper structure orientation prior to AC pulse significantly improved fusion between one-hemicytoplast + one-cell structures, obtaining an improvement from 54% with random alignment to 94% fusion when alignment was well checked. As structural alignment in our study was properly and carefully controlled, lower fusion rates may have been more related to the response of each type of structure to the DC pulse. There is a direct relationship between the size of the structures being fused; in general, the lower this ratio, the higher the response to alignment and fusion (Teissié et al., 1999). This is particularly true for cloning, in which fusion of a somatic cell to a cytoplast or hemicytoplast is not as efficient as fusion between cytoplasts or hemicytoplasts. In this way, the DC pulse amplitude is usually increased for the fusion of one-hemicitoplast + one-cell structures, as shown by Vajta et al. (2001). However, under our conditions, the increase in DC pulse amplitude usually resulted in higher rates of cytoplast lysis or subsequent degeneration. As electrofusion parameters were set in a pilot experiment, it appears that proper conditions fell within a narrow window for our apparatus settings. Nevertheless, our fusion strategy for reconstruction of clone embryos by attaching a somatic cell on a hemicytoplast, followed by a second hemicytoplast, creating a linear arrangement, with the donor cell staying on the edge, has proven more effective for subsequent membrane fusion than reconstruction methods described by Vajta et al. (2003), in which the donor cell is placed in between the adhered hemicytoplasts (data not shown).

In summary, this study was designed to determine the effect of the cytoplasmic volume on embryo development and cell density after aggregation or fusion of clone or parthenote embryos. Generally, the increase in cytoplasmic volume by fusion or by aggregation improved embryo development and cell number in clone and parthenote embryos, with clone embryos having more cells and higher quality than parthenotes. Interestingly, developmental potential was not affected by a reduction in half of the normal embryo volume, but cell density in developing blastocysts was significantly reduced. Overall, embryo aggregation appeared to improve development and cell density, on a per WOW basis, but no additive effect was observed on blastocyst yield or cell number, on a hemicytoplast basis. However, the aggregation of two clone embryos (2×100%) had an additive effect on the likelihood of development to the blastocysts stage, in addition to an increase in cell density and kinetics of embryo development, with a positive reflection on embryo quality.

The benefit of embryo aggregation on *in vitro* and *in vivo* development of clone embryos still needs to be further elucidated. Our efforts to understand the effects of the heteroplasmy, and the role of the ooplasmic components and volume on subsequent development, ultimately intend to increase our knowledge on the process of nuclear reprogramming, which may benefit studies on aging, cancer, disease syndromes, and normal processes in embryo development and abnormalities seen after embryo manipulations, such as cloning by somatic cell nuclear transfer.

Acknowledgments

This work was partially supported by grant no. 192058/Procad II from Capes/Brazil. The authors thank Frigorífico Verdi Ltda., from Pouso Redondo, SC, and Frigorífico El'Golli Ltda., from Taió, SC, for the provision of bovine ovaries. We also thank Dr. Gary B. Anderson for his unvaluable comments and suggestions after reviewing the final version of this manuscript.

Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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