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Analysis of factors contributing to the efficiency of the *in vitro* production of transgenic goat embryos (*Capra hircus*) by handmade cloning (HMC)

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ABSTRACT

Cloning by Somatic Cell Nuclear Transfer (SCNT) still is challenging and inefficient. Recently, the handmade cloning (HMC) procedures have been successfully applied to livestock species. The aim of this study was to compare the effect of distinct oocyte sources (*in vivo vs. post-mortem*) and the final cytoplasmic embryo volume (~85% or 2 × 50%) on fusion rates and on the developmental potential of Day-1 or Day-7 cloned transgenic goat embryos produced by HMC procedures. Cloned embryos were reconstructed by HMC using skin fibroblast donor cells established from a transgenic goat. Oocytes were obtained *in vivo* by laparoscopic oocyte recovery (LOR) from hormonally stimulated females or *post-mortem* from slaughterhouse ovaries from nonstimulated goats, resulting in no differences in the number of aspirated follicles, *cumulus*-oocyte complexes (COCs), and viable COCs per goat. However, the COC recovery rate was higher for slaughterhouse ovaries (86.0%) than for LOR (73.0%). Also, cytoplasmic volume (~85% *vs.* 2 × 50%) had no effect on fusion rates after embryo reconstruction. Using slaughterhouse ovaries for cloning, a total of 18.0% (27/150) and 12.7% (19/150) of the *in vitro*-cultured embryos developed to the compact morula and blastocyst stages on Day 7. However, no recipients became pregnant on Day 30 following the transfer of Day-1 or Day-7 embryos. In conclusion, the use of slaughterhouse ovaries was as valuable to supply oocytes for the production of cloned goat embryos by HMC as the *in vivo* approach. The HMC was proven a simple alternative for the production of cloned transgenic goat embryos.

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1. Introduction

After the birth of the first cloned mammal using adult sheep epithelial cells as donor nucleus (Wilmut et al., 1997), Somatic Cell Nuclear Transfer (SCNT) became a technology that has been successfully applied to more than 20 animal species, including a range of livestock species such as cattle (Cibelli et al., 1998), goats (Baguisi et al., 1999), and pigs (Polejaeva et al., 2000). The use of cloning has since been exploited to study interactions between the nucleus and the cytoplasm during differentiation and to investigate the effects of SCNT on epigenetics and nuclear reprogramming. Furthermore, SCNT has been shown to have applications in the production of large-scale

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numbers of genetically identical animals (Stice and Keefer, 1993), and also for genetic conservation (Campbell et al., 2007). In addition, current advances in the production of recombinant proteins have also had an important effect on the pharmaceutical industry. In fact, a range of distinct therapeutic proteins have been produced in the milk of transgenic animals (Baguisi et al., 1999; Baldassarre et al., 2004b), making cloning one of the most important supportive technologies for transgenesis.

Although SCNT is being performed in many laboratories, successful application of the cloning technology is still a difficult and demanding task due to many factors, including the stage of the donor cell cycle, the donor cell type, the embryo culture system, imprinting defects and reprogramming failures, inefficient embryo activation protocols, lab-to-lab variation, and the oocyte source and quality at the beginning of maturation (Campbell et al., 2005). Traditional SCNT animal cloning employs sophisticated pieces of equipment, tailored micropipettes, and requires a high level of technical skill. On the other hand, the handmade cloning (HMC) procedures have been developed exactly to circumvent the need of micromanipulators by using zona-free oocytes (Peura et al., 1998; Vajta et al., 2001, 2003). The HMC procedures are considered a technology with equivalent efficiency to traditional SCNT cloning (Tecirlioglu et al., 2005), indicating that it may be readily used as an alternative method for the generation of cloned offspring in various animal species. In goats, several studies on cloning have been successfully carried out using either micromanipulation systems to produce zona-intact (Baguisi et al., 1999; Lan et al., 2006) or zona-free (Nasr-Esfahani et al., 2011) cloned embryos, or using the non-micromanipulation (HMC) system for the production of zona-free cloned embryos (Akshey et al., 2010). However, the optimization of the distinct steps in the cloning procedures are still needed to attain a higher HMC efficiency in the goat.

As a transgenic female goat founder was obtained by pronuclear microinjection in 2008, which expressed acceptable levels of the human Granulocyte Colony Stimulating Factor (hG-CSF) in the milk (Freitas et al., 2012), our current goal is to expand the herd by means of cloning procedures using the hG-CSF founder goat. Therefore, using skin fibroblast primary culture cells from the transgenic founder goat as nucleus donor cells (karyoplasts), the aim of this study was to optimize the HMC cloning procedure for the goat by the comparison of the effect of the oocyte source (*in vivo*, from hormonally stimulated goats *vs. post-mortem*, by using slaughterhouse ovaries from non-stimulated females), and the final cytoplasmic volume schemes for embryo reconstruction (∼85% or 2 × 50%) on fusion rates and on the *in vitro* and *in vivo* developmental potential of Day-1 or Day-7 cloned transgenic goat embryos obtained by HMC procedures.

2. Materials and methods

2.1. Animal care and biosafety

Animal use and care were approved by the Animal Ethics Committee of the Ceará State University (CEUA/UECE, no. 09144595-7/50), in compliance with the Brazilian Biosafety Technical National Committee (CTNBio, no. 0288/06). Additionally, all trials were conducted in accordance with the guidelines for animal care (ASAB, 2006).

2.2. Chemicals, reagents and media

Unless indicated otherwise, all chemicals and reagents were from Sigma–Aldrich (St. Louis, USA) and the media for cell culture from Gibco-BRL (Carlsbad, USA).

2.3. Experimental design

Two independents experiments were carried out to compare the effect of the source of oocytes as cytoplasts for cloning procedures, and to evaluate the HMC efficiency for the production of viable Day-1 or Day-7 goat cloned embryos for the establishment of pregnancies following transfers to female recipients, as described below. In Experiment 1, we compared the efficiency between *in vivo vs. post-mortem* goat oocyte retrieval systems by laparoscopy (LOR) or by the aspiration of slaughterhouse ovaries, respectively. All oocytes obtained in this experiment were used to optimize the HMC cloning procedures to the goat. In Experiment 2, using oocytes from slaughterhouse ovaries according to results obtained from the first experiment, we evaluated the effect of the final cytoplasmic volume schemes (∼85 or 2 × 50%) on fusion rates. Additionally, we observed the *in vitro* and *in vivo* developmental potential of transgenic goat embryos cloned by HMC and subsequently transferred to synchronous female recipients. During the study, we also characterized qualitatively the somatic cell growth pattern of the ear fibroblast cells derived from the hG-CSF transgenic female goat by the analysis of the morphological cell features following the establishment of the cell cultures.

2.4. Generation and establishment of somatic cell primary cultures

2.4.1. Establishment and expansion of primary cell cultures

Cell samples were derived from primary skin cell cultures following standard procedures (Ribeiro et al., 2009). An ear biopsy was aseptically collected from an adult hG-CSF transgenic female Canindé founder goat (Freitas et al., 2012). The ear tissue was diced into 2–3 mm pieces, placed in 35-mm tissue culture dishes containing Dulbecco's modified Eagle's medium (DMEM) 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/mL amphotericin B and 10% fetal calf serum (FCS), and cultured for 7–14 days at 38.5 °C, 5% CO_2 and high humidity until near confluence. Dermal fibroblast-like cultures that reached 90% confluence were dispersed with a 0.25% trypsin/5 mM EDTA solution and subcultured at a 1:3 ratio. All cell cultures were established, expanded and maintained in culture until needed for analysis, or were cryopreserved in 0.25 mL plastic straws for further utilization, following established procedures (Urio et al., 2011).

2.4.2. Cell growth profile

Frozen-thawed transgenic goat cells on the 3rd or 4th passages were seeded in 4-well plates at cell densities of 1.0×10^4, 3×10^4, and 1.0×10^5 cells/well for the determination of the population doubling time (PDT). Cells from two wells were trypsinized and counted at 24 h intervals for up to 72 h of culture. The average cell counts at each time point were then plotted against time, and the PDT was determined based on the obtained cell growth profile (Costa et al., 2005).

2.5. Oocyte source and in vitro maturation (IVM) – Experiment 1

The *cumulus*-oocyte complexes (COCs) used in this experiment were recovered either *in vivo* from groups of superovulated live females, *via* laparoscopic oocyte retrieval (LOR), or *post-mortem* from ovaries collected from non-stimulated females at a local slaughterhouse. For the LOR, the estrous cycle of groups of goats was synchronized using sponges (Progespon, Syntex, Buenos Aires, Argentina) impregnated with 60 mg medroxyprogesterone acetate (MAP) inserted intravaginally for 11 days. On day 8, a 50-μg D-cloprostenol (Ciosin, Ford Dodge Saúde Animal, Campinas, Brazil) injection was given intramuscularly (IM), along with the onset of the ovarian stimulation, which was carried out using 120 mg pFSH (Folltropin-V, Vetrepharm, Ontario, Canada) distributed in five IM injections (30/30, 20/20 and 20 mg), 12 h apart. Animals were fasted for 24 h prior to the LOR, which was performed at the time of sponge removal,

according to Pereira et al. (2012). For the *post-mortem* oocyte retrieval, ovaries from well fed goats were collected at a local slaughterhouse and transported to the laboratory in saline at 30 °C. For both retrieval systems (LOR and slaughterhouse), all follicles greater than 2 mm were counted and aspirated using an aspiration system proper for small ruminants (WTA, Cravinhos, Brazil).

Retrieved immature goat COCs from both oocyte retrieval groups were classified based on cellular vestments and cytoplasmic uniformity (Almeida et al., 2011). Only GI to GIII COCs were *in vitro*-matured in TCM199 supplemented with 0.022 μg/mL sodium pyruvate, 10,000 IU penicillin, 10,000 μg/mL streptomycin sulfate, 25 μg/mL amphotericin B, 10% FCS, 10 ng/mL EGF, 5 μg/mL FSH, 10 μg/mL LH, 1 μg/mL 17β-estradiol and 100 μM cysteamine for 20 h at 38.5 °C, 5% CO_2 and high humidity. Following IVM, *cumulus* cells were removed by successive pipetting, and matured oocytes (MII) with a first polar body (PB) were selected for enucleation.

2.6. In vitro and in vivo development of cloned goat embryos by handmade cloning (HMC) – Experiment 2

The preparation of recipient cytoplasts, donor cell cultures and nuclear transfer procedures by HMC were adapted from procedures in cattle (Ribeiro et al., 2009; Gerger et al., 2010). The COCs for this experiment were obtained from slaughterhouse ovaries and were subjected to IVM as already described above.

2.6.1. Processing of IVM oocytes for preparation of cytoplasts

In vitro-matured MII oocytes were briefly exposed to a 0.5% protease solution for zona pellucida removal, followed by a rinse in pure FCS and multiple washes in holding medium (HM: TCM199 with 0.022 μg/mL sodium pyruvate, 10,000 IU penicillin, 10,000 μg/mL streptomycin sulfate, 25 μg/mL amphotericin B, 20% FCS). Zona-free oocytes were immediately used for SCNT. Briefly, zona-free oocytes were hand-bisected in 2.5 μg/mL cytochalasin B with a bisection blade (Ultra-Sharp Splitting Blades, Bioniche, USA) under a stereomicroscope. When the PB was still attached to the oocyte, which indicates the probable location of the MII plate into the oocyte, only about 15% of the cytoplasm was manually excised at the PB position, leaving a larger demi-oocyte with approximately 85% of its original cytoplasmic volume. In the absence of an attached PB, oocytes were bisected in halves of equal volumes (50%). Then, hemi-oocytes (50% and 85%) were screened under UV light in 10 μg/mL bisbenzimide (Hoechst 33342) in HM for the selection of enucleated cytoplasts.

2.6.2. Donor cell preparation for nuclear transfer

Ear fibroblast cells derived from the hG-CSF transgenic goat were used as the donor cell for the production of cloned embryos, following procedures adapted from Ribeiro et al. (2009) and Gerger et al. (2010). Frozen-thawed cells at the 2nd or 3rd passages were thawed 2 days prior to cloning and were cultured so that a high confluence level (>95%) could be attained 12–24 h before their use as nucleus donor cells.

2.6.3. Membrane fusion, chemical activation and in vitro embryo culture

After a brief exposure to a 500-μg/mL phytohemoagglutinin solution, cloned embryos were reconstructed by the adhesion of either one demi-cytoplast (~85% cytoplasmic volume) or two hemi-cytoplasts (2 × 50% cytoplasmic volume) to a single fibroblast donor cell.

Reconstructed structures were electrofused by two 1.1-kV/cm DC pulses for 5 μs (~26 h post-IVM), after a brief exposure to a 7.0-V pre-fusion AC pulse in electrofusion medium (300 mM mannitol, 0.1 mM $MgSO_4 \cdot 7H_2O$, 0.05 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM HEPES, 0.01% PVA) in a 0.22-mm micro-fusion chamber (Micro chamber, Eppendorf, Hamburg, Germany) coupled to an electrofusion apparatus (Multiporator, Eppendorf, Hamburg, Germany). Fusion was assessed within 30–60 min. Fused embryos were chemically activated 2–3 h post-fusion in 5 μM ionomycin in HM for 5 min, followed by a 4 h incubation in 2 mM 6-dimethylaminopurine (6-DMAP) in modified SOFaa supplemented with 0.4% BSA and 2% FCS. Resulting activated cloned embryos were *in vitro*-cultured in 300 μL modified SOFaa medium supplemented with 0.4% BSA and 2% FCS, under mineral oil. Cloned embryos were *in vitro*-cultured (IVC) individually in microwells using the well-of-the-well (WOW) system, based on Vajta et al. (2000) and modified by Feltrin et al. (2006), at 38.5 °C, in humidified air and 5% CO_2, 5% O_2 and 90% N_2 for up to 24 h or for 7 days of culture.

2.6.4. Zona free SCNT-embryo transfer

After up to 24 h of IVC, groups of transgenic cloned 1-cell stage goat embryos were transferred into the oviducts of synchronous female recipients on Day 1 of the estrous cycle, whereas other groups of transgenic embryos were *in vitro*-cultured for up to 7 days to the morula and blastocyst stages prior to the uterine transfer to synchronous recipients, on Day 7 of the estrous cycle. Crossbreed goat recipients were estrus-synchronized using intravaginal MAP-impregnated sponges for 10 days, associated with an injection of 400 IU eCG (Novormon, Syntex, Buenos Aires, Argentina) and 75 μg D-cloprostenol (Ciosin, Ford Dodge Saúde Animal, Campinas, Brazil) at the 8th day of the protocol. Prior to the embryo transfer, the recipient goats were fasted and anesthetized as described by Freitas et al. (2007). A laparoscopic examination was performed to confirm the presence of at least one recent ovulation or corpus hemorragicum (CH) on Day 1 or a mature corpus luteum (CL) on Day 7, according to the day of the embryo transfer. Day-1 cloned embryos were transferred into the oviduct, whereas Day-7 cloned morulae/blastocysts were transferred into the uterine horn, with the transfers being ipsilateral to the CH- or CL-bearing ovary. Pregnancy diagnosis was performed per rectum by ultrasonography on Days 28 and 35 of development, using a Falco 100 scanner (Pie Medical, Maastricht, Netherlands) equipped with a transrectal 6–8 MHz linear array transducer.

2.7. Data analyses

For Experiment 1, data regarding the mean number of follicles, total COCs, and viable COCs, on a per female basis, and the recovery efficiency (COCs per number of follicles) were analyzed using the Student's *t*-test or the χ^2 test, for $P < 0.05$. For Experiment 2, maturation and fusion rates were compared by the χ^2 test ($P < 0.05$) and were evaluated for embryos transferred both on Days 1 or 7. Cleavage and morula/blastocyst rates were also compared by the χ^2 test ($P < 0.05$) and were obviously determined only for embryos *in vitro*-cultured for 7 days and transferred on Day 7 of development.

3. Results

3.1. In vitro growth pattern of the transgenic donor cells

Seven to 14 days after the tissue explants were placed in culture plates, fibroblastic cells were observed to outgrow from the edges of the tissue pieces (Fig. 1A). Cells showed typical fibrous and fusiform morphologies with centered oval-shaped nuclei (Fig. 1B), with the presence of groups of isolated colonies of epithelial cells. Cells then continued to proliferate and were subcultured up to 90–95% confluence (Fig. 1C). Fibroblasts grew rapidly and gradually replaced the epithelial cells in subcultures. After cryopreservation, transgenic cells presented the same characteristics of the fresh cells during *in vitro* culture (Fig. 1D). The growth kinetic of the transgenic cells was demonstrated by counting total cells during 72 h of culture, resulting in an estimated PDT of 23 h.

3.2. Retrieval efficiency of goat cumulus-oocyte complexes obtained in vivo by laparoscopy (LOR) or post-mortem from slaughterhouse ovaries

Data regarding COC retrieval after three sessions of LOR performed in 36 hormonally stimulated donor females (12 females per session) and five replications with slaughterhouse ovaries from 63 nonstimulated goats (*post-mortem* oocyte retrieval) are presented in Table 1. The mean number, per goat, of aspirated follicles (16.3 *vs.*16.3), total COCs

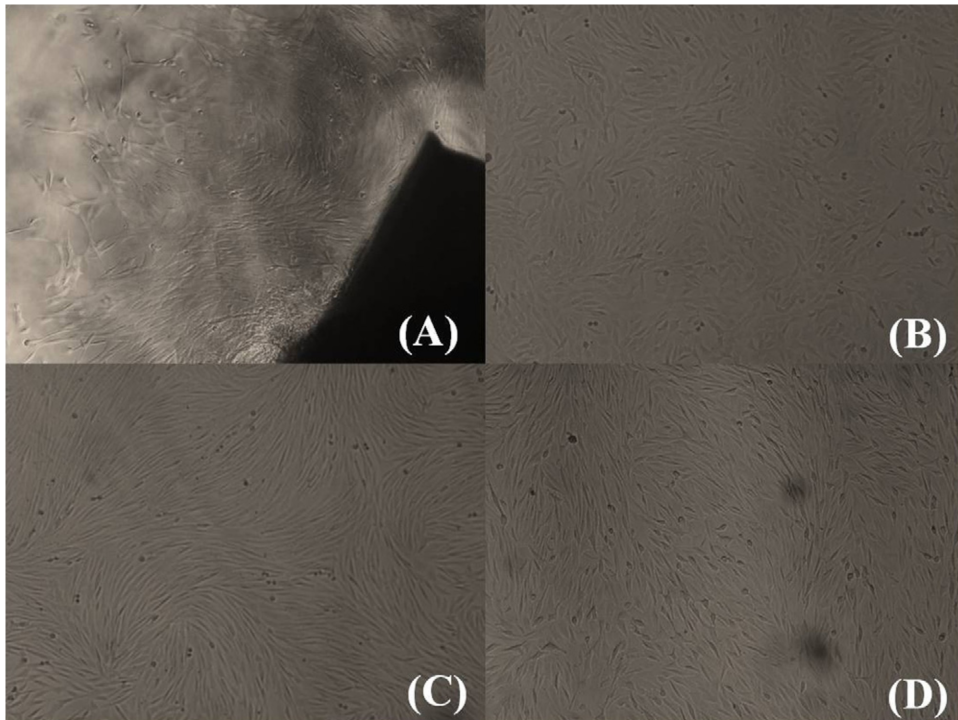

Fig. 1. Primary cultures of ear-derived goat tissue explants. (A) Edge of an ear explant, 7–14 days after the onset of culture, with fibroblast cell outgrowth. Fibroblast cells (B) at near confluence, (C) at confluence, prior to cryopreservation, and (D) 24 h in culture after thawing (200×).

(11.9 *vs.*14.0), and viable COCs (10.7 *vs.*11.4) did not differ between the *in vivo* and the *post-mortem* groups. However, the COC recovery rate for slaughterhouse ovaries was higher than for the LOR procedure ($P < 0.05$). When the number of viable COCs was compared, based on the morphological quality (Table 2), a significant higher proportion of COCs of better quality (Grade II, 63.2%) was obtained *in vivo* by laparoscopy, from hormonally stimulated goats ($P < 0.05$), than from slaughterhouse ovaries (*post-mortem*) obtained from nonstimulated goats, which had a similar proportion of Grades II and III COCs within the group (32.2 and 33.8%, respectively).

Table 1
Comparison of the recovery efficiency of goat *cumulus*-oocyte complexes (COCs) obtained *in vivo* by laparoscopy or *post-mortem* from slaughterhouse ovaries (mean ± SD) – Experiment 1.

Parameter	COC retrieval by follicular aspiration	
	In vivo (LOR)	*Post-mortem*
No. of females	36	63
No. of aspirated follicles	588	1029
Mean (±SD) number of aspirated follicles per goat	16.3 ± 0.2[a]	16.3 ± 3.4[a]
No. of recovered COCs oocytes	429	885
Mean (±SD) number of recovered COCs per goat	11.9 ± 2.0[a]	14.0 ± 3.0[a]
COC recovery rate (%)	73.0[a]	86.0[b]
No. of viable COCs	385	720
Mean (±SD) number of viable COCs per goat	10.7 ± 1.3[a]	11.4 ± 3.0[a]

[a,b] Numbers in the same row without common superscripts differ, $P < 0.05$.
LOR, laparoscopic oocyte recovery.

3.3. In vitro production of transgenic cloned goat embryos and embryo transfer

After six replications, the mean COC recovery rate from slaughterhouse goat ovaries (Fig. 2A) was 82.5% (Table 3), and the maturation rate, based on PB selection (Fig. 2B and C), was 83.2% (1046/1257). Selected COCs were used for the production of cloned embryos that were *in vitro*-cultured either for 1 day or for 7 days prior to transfer. No differences were observed between the number of aspirated follicles, COC recovery rate, morphological quality or maturation rate between the sub-groups (Table 3).

Table 2
Total number of recovered oocytes and proportion of retrieved COCs based on morphological quality following the follicular aspiration of goat ovaries *in vivo* by laparoscopy or *post-mortem* from slaughterhouse goat ovaries – Experiment 1.

COC quality	COC retrieval by follicular aspiration			
	In vivo (LOR)		*Post-mortem*	
	n	%	*n*	%
Grade I	74	17.2[a]	136	15.4[a]
Grade II	271	63.2[a]	285	32.2[b]
Grade III	40	9.3[a]	299	33.8[b]
Grade IV	42	9.8[a]	157	17.7[a]
Total	429		885	

[a,b] Numbers in the same row without common superscripts differ, $P < 0.05$.
LOR, laparoscopic oocyte recovery.

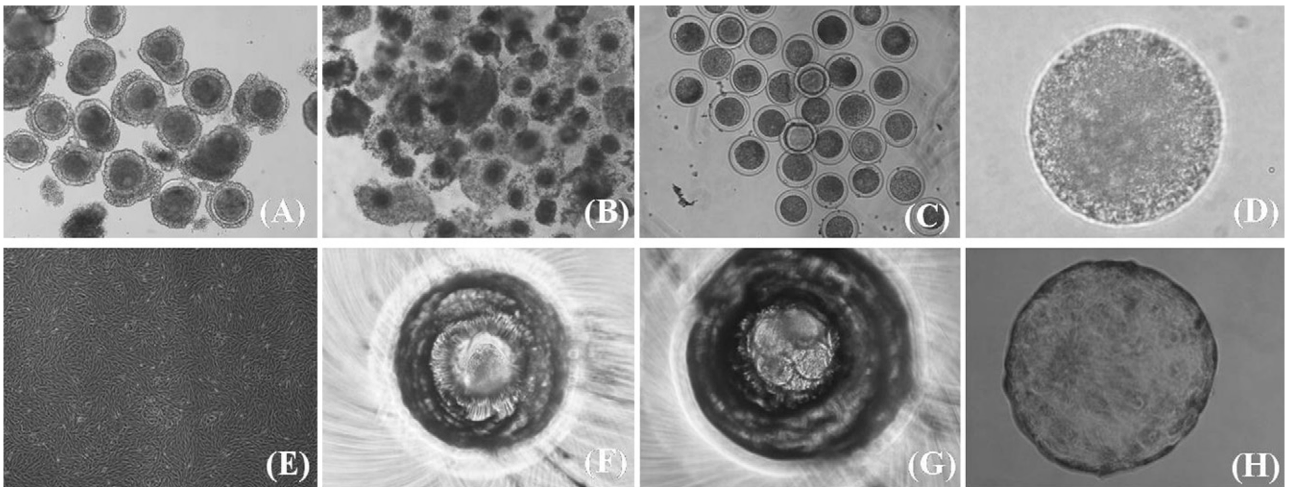

Fig. 2. *In vitro* production steps of goat handmade cloned embryos using donor cells from an hG-CSF transgenic goat. (A) Immature COCs from slaughterhouse ovaries prior to IVM (200×). (B) COCs in modified HM after 20 h of IVM (200×). (C) *In vitro*-matured oocytes (MII) based on PB selection after the removal of cumulus cells (200×). (D) Zona-free oocyte after the enzymatic removal of the zona pellucida (600×). (E) Skin fibroblast donor cells from a Canindé transgenic female at high confluence prior to use in cloning (200×). (F) Transgenic 1-cell stage cloned embryo on Day 1 of IVC into a microwell (WOW system) before the transfer to a synchronized recipient (400×). (G) Transgenic 4-cell stage cloned embryo on Day 2 of IVC (evaluation of cleavage rate) into a microwell (400×). (H) Transgenic cloned blastocyst on Day 7 of development prior to transfer to a synchronous female recipient (600×).

A total of 793 matured oocytes were submitted to zona removal by protease digestion (Fig. 2D), with 85.1% (793/945) survival rate. Fibroblast donor cells in culture were in high confluence (>95%) prior to use for cloning (Fig. 2E).

Results for *in vitro* and *in vivo* embryo developmental potential are summarized in Table 4. A total of 139 and 209 embryos were reconstructed for the transfers as 1-cell stage embryos on Day 1 or as morulae/blastocysts on Day 7, after 1 or 7 days of IVC, respectively. From those, 50 (36.0%) and 89 (64.0%), and 30 (14.4%) and 179 (85.6%) embryos were reconstructed using ~85% or 2 × 50% final cytoplasmic volume schemes for Day-1 or Day-7 embryos, respectively. No differences were seen in fusion rates between sub-groups, resulting in 30 (60.0%) and 79 (89.0%), and 21 (70.0%) and 152 (85.0%) fused embryos with ~85% or 2 × 50% of the final cytoplasmic volumes for Day-1 or Day-7 embryos, respectively. Embryo types, based on cytoplasmic volumes, were mixed for activation, initiated 27.5 h after the onset of the IVM.

For embryos that were *in vitro*-cultured for 7 days prior to the transfers, cleavage rate on Day 2 (Fig. 2G) and morula and blastocyst rates on Day 7 were 87.3%, 18.0% and 12.7% (Fig. 2H), respectively, with the production of 27 compact morulae and 19 blastocysts on Day 7 (46/171; 26.9%). For *in vivo* embryo development, a total of 88 and 13 cloned transgenic 1-cell stage embryos and Day-7 morulae/blastocysts were transferred into six synchronous recipients, respectively. No recipients receiving cloned embryos (Fig. 2F) were diagnosed pregnant upon ultrasound examination on Days 28 and 35.

4. Discussion

The birth of live goat clones using different cell types have been widely reported, including the birth of goats using primary fetal somatic cell line derived from a transgenic female fetus (Baguisi et al., 1999), dwarf goats cloned using fetal fibroblast cells (Keefer et al., 2001), dairy goats using *cumulus* and fetal fibroblast cells (Lan et al., 2006), BELE (Breed Early, Lactate Early) goats cloned using adult granulosa cells (Keefer et al., 2002), and Asian Yellow goats cloned using ear fibroblast cells (Chen et al., 2007). The knowledge of the donor nucleus cell lineage or type in use

Table 3
Number of aspirated follicles, COC recovery rate and oocyte maturation rate for goat COCs obtained from slaughterhouse ovaries used for cloning by HMC – Experiment 2.

IVC + ET[a]	Ovaries	Aspirated follicles	Recovered COCs		Oocyte quality						IVM rate		
					I + II		III		IV		Immature oocytes	MII oocytes	
	n	n	n	%	n	%	n	%	n	%	n	n	%
Day 1 + ET	50	374	349	93.3	168	48.1	111	31.8	24	6.9	321	268	83.5
Day 7 + ET	111	981	914	93.2	355	38.8	421	46.1	138	15.1	907	710	78.3
Total	161	1355	1263	93.2	523	41.4	532	42.1	162	12.8	1257	1046	83.2

[a] IVC + ET: days in *in vitro* culture prior to the embryo transfer.

Table 4
In vitro embryo development of hG-CSF (human Granulocyte Colony Stimulating Factor) transgenic goat embryos produced by handmade cloning (HMC), and number of embryos transferred per synchronous female recipient – Experiment 2.

IVC + ET	Reconstructed embryos	Fusion rate[A]		Embryos per total MII oocytes		Cleavage rate[B]		Morulae rate[C]		Blastocyst rate[C]		ET
	n	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*/recipient
Day 1 + ET	139	109	78.4	109/268	40.7[a]	–	–	–	–	–	–	88/5
Day 7 + ET	209	173	82.8	171/710	24.1[b]	131/150	87.3	27/150	18.0	19/150	12.7	13/1[D]
Total	348	282	81.0	282/1046	27.0	131/150	87.3	27/150	18.0	19/150	12.7	101/6

[a,b] Numbers in the same column without common superscripts differ, $P < 0.05$.
[A] Fusion of either one demi-cytoplast (85%) + donor cell or two hemi-cytoplasts (2 × 50%) + donor cell (see text for details).
[B] Assessment on Day 2 of development.
[C] Assessment on Day 7 of development.
[D] Transfer of seven compact morulae and six blastocysts.

for cloning are considered by many investigators as a key factor needed to increase nuclear reprogramming competence and, in turn, the cloning efficiency. In fact, several studies have proposed over the years the need of the synchronization/arrest of the cell cycle in the G0/G1 phase (Akshey et al., 2011; Choresca et al., 2009; Koo et al., 2009) or the evaluation of the competence of other donor cells other than fibroblasts from adult or fetal origin (Dutta et al., 2011). In our work, we also verified the growth profile of somatic cells from a transgenic goat. To date, studies on cell culture conditions and cell cycle synchronization from transgenic goats are rather limited (Memili et al., 2004). A standard analysis of the phenotype and PDT for ear fibroblast cells from an hG-CSF transgenic goat was performed in this study, demonstrating the cells' potential to be used as donor cells in our SCNT program. For that, cells were synchronized by contact inhibition through high confluence, leading to the successful *in vitro* production of cloned embryos by HMC. Even though we have not analyzed the cell cycle stage to verify the cell synchrony prior to cloning in this study, our results agree, at least in part, with findings by Boquest et al. (1999) in which more than 85% of cells were in G0/G1 when from highly confluent cultures (>95%), and by Gerger et al. (2010), who demonstrated a linear increase in blastocyst yield and a positive correlation between the *in vitro* developmental potential of HMC-derived cloned bovine embryos and the level of cell culture confluence at or near the plateau phase prior to cloning.

Thus far, several transgenic and non-transgenic live born goats have been produced by conventional cloning using micromanipulators (Baguisi et al., 1999; Behboodi et al., 2004; Keefer et al., 2002; Lan et al., 2006), and recently, a cloned goat have been produced (Nasr-Esfahani et al., 2011) using the simplified zona-free method proposed by Oback et al. (2003) in cattle. Even though *in vitro* (Akshey et al., 2008, 2010, 2011; Feltrin et al., 2012) and *in vivo* (Feltrin et al., 2012) embryo development has been attained in goats using the HMC procedures, results on the birth of HMC-derived goats are yet to be achieved. A few factors may contribute to the slower technological development of alternative cloning procedures in goats when compared with cattle, including the lower availability of good quality oocytes, and the less extensive knowledge of the cell biology, physiology, and manipulations and strict culture requirements for goat *in vitro* embryo production (Baldassarre et al., 2004a; Behboodi et al., 2004). Such factors delay the development of alternative procedures, as for cloning by HMC, with the discussion of technical results on the establishment of successful protocols being important for future studies.

The easy access to an abundant and inexpensive source of properly matured oocytes is one of the limiting factors in the commercial application of SCNT in farm animals (Baldassarre et al., 2004a). Although cloned transgenic goats have been produced using oocytes collected from hormonally stimulated donor animals (Baguisi et al., 1999), abattoir-derived ovaries is still a great and inexpensive source of oocytes for the production of transgenic cloned goats, and may be more cost effective for the generation of pharmaceutically important proteins. In general, abattoir oocytes may be easier to obtain and more cost effective than maintaining and stimulating a herd of donor goats. In turn, laparoscopic procedure for the aspiration of follicle from hormonally stimulated females has been proposed as an efficient method for the exponential propagation of goats of high genetic value (Cognie et al., 2004). In this study, we demonstrated that, on one hand, the *in vivo* approach resulted in higher quality oocytes, whereas on the other hand, a higher oocyte recovery rate was obtained from slaughterhouse ovaries. Nevertheless, both laparoscopic and aspiration of slaughterhouse ovaries were effective as sources of good quality oocytes for goat SCNT trials.

Reggio et al. (2001) demonstrated no significant differences in the percentage of matured oocytes successfully enucleated and reconstructed using oocytes from FSH-stimulated females *vs.* abattoir-derived ovaries. In the same study, the COC source had also no effect on embryo development and overall pregnancy rate. In our study, no differences in number of aspirated follicles, total retrieved COCs and viable COCs were seen between hormonally stimulated females subjected to laparoscopic *in vivo* follicular aspiration and slaughterhouse ovaries from nonstimulated females, which contrasted results by Reggio et al. (2001), who obtained an average of 24.4 and 1.6 oocytes per female from the superstimulated and nonstimulated animal groups, respectively. Interestingly, higher recovery efficiency was obtained from slaughterhouse ovaries in this study, which was likely due to a stricter control over

the follicular aspiration process than the remote laparoscopic approach. Also, visually undetected follicles may be aspirated when controlled by hand, improving the recovery efficiency. Despite the lower recovery efficiency, the *in vivo* aspiration of superstimulated goats resulted in a higher proportion of good quality oocytes. Likely, the hormonal stimulation provided better conditions for follicular development, resulting in COCs with improved quality than in the case of nonstimulated females (Sirard et al., 2006). However, to be effective, the laparoscopic *in vivo* oocyte retrieval requires specific instrumentation, highly trained personnel, and usually, hormonal stimulation of the donor females, which makes the procedure more laborious, expensive and less cost effective than the use of slaughterhouse ovaries.

To be suitable for the goat, the HMC procedure requires specific adaptations in its steps (Nasr-Esfahani et al., 2011). Here, we also observed that zona-free oocyte and embryo manipulations presented singular conditions in goats when compared with cattle. When placed in *in vitro* maturation, immature oocytes from different livestock species are known to take different time periods to reach the metaphase II stage. In the present study, oocytes used for cloning by HMC were matured for 20 h before being processed for cloning. In general, the IVM process requires a period of around 24 h for its completion, although in goats, a slightly longer period of 27 h has been observed (Cognie et al., 2004). Meanwhile, in our cloning procedures, the beginning of embryonic reconstruction started usually 20–21 h after IVM so that embryonic activation could occur around 27 h after then onset of IVM. Consequently, the period of oocyte/zygote manipulation in this work was carried out within the window of biological viability already reported for the goat.

The enzymatic digestion is considered the method of choice in domestic livestock species for the removal of the zona pellucida. Interestingly, we observed that goat oocytes appeared to be more sensitive to protease digestion, when compared with our previous experience in cattle (Ribeiro et al., 2009; Gerger et al., 2010). Consequently, in the present study, we used enzymatic digestion with protease at a concentration of 0.25%, differently of the 0.5% concentration used for bovine oocytes (Gerger et al., 2010; Vajta et al., 2004). In another study with goat oocytes, Nasr-Esfahani et al. (2011), observed that 0.25% protease in the presence of 10% serum in medium was ideal for zona removal after 1 min incubation, after evaluating the protease concentration in a range from 1 to 5 mg/mL (0.1–0.5%) for the zona removal. Moreover, using the 0.25% protease solution, oocytes with enlarged but not completely dissolved zonae were released from the zona by pipetting with a narrow bored pipette. Additionally, we also used the same concentration of protease as Akshey et al. (2010) for zona-free goat embryos. Rates of survival following zona digestion and rates of development to the morula and blastocyst stages reflected well the effectiveness of the lower concentration of protease used in this study.

The presence of an attached PB on the zona-free oocyte is very useful as a guide for the removal of a smaller amount of cytoplasm from the oocyte, adjacent to the PB, during manual enucleation. Using procedures adapted from cattle (Ribeiro et al., 2009), the manual section of goat oocytes resulted in 82 demi-cytoplasts and 231 hemi-cytoplasts (with ~85% and ~50% of the cytoplasmic volume) out of 648 matured oocytes. Thus, the PB-oriented bisection that results in ~85% cytoplasts improved the embryo yield after cloning by HMC (especially in the Group Day 1 + ET, as presented in Table 4), rendering it advantageous for species such as the goat, for which oocyte supply and numbers are usually limiting factors. However, the reduction in cytoplasmic volume, even if minimal, may have a detrimental effect on further embryo development, as demonstrated by others in cattle (Peura et al., 1998; Westhusin et al., 1996; Ribeiro et al., 2009). Nevertheless, oftentimes the PB detaches from zona-free oocytes during the manipulation for the zona removal. Consequently, such oocytes must be bisected in two similar halves, unless alternative approaches are applied to minimize the loss of cytoplasm. In some species, such as in mice and cattle, the use of chemical supplements in the medium, like sucrose and demecolcine, is helpful for the appearance of a clear protrusion cone on the oocyte surface in the region next to the MII plate (Vajta et al., 2001).

The *in vitro* culture (IVC) conditions have a crucial impact on the development not only of *in vitro*-fertilized but also of SCNT-derived embryos (McEvoy, 2003). Several culture systems, such as the WOW system (Vajta et al., 2001), agarose gel (Peura and Vajta, 2003), flat surface (Akshey et al., 2010), and microdrops (Oback et al., 2003) have been developed for the IVC of zona-free embryos, with a significant variation in terms of blastocyst production efficiency. The WOW system is an effective approach to culture zona-free embryos, as it provides a constant *in vitro* microenvironment due to its suitable size surrounding the embryo, also avoiding the formation of chimeras when direct contact between individual zona-free embryos occurs (Du et al., 2007). In cattle, a blastocyst yield of about 50% was observed using the WOW system (Vajta, 2007), which clearly demonstrates its efficiency for the IVC of zona-free cloned embryos. Moreover, the lack of zona pellucida as a result of HMC procedures does not affect either the development to blastocysts (Rodríguez et al., 2008) or the birth of cloned bovine, swine and mice (Vajta et al., 2004; Ribas et al., 2005; Du et al., 2007). Using a modified WOW system, goat blastocyst rates observed in this study were equivalent to results obtained by Akshey et al. (2010) in goats (12.7% *vs.* 15.8%, respectively). Dutta et al. (2011), using adult fibroblast cells, and Feltrin et al. (2012), using fibroblasts and bone marrow-derived mesenchyimal stem cells from adult lysozyme transgenic goats, obtained cloned blastocyst rates of around 30% by the HMC procedure. On other hand, in preliminary experiments, parthenogenetic morula/blastocyst rate of 21.1% was obtained using goat oocytes with zona pellucida activated chemically (unpublished data).

Pregnancies and live born goats have been reported by cloning using conventional (Baguisi et al., 1999; Keefer et al., 2002; Behboodi et al., 2004) and zona-free SCNT (Nasr-Esfahani et al., 2011) procedures, as previously described. To produce live cloned goats, reconstructed embryos must be transferred into the oviduct or the uterus of synchronous recipients. For the intrauterine transfer,

embryos must be in further stages of development, which can be attained either by the transient *in vivo* culture into the oviducts of a host female (Wilmut et al., 1997), which is usually unpractical, or after the IVC in the laboratory. The IVC of cloned embryos is one of the most important stages affecting the preimplantation development, pregnancy and the number of offspring generated (Campbell et al., 2007). Although cloned goat embryos can develop to the blastocyst stage after IVC, there are only few births of live kids following the transfer of *in vitro*-cultured cloned blastocysts (Nasr-Esfahani et al., 2011). Ohkoshi et al. (2003) produced the first SCNT-derived cloned goat following the transfer of *in vitro*-produced blastocysts, and despite the lower efficiency, Tang et al. (2011) confirmed that *in vitro*-cultured SCNT goat blastocysts can develop to term. However, studies regarding an optimal embryo culture system for goat embryos are still pending. To the current knowledge, most of the viable cloned goats have been derived from the transfer of early stage embryos to the oviduct of recipients. Most viable cloned goats were derived from 1- to 4-cell stage embryos when transferred to the oviducts of recipients, with 7–10 cloned embryos transferred to each oviduct; pregnancy rates on Days 30–50 ranged from 17 to 50%, depending on the type of donor cells, oocyte origin, and culture conditions (Keefer et al., 2001, 2002; Behboodi et al., 2005; Lan et al., 2006). In this light, the number of goat embryos transferred per female seems to affect the survival rate of embryos in cloning programs (Liu et al., 2011). The increase in the number of transferred embryos may be beneficial for producing more live offspring with fewer recipients (20–30 cloned embryos per recipient) in goat cloning (Liu et al., 2011). Reggio et al. (2001) produced five transgenic cloned goats, using an average of eight 2- to 4-cell stage embryos/female (ranging from 1 to 15 embryos/female). In the limited trial carried out in our study, we also analyzed the *in vivo* developmental competence of cloned embryos by the transfer of a mean number of 17.6 zona-free 1-cell stage HMC-derived embryos and 13 cloned morulae/blastocyts per synchronous female recipients, resulting in no detected pregnancies by ultrasonography.

Even though no recipients receiving reconstructed cloned 1-cell stage goat embryos became pregnant in this study, and as only a few replications with morula and blastocyst transfers were performed, the negative results may be more related to the low number of transfers performed and to factors unrelated to the zona-free condition of the transferred embryos. Zona-free blastocysts have been extensively and successfully transferred in cattle (Peura et al., 1998; Oback et al., 2003; Vajta et al., 2004), pigs (Du et al., 2007), and even goats (Nasr-Esfahani et al., 2011), resulting in live born animals. However, a current dogma in developmental biology postulates that the transfer of embryos into the oviducts at early stages requires the presence of the zona pellucida or similar protective barrier, such as agar embedding, for the protection of embryos from the recipient's immune system (Loi et al., 1999). One of the main limitations of the zona-free method, then, would be the fragility of the zona-free oocyte and early embryos, and the requirement that embryos have to reach at least the compact morula or early blastocyst stages *in vitro* to avoid disintegration or an immune system attack in the oviduct without the protective layer of the zona pellucida (Du et al., 2005; Vajta et al., 2005). Considering that no pregnancies were obtained in this study following the transfer of HMC-derived embryos, and taking the factors above into consideration, it is feasible to consider that the transfers of zona-free 1-cell stage embryos into the oviducts were expected to result in failure. However, a similar study carried out in pigs to evaluate the *in vivo* developmental viability of porcine handmade cloned embryos showed that the transfer of zona-free 1-cell stage embryos directly into the oviduct of a synchronous sow, without zona pellucida or agar embedding, can in fact result in a positive pregnancy outcome (Ohlweiler et al., 2010). However, such possibility still needs to be tested in other species, such as the goat.

In summary, skin-derived somatic cells from an hG-CSF transgenic goat were successfully used as karyoplasts for the *in vitro* production of cloned embryos by HMC procedures. Also, despite the lower COC recovery efficiency, our preliminary results indicated that the *in vivo* laparoscopic procedure was as efficient as the use of slaughterhouse ovaries for the retrieval of goat COCs, with both methods rendering a similar number of good quality oocytes, on a per goat basis. Nevertheless, the use of slaughterhouse ovaries from nonstimulated goat females appeared to be as effective to provide oocytes for use as cytoplasts for cloning as the *in vivo* laparoscopic approach from hormonally stimulated females, making the former a more advantageous procedure than the latter due to its simplicity and rather inexpensive outcome. Further studies are still underway for the production of live born offspring by HMC procedures in goats.

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