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# Effects of oocyte source, cell origin, and embryo reconstruction procedures on *in vitro* and *in vivo* embryo survival after goat cloning

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## Abstract

The birth of cloned goats has been well documented, but the overall goat cloning efficiency by somatic cell nuclear transfer procedures is still low, which may be further intensified in extreme environments. The aim of this study was to produce cloned goats under the conditions of the Brazilian Semi-Arid region, in a transgenic program for the expression of human lysozyme in the milk to target childhood diarrhea and malnutrition, comparing the effects of oocyte source, cell type, and embryo reconstruction procedures on *in vitro* and *in vivo* embryo survival after cloning by micromanipulation or by handmade cloning. The use of *in vitro*-matured oocytes resulted in more viable embryos after cloning than *in vivo*-matured cytoplasts, but no differences in pregnancy rates on day 23 were seen between oocyte sources (77.5 *vs.* 77.8%, respectively). The presence or absence of the zona pellucida for embryo reconstruction (78.8 *vs.* 76.0%, respectively) did not affect pregnancy outcome after transfer. However, pregnancy rate on day 23 was higher for embryos chemically activated by a conventional than a modified protocol (88.1 *vs.* 50.0%), and for embryos reconstructed with mesenchymal stem cells and fetal fibroblasts (100.0 and 93.3%) than with adult fibroblasts (64.7%). Although most pregnancies were lost, the birth of a cloned female was obtained from embryos reconstructed by micromanipulation using non-transgenic control cells and *in vitro*-matured oocytes with intact zona pellucida, after conventional activation and transfer at the 1-cell stage.

**Keywords**: goats, oocyte maturation, somatic cell nuclear transfer, zona-free embryos.

## Introduction

Childhood diarrhea and malnutrition still are some of the major social problems in developing countries. This is especially true in less favorable regions such as the Semi-Arid area of Brazil, resulting in thousands of infant deaths worldwide each year (Boccolini *et al*., 2012). Several epidemiological studies have already demonstrated the benefits of breast feeding for the infant´s health, including passive immune-defense against infections by pathogenic microorganisms, growth stimuli to benign agents in the intestinal microbiota, development and maturation of the gastrointestinal tract, protection against asthma and allergies, and anti-inflammatory effects (Lönnerdal, 2003; Oddy, 2017). The positive effects of human milk to breastfed children are reflected in an improved general health, adequate growth and development including epigenetic beneficial changes, lower susceptibility to chronic and acute diseases during and after childhood, (American Academy of Pediatrics, 1997, Verduci *et al*., 2014), and lower incidence of infections of the gastrointestinal, respiratory and urinary tracts (Levy, 1998). Recently, breastfeeding for at least 12 months also been shown to be associated with improvement in children neurodevelopment, an increase in IQ scores, more schooling and higher salaries as adults (Victora *et al*., 2015; Lechner and Vohr, 2017). Such effects on the health of the young have been attributed to the presence of immunocompounds in human breast milk, such as lysozyme, lactoferrin and secretory immunoglobulin A, or IgA (Levy, 1998; Hassiotou and Geddes, 2015). The antimicrobial effects of human lysozyme and lactoferrin are considered as integral part of the passive immunity and defense against bacteria, viruses, parasites and fungi that is passed on to children through human breast milk (Mountzouris *et al*., 2002; Chow *et al*., 2016; Lönnerdal, 2016). Unfortunately, breastfeeding and the supply of such compounds to the infant are not permanent, which normally causes an impact on the child's health in unassisted populations. In contrast, the milk produced by livestock, such as goats, can be easily and continuously obtained, and used as a substitute for the nutritional properties of breast milk. However, lysozyme and lactoferrin are present in insufficient

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concentrations in animal´s milk to provide effective protection to humans (Stenfors *et al*., 2002; Krol *et al*., 2010). Therefore, the production of human immunocompounds in the milk of domestic animals through genetic engineering could contribute to human gastrointestinal health by modulating the resistance and susceptibility to various diseases, such as childhood diarrhea (Maga and Murray, 1995; Maga *et al.*, 2006a, b). Because of this potential, the production of transgenic goats to express human lysozyme (hLZ) in the milk may have a great impact on society (Maga *et al*., 2006a, b; Cooper *et al*., 2015), especially for the population of less favorable areas in the world, such as the Brazilian Semi-Arid region. For such purposes, cloning by somatic cell nuclear transfer (SCNT) may be useful for the production of goats for the lysozyme and lactoferrin transgenic models (Meng *et al*., 2012).

Goat cloning by SCNT has been established since late 1990s (Baguisi *et al*., 1999), but the successful application of the technology is still challenging, which is translated by the low overall efficiency (< 1 to 5%) of the process as a whole (Baldassare *et al*., 2004; Gavin *et al*., 2013) in more favorable regions of the world. Technical and biological aspects associated with such low efficiency are further intensified when facing climatically extreme and more challenging environments, such as the Brazilian Semi-Arid region. Even though goats are generally considered more adaptable to adverse conditions than other domestic animals, high temperatures and low rainfall also affect this species through the lack of good quality food, excessive temperatures, and the presence of toxic plants, among other factors to which the animals are constantly exposed to (Carneiro, 2008; Chaves *et al*., 2011). As a consequence, an overall decrease in reproductive efficiency may occur (Chaves et al., 2010, 2011), which can make the production of a cloned animal even more challenging. The adjustment of SCNT cloning procedures in goats, therefore, gains crucial importance in this specific environment, given that no reports of cloned goats produced in Brazil and between parallels 30°N and 30°S in the world have been available prior to this study and to our previous recent report (Martins *et al*., 2016).

The aim of this study was to optimize goat cloning procedures under the conditions of the Brazilian Semi-Arid region, using somatic donor cells transgenic for the hLZ gene, through experiments evaluating the *in vitro* and *in vivo* survival of goat embryos cloned by micromanipulation or Handmade Cloning (HMC), comparing different cytoplast sources (*in vivo*- or *in vitro*-matured oocytes), karyoplast types (adult fibroblasts, fetal fibroblasts and mesenchymal stem cells), and manipulation and reconstruction procedures for the production of goat cloned embryos.

## Materials and Methods

All reagents and the water used for medium preparation were from Sigma Chemical Co. (St Louis, MO, USA), unless stated otherwise.

*Cytoplast source:* in vitro-*matured and* in vivo-*matured oocytes*

Two cytoplast (oocytes) sources were compared for the production of cloned goat embryos, either by micromanipulation or by HMC procedures, as below.

In vitro *maturation*

Goat ovaries were obtained *post-mortem* from pubertal adult goats and transported in DPBS (Nutricell, São Paulo, Brazil) to the laboratory in an insulated container at 33°C. *Cumulus*-oocyte complexes (COCs) were obtained by ovary slicing. Viable COCs, selected based on morphological quality adapted from Leibfried and First (1979), were *in vitro*-matured (IVM) for 22 ± 2 h, according to Pereira *et al*. (2013).

In vivo *maturation*.

Healthy pubertal adult goats were subjected to ovarian stimulation for the collection of *in vivo*-matured oocytes. For that, an intravaginal progesterone insert (Eazi-Breed CIDR®, Laboratórios Pfizer Ltda., Brazil) was placed on day 0, with the replacement by a new one after 6 days. On day 10, a total of 180 mg pFSH (Folltropin-V®, Bioniche, USA) was given IM, twice a day, for 3 days (36 and 36, 36 and 36, 18 and 18 mg, respectively). The progesterone insert was removed at the last FSH dose (day 12), and approximately 15 h after removal, a dose of 0.025 mg of gonadorelin acetate (Gestran®, ARSA S.R.L., Argentina), an analogue of GnRH, was given IM. Twenty-two hours (22 h) following the GnRH dose, ovaries were exteriorized by laparoscopy for the aspiration of >4 mm follicles with a 10 ml syringe attached to an 18 G needle. Recovered oocytes were selected according to the expansion of the cumulus cells and the presence of the first polar body (PB) under a stereomicroscope.

For both oocyte sources, after the removal of the cumulus cells and selection of matured oocytes (PB selection), a group of oocytes from each source was subjected to enzymatic zona pellucida (ZP) removal in 0.5% protease (P8811) solution, according to Ribeiro *et al.* (2009) and Pereira *et al*. (2013), for subsequent embryo reconstruction by micromanipulation without ZP or by Handmade Cloning (HMC), as described below. The other group of oocytes from each source was kept with intact ZP for cloning by micromanipulation with ZP.

*Somatic cell nucleus donors for cloning, and cell cycle analysis*

*Type of karyoplasts*

The somatic cells (karyoplasts) used for cloning were isolated from goats from the University of California, Davis, USA (CTNBio/Brazil 3467/2012), from a human lysozyme (hLZ) transgenic line. Mesenchymal stem cells (MSCs), adult fibroblast cells

(AF), and fetal fibroblast cells (FF) were used for cloning by micromanipulation, whereas only MSCs and AF were used for Handmade Cloning, as below. Briefly, MSCs were isolated from the bone marrow of a neonate male; FF from a 40-day male fetus; and AF were obtained after the ear biopsy of a pubertal adult female, according to Baguisi *et al.* (1999), Monaco *et al*. (2009) and Gerger *et al*. (2010), respectively. The MSCs were used at 60-70% confluence (passage 4), the FF at 80-90% confluence (passage 4), and the AF (passage 3) at >95% confluence. Except for MSCs, the FF and AF cell cycles were synchronized by contact inhibition (high confluence) after 3 to 5 days of *in vitro* culture.

In a few cloning procedures (n = 3), fetal fibroblast cells obtained from a 40-day non-transgenic female fetus were used at low passage (P2) and high confluence (>95%) as controls for cloning procedures and to evaluate *in vitro* and *in vivo* embryo survival. Due to the low frequency of use of such cells for cloning, data after the use of control cells are not presented in comparative form with the other transgenic hLZ cells lineages.

*Analysis of the cell cycle*

A portion of the MSCs, AF, and FF cells was used for cloning by micromanipulation, while the remaining cells were processed for the determination of the cell cycle phase through flow cytometry. Cultures of MSC, FF and AF cells were isolated with 0.25% trypsin-EDTA and centrifuged twice in DPBS. Then, cell were treated with 10 mg/ml RNase A (R4875) and 100 µg/ml propidium iodide (PI, P4170) in a 2.94% sodium citrate solution and 0.1% Triton™ X-100 (T8787), for 30 min at RT. Cells were then centrifuged at 1500 g for 5 min, at 4°C, re-suspended in DPBS and immediately placed in a container with ice for the determination of the cell cycle phase (G0/G1, S, G2/M) by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA, USA). Histogram plots were created using the Cell Quest software (Becton Dickinson). Percentage of cells within the various phases of the cell cycle were calculated using Cell Quest by gating G0/G1, S, and G2/M cell populations, with a scatterplot of red fluorescence (FL2-A x FL2-W).

*Experiment 1: Production of goat cloned embryos by micromanipulation: effects of the enucleation of* in vitro- *or* in vivo-*matured oocytes with or without the zona pellucida, reconstruction with distinct karyoplast types by membrane fusion or cellular micro-injection, and embryo activation with or without cytochalasin B*

*Enucleation with or without ZP*

Groups of zona-intact (ZI) and zona-free (ZF) *in vitro*- or *in vivo*-matured oocytes were enucleated by micromanipulation. For that, oocytes were first incubated for 15 min in TCM-HEPES supplemented with 5 µg/ml cytochalasin B (C6762) and 5 µg/ml Hoechst 33342 (B2883). For ZI oocytes, conventional micromanipulation procedures were performed, according to Baguisi *et al*. (1999) and Keefer *et al*. (2001). For ZF oocytes, enucleation by micromanipulation was performed according to Oback *et al*. (2003).

*Reconstruction by cell fusion (CF) or by donor cell microinjection (CI)*

For the reconstruction of ZI embryos, the nucleus donor cells (MSC, AF or FF) were either transferred by micromanipulation to the perivitelline space of enucleated goat oocytes (reconstruction by cell fusion, CF), or were injected directly into the ooplasm (reconstruction by cell injection, CI), according to Keefer *et al*. (2001) and Chen *et al*. (2007), respectively. Prior to injection into the ooplasm, cells were consecutively pipetted with a 12 µm reconstruction pipette until a deformation of the cell membranes was visible.

For the reconstruction of ZF embryos, enucleated structures were incubated for 2 to 3 min in 500-µg/ml phytohemagglutinin (PHA) solution so that the cytoplast could adhere to the karyoplast, under stereomicroscope. All ZF embryos were reconstructed by membrane fusion.

For membrane fusion (ZI-CF and ZF-CF), reconstructed complexes were rinsed in fusion medium (Ribeiro *et al*., 2009), and then subjected to membrane fusion in an electrofusion apparatus (BTX Electro Cell Manipulator 200, Biotechnologies & Experimental Research Inc., USA San Diego, CA, USA) coupled to a 320 µm fusion chamber (BTX453, BTX Instruments, Genetronics, San Diego, CA, USA). The ZI structures were fused by two 2-kV/cm DC pulses for 20 µs, whereas the ZF structures received two 1-kV/cm DC pulses for 20 µs. Fusion rates were assessed 45 to 60 min after fusion. Non-fused structures were subjected to a second round of electrofusion.

*Use of cytochalasin B during the embryonic activation*

Reconstructed embryos were submitted to two different protocols for embryo activation, based on Dutta *et al*. (2011), for protocol 1, and on Wells *et al*. (2011), for protocol 2, as follows. For the conventional protocol, or protocol 1, cloned embryos were exposed for 5 min to 5-µm ionomycin solution (I0634). Embryos were then incubated at 38.5ºC for 4 h in TCM199 supplemented with 2 mm 6-DMAP (D2629). For the modified protocol, or protocol 2, structures were incubated for 2 h in 2.5 µg/ml cytochalasin B (CB) immediately after fusion evaluation, followed by the activation in 5 µm ionomycin for 1 min. Then, embryos were incubated in 2 mm 6-DMAP for 4 h. Finally, cloned embryos were *in vitro*-cultured, as described below.

*Experiment 2: Production of goat cloned embryos by handmade cloning: effects of the cytoplast source, karyoplast type, and final embryonic cytoplasmatic volume*

Procedures for HMC were adapted from

Ribeiro *et al*. (2009) for cattle and Pereira *et al*. (2013) for goats.

*Cytoplast source*

*In vitro*- and *in vivo*-matured COCs were subjected to enzymatic removal of the zona pellucida in 0.25% protease, as above.

*Embryonic cytoplasmatic volume*

Zona-free oocytes were sectioned manually in 2.5 µg/ml cytochalasin B, depending on the presence or absence of the polar body (PB) or a protrusion cone (PC), indicative of the location of the MII plate. Oocytes without PB or PC were bissected in halves of equal sizes and volumes (50% of the volume), whereas oocytes with PB or PC were sectioned at the extremity next to the PB or PC, resulting in portions of approximately 85 and 15% of the original volume, with the smaller portion containing the MII plate. All hemi-oocytes were selected by the presence (nucleated) or absence (enucleated) of the MII plate under UV light, in TCM199 + 10% of FBS + 10 µg/ml Hoechst 33342. Embryos were reconstructed either with two 50% hemi-oocytes + donor cell (50% + 50% + cell) or one 85% hemi-oocyte + donor cell (85% + cell).

*Karyoplast type*

Single hLZ-derived MSC or AF cells were used as karyoplasts for embryo reconstruction by attachment to enucleated *in vivo*- or *in vitro*-matured ZF hemi-oocytes with 50 or 85% cytoplasmatic volume, after a brief exposure to PHA solution, as aforementioned. Structures reconstructed by HMC were fused under the same fusion procedures as described above for ZF-embryos, followed by chemical activation by the conventional protocol (protocol 1), as above. HMC-derived embryos were *in vitro*-cultured, as described below.

*In vitro culture (IVC)*

Cloned embryos reconstructed by micromanipulation or by HMC were *in vitro*-cultured in modified SOFaa medium (Holm *et al*., 1999) supplemented with 5% FBS + 0.3% BSA and 1% ITS, at 38.5°C with 100% relative humidity, and a gas mixture containing 5% $CO_2$, 5% $O_2$ and 90% $N_2$ (Ribeiro *et al*., 2009). For ZI cloned embryos, 15 to 20 structures were cultured in 100 µl drops; in turn, ZF cloned embryos were cultured in a modified WOW system (Vajta *et al*., 2000; Feltrin *et al*., 2006) in 4-well dishes containing 500 µl IVC medium. Prior to transfer to synchronous female recipients, cloned embryos reconstructed by micromanipulation were *in vitro*-cultured for approximately 18 h, whereas embryos reconstructed by HMC were *in vitro*-cultured for 7 days to the blastocyst stage.

In some procedures (n = 6), groups of ZI oocytes were kept under the same conditions as the structures reconstructed during cloning, to be chemically activated (conventional protocol, or protocol 1) and *in vitro*-cultured for 7 days, under the same conditions described above (control group by parthenogenesis).

*Estrous cycle synchronization of recipient females*

Recipient females were estrus synchronized by the use of intravaginal progesterone inserts (Eazi-Breed CIDR®, Laboratórios Pfizer Ltda., Brazil) for 10 days. At insert removal, 300 IU eCG (Folligon®, Intervet, Brazil) and 0.075 mg PGF2α (Prolise®, ARSA S.R.L., Argentina) were given IM. A dose of 0.025 mg gonadorelin acetate (Gestran®, ARSA S.R.L., Argentina) and 25 mg LH (Lutropin®, Bioniche, Brazil) were given IM 36 and 60 h after eCG administration, respectively.

*Embryo transfer (ET) and pregnancy diagnosis: embryos reconstructed by micromanipulation*

Cloned embryos at the 1-cell stage were transferred to the oviduct of recipient females on day 1 of the cycle by semi-laparoscopy, approximately 8 h after the LH dose. The mean number of embryos transferred per female was 13.4, with a variation of 11 to 25 embryos per recipient. On the 4th day after the embryo transfer, an intravaginal progesterone insert (Eazi-Breed CIDR®, Laboratórios Pfizer Ltda., Brazil) was placed in the female recipients and remained until pregnancy diagnosis. The progesterone insert was replaced weekly until day 140 of the pregnancy, or until the detection of a non-viable pregnancy (no pregnancy after diagnosis or after detection of conceptus death).

*Embryo transfer (ET) and pregnancy diagnosis: embryos reconstructed by HMC*

Cloned goat embryos on days 7 of development were transferred to synchronous female recipients (4 to 6 embryos/female), by semi-laparoscopy, to the uterine horn ipsilateral to the ovary with a functional corpus luteum, according to Melican and Gavin (2008). This group of female recipients did not receive any progesterone supplementation (intravaginal inserts) after the transfer of embryos.

Pregnancy diagnosis was performed on day 23 by rectal ultrasonography using a 6 MHz linear transducer. Pregnancies were monitored by ultrasound scanning every 3-4 days until no sign of pregnancy was displayed or for confirmation of pregnancy viability. For viable pregnancies, a transabdominal ultrasound examination was repeated at weekly intervals from the 35th day of pregnancy through term. The presence of one or more embryos or fetuses, detectable heart beat, embryonic or fetal membranes, and placentomes were examined qualitatively.

*Data analysis*

Data relative to *in vitro* survival, fusion and

pregnancy rates were compared between the experimental groups by the Chi-square test (Minitab, State College, PA, USA), for $P < 0.05$, for cloning by micromanipulation (experiment 1) or by HMC (experiment 2), considering oocyte source (*in vivo vs. in vitro*) and cell type (MSC *vs.* FF *vs.* AF for micromanipulation, and MSC *vs.* AF for cloning by HMC). For cloning by micromanipulation, the analyses also considered the type of manipulation (ZI *vs.* ZF), reconstruction (CF *vs.* CI), embryonic activation protocol (1 *vs.* 2), and the proportion of cells at different phases of the cell cycle (G0/G1 *vs.* S *vs.* G2/M), whereas for cloning by HMC, the analyses also included data on the final cytoplasmic volume (85 *vs.* 100%). Data regarding the number of retrieved COCs per animal, for both cloning methods, were compared by the Students´ test ($P < 0.05$).

## Results

*Experiment 1: Embryo reconstruction by micromanipulation*

*Cytoplast source*

For *in vitro*-matured oocytes, after 16 replications, a total of 4,138 immature COCs (19.9 COCs/goat) were recovered by *post-mortem* ovary slicing from 415 ovaries collected from non-stimulated slaughterhouse does. Upon morphological selection, 20 grade I (1.0%), 380 grade II (18.9%), 1,255 grade III (62.4%) and 509 grade IV (20.2%) oocytes (2,164 viable COCs, 52.3%) were *in vitro*-matured (10.4 COCs/female). After IVM, the maturation rate, based on the presence of the PB, was 83.6% (17/20), 60.2% (228/380), 43.5% (546/1255) and 30.2% (153/509), for grades I, II, III and IV COCs, respectively, for a total maturation rate of 48.4% (1,047/2,164) and mean of 5.0 matured oocytes/female. For *in vivo*-matured oocytes, after seven replications, a total of 974 COCs (14.3 COCs/female) were recovered after the *in vivo* aspiration of pre-ovulatory follicles from 136 ovaries from pFSH-stimulated females. Upon selection, 937 COCs (96.2%) had cumulus cells expansion (13.8 oocytes/goat), and 741 oocytes displayed the extrusion of the 1st PB, resulting in a maturation rate of 52.6% and 7.3 mature oocytes/female. The maturation rate and the number of matured oocytes/female were significantly higher in the group of *in vivo*-matured oocytes when compared with the group of *in vitro*-matured COCs ($P < 0.05$). However, when used for embryo reconstruction by micromanipulation, survival rate after enucleation and after reconstruction *per se* was higher in the *in vitro*-matured group than the *in vivo*-matured counterpart (Table 1), with no differences observed in pregnancy rates between groups.

A total of 120 *in vitro*-matured oocytes obtained from samples from each replication were chemically activated and *in vitro*-cultured as control for the manipulation process *per se* and for oocyte quality/competence, from which, 75 cleaved (62.5%) and 22 reached the morula/blastocyst stages (18.3%) on day 7 of development.

Table 1. Survival rates after manipulation and embryo reconstruction following cloning by micromanipulation in goats using oocytes obtained either by *in vitro*-maturation after *postmortem* oocyte collection from nonstimulated females, or by *in vivo*-maturation after *in vivo* oocyte collection from FSH-stimulated females.

| Oocyte source | *Postmortem collection*, nonstimulated does, *in vitro*-matured oocytes | | *In vivo collection*, FSH-stimulated does, *in vivo*-matured oocytes | |
|---|---|---|---|---|
| Survival | n | % | n | % |
| Micromanipulated oocytes | 903 | - | 444 | - |
| Enucleated oocytes | 808 | 89.5[a] | 362 | 81.5[b] |
| Reconstructed embryos | 793 | 98.1[a] | 340 | 93.9[b] |
| Fusion or cell injection | 700 | 88.3[a] | 282 | 82.9[b] |
| Viable embryos in IVC | 649 | 92.7[a] | 275 | 97.5[b] |
| Viable embryos transferred | 561 | 86.4[a] | 221 | 80.3[b] |
| Recipient females diagnosed pregnant on day 23, from total | 31/40 | 77.5[a] | 14/18 | 77.8[a] |

[a,b]numbers in each row followed by different superscripts differ, for $P < 0.05$.

*Type of karyoplast and cell cycle phase*

No differences were observed in fusion rates and in the number of viable embryos for IVC between cell types used for the production of cloned embryos (MSC, FF, AF). After the IVC, the number of viable embryos was greater in the group derived from FF cells than in the other groups. However, pregnancy rate was higher in the groups of embryos produced using FF and MSCs when compared with AF cells (Table 2).

Differences ($P < 0.05$) were detected between cell types regarding the distribution in the phases of the cell cycle, as depicted in Figure 1. The synchronization of the cell cycle in the G0/G1 phase in the adult fibroblast (AF) group was greater than in the fetal fibroblast (FF) group, which, in turn, was greater than for mesenchymal stem cells (MSC), which showed a relationship with the mean cell confluence for each cell type (>95, 80-90 and 60-70%, respectively) when used for cloning.

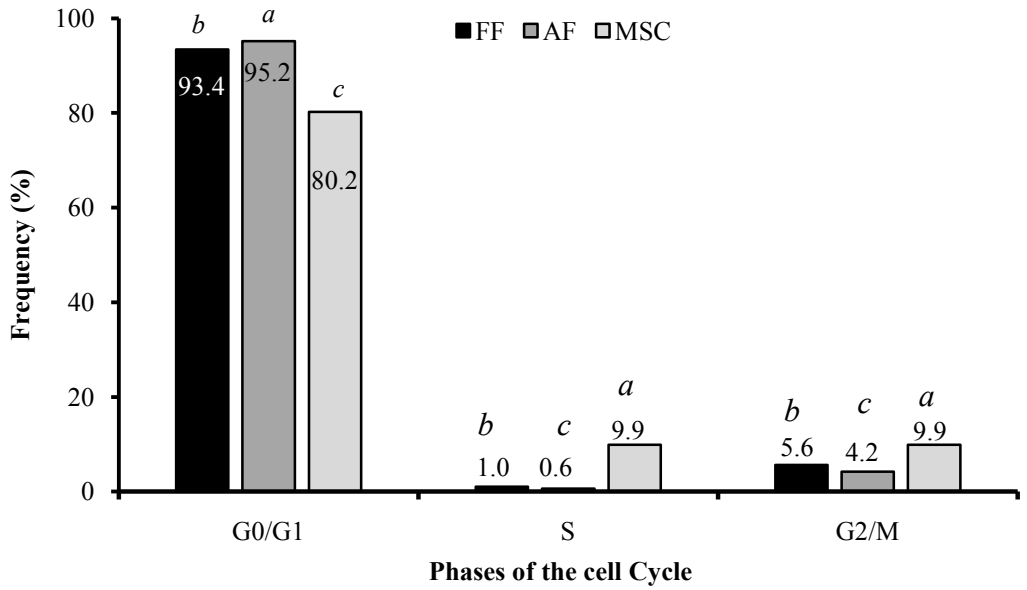


Figure 1. Distribution of cells in the phases of the cell cycle (G0/G1, S, G2/M) in goat donor cells used for cloning. FF: fetal fibroblasts, 80-90% confluence. AF: adult fibroblasts, >95% confluence. MSC: mesenchymal stem cells, 60-70% confluence. Numbers within or above columns indicate values in percentage. [a,b,c]columns in each phase of the cell cycle with distinct superscripts differ ($P < 0.05$).

Table 2. *In vitro* and *in vivo* survival of reconstructed embryos using fetal fibroblasts (FF), adult fibroblast (AF), or bone marrow-derived mesenchymal stem cells (MSC) as nucleus donor cells for cloning by micromanipulation in goats.

| Cell type | FF | | AF | | MSC | |
|---|---|---|---|---|---|---|
| | n | % | n | % | n | % |
| Reconstructed embryos | 251 | - | 686 | - | 196 | - |
| Fusion efficiency | 223 | 88.8[a] | 594 | 86.6[a] | 172 | 87.7[a] |
| Viable embryos in IVC | 208 | 93.2[a] | 548 | 92.2[a] | 164 | 95.3[a] |
| Viable embryos transferred | 192 | 92.3[a] | 452 | 82.5[b] | 138 | 84.1[b] |
| Recipient females diagnosed pregnant on day 23 | 14/15 | 93.3[a] | 22/34 | 64.7[b] | 9/9 | 100.0[a] |

[a,b]numbers in each row followed by different superscripts differ, for $P < 0.05$.

*Removal of zona pellucida*

Considering the presence or removal of the ZP, the post-enucleation survival, the fusion/microinjection rate and the number of viable embryos after reconstruction by micromanipulation were higher in the ZI group when compared to the ZF group. However, no differences were seen in pregnancy rates between groups (Table 3).

*Method for nucleus donor transfer*

The survival rate after reconstruction by micromanipulation was greater in the group of cell microinjection (CI) into the ooplasm when compared with the group of cell fusion (CF), irrespective of the presence (ZI) or absence (ZF) of the ZP ($P < 0.05$). However, no significant differences in pregnancy rates were observed between groups (Table 4).

Table 3. Effect of the presence (ZI) or removal (ZF) of the zona pellucida on survival after enucleation and embryo reconstruction by micromanipulation procedures for cloning by SCNT in goats.

| Micromanipulation method | Zona-intact oocytes (ZI) | | Zona-free oocytes (ZF) | |
|---|---|---|---|---|
| | n | % | n | % |
| Micromanipulated oocytes | 675 | - | 672 | - |
| Enucleated oocytes | 603 | 89.3[a] | 567 | 84.4[b] |
| Reconstructed embryos | 580 | 96.2[a] | 553 | 97.5[a] |
| Fusion or cell injection | 535 | 92.2[a] | 449 | 81.2[b] |
| Viable embryos in IVC | 517 | 99.2[a] | 407 | 90.6[b] |
| Viable embryos transferred | 445 | 86.0[a] | 337 | 82.8[a] |
| Recipient females diagnosed pregnant on Day 23, from total | 26/33 | 78.8[a] | 19/25 | 76.0[a] |

[a,b]numbers in each row followed by different superscripts differ, for $P < 0.05$.

Table 4. *In vitro* survival and pregnancy outcome of goat cloned embryos after embryo reconstruction by micromanipulation and transfer to female recipients on day 1 of development

| Oocyte type | Cell fusion (CF) or cell injection (CI) | Reconstructed structures | Survival after CF or CI | | Transferred embryos | Recipients | Pregnancy rate (day 23) | |
|---|---|---|---|---|---|---|---|---|
| | | n | n | % | n | n | n | % |
| ZI | CI | 232 | 222 | 95.6[a] | 193 | 14 | 11 | 78.5[a] |
| ZI | CF | 348 | 295 | 84.7[b] | 252 | 19 | 15 | 78.9[a] |
| ZF | CF | 553 | 407 | 73.6 | 337 | 25 | 19 | 76.0[a] |

[a,b]numbers in each column followed by different superscripts differ, for P < 0.05. ZI: zona intact; ZF: zona free; CF: cell fusion; CI: cell injection.

*Activation protocol*

When comparing the activation protocol, pregnancy rates on day 23 after the reconstruction by micromanipulation was higher in the group of embryos activated by the Conventional Protocol, or protocol 1, when compared with the activation protocol with CB, or protocol 2 (Table 5).

Table 5. *In vitro* survival and pregnancy outcome of goat cloned embryos after embryo reconstruction by micromanipulation and embryo activation using either a conventional or a modified activation protocol.

| Activation protocol | Conventional (1) | | Modified (2) | |
|---|---|---|---|---|
| | n | % | n | % |
| Activated embryos | 727 | - | 255 | - |
| Viable embryos in IVC | 687 | 94.5[a] | 237 | 92.9[a] |
| Viable embryos transferred | 591 | 86.0[a] | 191 | 80.6[b] |
| Recipient females diagnosed pregnant on day 23 | 37/42 | 88.1[a] | 8/16 | 50.0[b] |

[a,b]numbers in each row followed by different superscripts differ, for P < 0.05.

*Experiment 2: Embryo reconstruction by handmade cloning*

*Cytoplast source*

The summary of results after reconstruction by HMC, after 10 replications, is presented in Tables 6 and 7. A total of 50 and 106 goats (99 and 211 ovaries, respectively) were used to obtain *in vivo*- and *in vitro*-matured oocytes, respectively. No differences were observed in the mean number of recovered COCs (17.8 *vs.* 20.6/female) and viable oocytes (14.2 *vs.* 13.1/female) between non-stimulated donor females (*in vitro* maturation) and hormonally superstimulated does (*in vivo* maturation), respectively. The recovery rate for total COCs was higher from non-stimulated goats (1,880/2,095 follicles, 89.7%) than from the hormonally stimulated group (1,019/1,178 follicles, 86.5%). Moreover, the proportion of viable COCs and the oocyte maturation rate (presence of polar body) were significantly higher in the *in vitro* group than in the *in vivo* group (Table 6). However, the source of oocytes was not associated with differences in *in vitro* (Table 7) or *in vivo* development of cloned goat embryos, irrespective of the other experimental variables, resulting in similar fusion (76/115, 66.1% *vs.* 193/274, 70.4%), re-fusion (18/35, 51.4% *vs.* 34/77, 44.2%), cleavage (66/94, 70.2% *vs.* 163/227, 71.8%) and blastocyst (26/94, 27.7% *vs.* 70/227, 30.8%) rates for *in vivo*- *vs. in vitro*-matured oocytes, respectively.

Table 6. Recovery and maturation rates using oocytes obtained *in vivo* from pFSH-stimulated females (*in vivo* maturation) or *postmortem* from nonstimulated females (*in vitro* maturation) for embryo reconstruction by Handmade Cloning.

| Oocyte source | Total COCs | Viable COCs | | Oocytes with polar body* | |
|---|---|---|---|---|---|
| | n | N | % | n | % |
| *In vivo* | 1,019 | 703 | 69.0[b] | 312 | 44.4[b] |
| *In vitro* | 1,880 | 1,380 | 73.4[a] | 727 | 52.7[a] |
| Total | 2,899 | 2,083 | 71.9 | 1,039 | 49.9 |

[a,b]numbers in each row followed by different superscripts differ, for P < 0.05. *Based on the number of recovered viable COCs.

*Type of karyoplast*

In general, the type of karyoplast or cytoplast did not affect any *in vitro* and *in vivo* embryonic development parameter, with no significant differences detected between groups regarding *in vitro* embryo development until day 7 (Table 7). When analyzed separately, embryo reconstruction using mesenchymal stem cells (MSC) resulted in higher fusion rates than using adult fibroblasts (160/198, 80.8% *vs.* 119/191, 62.3%, respectively), with no differences in re-fusion (24/43, 55.8% *vs.* 28/69, 40.6%), cleavage (128/174, 73.6% *vs.* 101/147, 68.7%) and blastocyst (58/174, 33.3% *vs.* 38/147, 25.9%) rates.

Table 7. *In vitro* development of goat embryos cloned by Handmade Cloning.

| Oocyte source | Donor cell type | Cytoplasmic volume | Cloned embryos | Fusion | | Re-fusion | | Cleavage rate | | Blastocyst rate | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | n | % | n | % | n | % | n | % |
| *In vivo* | MSC | 100% | 32 | 27 | 84.4[a] | 2/5 | 40.0[a] | 23/29 | 79.3[a] | 11/29 | 37.9[a] |
| | | 85% | 32 | 18 | 58.1[b] | 8/13 | 61.5[a] | 17/26 | 65.4[a] | 6/26 | 23.1[a] |
| | AF | 100% | 27 | 18 | 66.7[ab] | 4/9 | 44.4[a] | 16/22 | 72.7[a] | 6/22 | 27.3[a] |
| | | 85% | 24 | 13 | 54.2[b] | 4/8 | 50.0[a] | 10/17 | 58.8[a] | 3/17 | 17.3[a] |
| *In vitro* | MSC | 100% | 92 | 72 | 78.3[a] | 11/18 | 61.1[a] | 63/83 | 75.9[a] | 29/83 | 34.9[a] |
| | | 85% | 42 | 33 | 78.6[b] | 3/7 | 42.9[a] | 25/36 | 69.4[a] | 12/36 | 33.3[a] |
| | AF | 100% | 88 | 54 | 61.4[ab] | 13/36 | 36.1[a] | 50/68 | 73.5[a] | 19/68 | 27.9[a] |
| | | 85% | 52 | 34 | 65.4[ab] | 7/16 | 45.8[a] | 25/40 | 62.5[a] | 10/40 | 25.0[a] |
| Total | | | 389 | 269 | 69.3 | 52/119 | 43.7 | 229/321 | 71.3 | 96/321 | 29.9 |

[a,b]numbers in each column followed by different superscripts differ, for $P < 0.05$. MSC: mesenchymal stem cells. AF adult fibroblasts.

*Final cytoplasmic volume*

No differences were observed in final cytoplasmic volume for embryo reconstruction for fusion (171/239, 71.5% *vs.* 98/150, 65.3%), re-fusion (30/68, 44.1% *vs.* 22/44, 50.0%), and blastocyst (65/202, 32.2% *vs.* 31/119, 26.1%) rates, irrespective of the oocyte source and cell type used for cloning. However, the final cytoplasmic volume of 100% (50% + 50% + donor cell) resulted in higher cleavage rates (152/202, 75.2%) than with 85% (77/119, 64.7%). Embryo reconstruction using oocytes with 85% of the final cytoplasmic volume and MSCs resulted in lower fusion rates than after the reconstitution of 100% of the oocyte volume and MSCs, with no differences between groups when AF cells were used for cloning, independently of the cytoplasmic volume and oocyte source (Table 7). Also, no differences were observed between groups in re-fusion, cleavage and blastocyst rates.

In vivo *embryo development and birth of a cloned goat*

*Embryos reconstructed by micromanipulation*

Collectively, 782 cloned embryo were transferred on day 1 of development to the oviduct of 58 synchronous recipient females, resulting in 45 pregnancies (45/58, 77.0%) on day 23 of gestation. However, all established pregnancies with transgenic human lysozyme (hLZ) cells, in all groups and sub-groups, were lost before the fetal phase (up to day 45) of gestation. Nevertheless, after the induction of parturition following our established protocol (Chavatte-Palmer *et al*., 2013), a viable cloned female was born by elective Caesarean section after 147 days of gestation from the transfer of 27 embryos cloned with non-transgenic control cells to two female recipients. The cloned female was generated from the reconstruction by micromanipulation with control cells (non-transgenic fetal fibroblasts), using *in vitro*-matured oocytes with zona pellucida (ZI), reconstructed by membrane fusion (CF), activated by the conventional protocol (P1) and transferred on day 1 of development to the oviduct of a female recipient that had received a vaginal progesterone insert throughout pregnancy.

*Embryos reconstructed by HMC*.

A total of 96 goat blastocysts were transferred on day 7 of development to the uterus of 19 synchronous females. The pregnancy diagnosis, performed by ultrasound on day 23 of gestation, resulted in three pregnancies originated from *in vivo*- (n = 1) and *in vitro*-matured (n = 2) oocytes, from which, two were obtained using mesenchymal stem cells and one through the use of adult fibroblasts. The three pregnancies were lost before day 45 of gestation.

## Discussion

Although cloning by SCNT is well established in goats, with birth rates similar to those found in other species, there are no reports of cloned goats born in the tropics of the world, between parallels 30°N and 30°S, with all cloned goats born in countries under temperate or subtropical climates (Baguisi *et al*., 1999; Keefer *et al*., 2001; Reggio *et al*., 2001; Ohkoshi *et al*., 2003; Lan *et al*., 2006; Chen *et al*., 2007; Folch *et al*., 2009; Akshey *et al*., 2010; Colato *et al*., 2011; Liu *et al.,* 2011; Nasr-Esfahani *et al*., 2011; Wells *et al.,* 2011; Meng *et al*., 2012; An *et al*., 2012; Zhou *et al*., 2013; Yuan *et al.,* 2014; Feng *et al*., 2015; Hosseini *et al*., 2015; Zhang *et al*., 2015; Yang *et al*., 2016a; Bai *et al*., 2017). This fact may be associated with the low productive and reproductive indexes of goat herds in tropical countries, such as in the Brazilian Semi-Arid region, where the annual birth rate in goats does not exceed 20% (Guimarães, 2006).

The oocyte quality and competence play a

crucial role in the success of a cloning program, since the ooplasm is responsible for reprogramming the nucleus donor, which has an important effect on subsequent development (Fissore *et al*., 1999; Kelly *et al*., 2007; Mohapatra *et al*., 2015). Normally, better quality oocytes, usually grades I (GI) and II (GII), are selected for *in vitro* maturation (Chen *et al*., 2007; Tang *et al*., 2011). When analyzing data regarding the quality of immature COCs in this study, less than 20% of selected COCs were rated as GI and GII, with a large portion of the COCs lacking or having little cumulus cells vestment. Previous studies have shown that body condition score, physiological conditions of the egg donor, breed, age and individual variations directly interfere with the quality of the recovered COCs (Edwards and Hansen, 1996; Vinoles *et al*., 2002; Fatehi *et al*., 2005; Cecconi *et al*., 2007). In addition, the absence of significant variation in the photoperiod throughout the year, the low rainfall, or even the high temperatures in equatorial or tropical zones, may cause a reduction in the quality of goat COCs (Jordan, 2003; Chaves *et al*., 2010, 2011). According to Roth and Hansen (2004), inter- and intra-cellular components define how an oocyte will react to effects from the environment and high temperatures, even if within physiological ranges, potentially even being a stimulus to apoptosis in mammalian oocytes. A fact that corroborates this assertion is that the rate of development of parthenogenetic embryos to the morula and blastocyst stages obtained in our experiment was 18.3%, a low value for oocytes previously selected for the presence of the 1st PB extrusion and cytoplasm morphology when compared with studies by Apimeteetumrong *et al.* (2004) and Nasr-Esfahani *et al.* (2011), who obtained 42.3 and 54.9% of parthenogenetic development to the morula and blastocyst stages, and to the blastocyst stage, respectively. The animal response to climatic elements and factors may play an influence in the results of this study, resulting in a low overall efficiency of cloning under our conditions. Nevertheless, we obtained approximately 30% of embryonic development to the blastocyst stage after VC of embryos cloned by HMC. However, these embryos exhibited low morphological quality (data not shown), which is commonly reflected in lower rates of *in vivo* development (Pereira *et al*., 2013), as observed in this study.

Based on the morphological features of the collected COCs, one of the alternatives attempted to improve results was the use of *in vivo*-matured oocytes. However, this issue seems to be controversial, since while Reggio *et al*. (2001) found no differences between *in vitro*- and *in vivo*-matured oocytes, showing that both oocyte sources were similar in competence to support *in vivo* development after goat cloning, whereas Behboodi *et al.* (2004) and Martins *et al*. (2016) did not obtain pregnancies after the transfer of cloned embryos using *in vitro*-matured oocytes. Unlike Reggio *et al*. (2011), our experiment found that nuclear maturation rate was higher in the group of *in vitro*-matured oocytes than the *in vivo*-matured counterparts. However, the nuclear maturation rate only takes into account the extrusion of the 1st PB, which is not the only factor to be considered to determine oocyte quality and competence. As for findings by Reggio *et al*. (2011), no differences in pregnancy rates were observed between the oocyte source, which may indicate that the *in vitro* protocols for nuclear maturation are rather well established for goats, with attained pregnancy rates similar (Baldassare *et al*., 2004) to those found for other species.

In this study, two distinct SCNT cloning micromanipulation methods were compared for the production of clone embryos, with the use of conventional cloning with (ZI) or without (ZF) zona pellucida (ZP). Cloning by micromanipulation with the ZP is in more widespread use in the world for goats, with the ZP maintained until the end of the procedure (Keefer *et al.*, 2001; Chavatte-Palmer *et al*., 2013). However, this technique requires greater skills from the operator than the zona-free method, since the presence of the ZP imposes an extra challenge for the aspiration of the MII plate and the PB (Peura, 2003). The ZF technique, on the other hand, should be an easier process, as the enucleation is more straightforward, which is further facilitated as there is no need for another micromanipulation step for the reconstruction of embryos by cell insertion (Booth *et al*., 2001; Hosseini *et al*., 2015). Although embryo production rates using both techniques are similar, the ZF procedure enables the production of a greater number of embryos per routine (Booth *et al*., 2001; Peura, 2003). In our case, the post-enucleation survival, the fusion/microinjection rate and the number of viable embryos were higher in the ZF group than the ZI group. However, no differences were observed in pregnancy rates between groups.

The cell type, cycle synchronization, lineage and time in culture, among other factors, are known to be crucial for the cloning outcome (Dominko *et al*., 1999; Yang *et al*., 2016b). Keefer *et al.* (2001) used three different GFP transgenic cell lines to clone goats, and found that only one line was capable of producing viable animals. In that same study, the group used five different lines of fetal fibroblasts, and only two were able to generate viable cloned animals. According to Baldassare *et al*. (2004), pregnancy rates varied from 0 to 89% when using different cell lines for SCNT cloning, demonstrating the high variability in results when different cell lines are used. In our experiment, after the IVC period, the number of embryos suitable for transfer was higher in the group of fetal fibroblasts (FF) than the other groups. However, pregnancy rates were higher for embryos produced with fetal fibroblasts (FF) and mesenchymal stem cells (MSC). In addition, similarly to what was observed by Chen *et al*. (2007), survival rate after reconstruction was greater when cells were microinjected in the ooplasm than cell fusion, irrespective of the presence or removal of the zona pellucida.

In the group of embryos produced by the HMC, the final cytoplasmic volume of 100% resulted in higher cleavage rates than with 85%. We have previously seen that the reduction of cytoplasmic volume to 50% of the final volume significantly

compromises *in vitro* development and embryo kinetics of cloned bovine embryos, with the reduction of the total number of cells in blastocysts (Ribeiro *et al*., 2009). Because the cytoplast plays a key role in chromatin remodeling, the effect of the cytoplasmic volume after cloning cannot be neglected. Previous studies also corroborate the effect of the reduction or increase of the cytoplasmic volume on embryonic development. The removal of 50 or 25% of ooplasm during enucleation compromised embryonic development and quality and the total number of cells in cloned bovine blastocysts (Westhusin *et al.*, 1996; Peura *et al*., 1998; Ribeiro *et al*., 2009). In conditions where the volume is reduced, the amount of ooplasmic components probably will not be sufficient to support cleavage, activation of the embryonic genome, or even cavitation, but since the cytoplasmatic volume does not increase during the first cycles of cell division, the total number of cells tends to be limited by the total volume of the developing embryo (Westhusin *et al*., 1996; Ribeiro *et al*., 2009).

Although embryonic vesicles have been observed from day 23 of development in all groups, no heart beats could be observed in most cases. Such nonviable structures often remained until day 50 of gestation, when the progesterone inserts were removed. After a few days, the structures could no longer be observed. These findings corroborate with Baguisi *et al.* (1999) and Zhang *et al*. (2010). According to Baguisi *et al*. (1999), more than 2/3 of their clone pregnancies were not viable, and such "embryonic structures" were observed in the uterus until day 55 of gestation. In our case, out of 45 embryonic structures, the heartbeat could only be observed in five cases. Several factors may have contributed to these findings, including failures in placentation and/or embryonic genome activation, or even in the enucleation process, which could lead to the transfer of polyploid embryos in rare cases (Baguisi *et al*., 1999). Collectively, the overall efficiency of cloning under our conditions was 0.11%, considering the number of transferred embryos (1/809) to obtain one live born animal, which is significantly lower than what was previously reported in the literature (Keefer *et al*., 2001, 2002; Baldassare *et al*., 2004), even for transgenic cloned kids (Gavin *et al*., 2013; Feng *et al.,* 2015).

The high rate of pregnancy losses observed in this study may have been caused by the cell types and lines used for cloning, the low oocyte quality and inefficient genomic reprogramming, and even by technical aspects inherent to SCNT cloning *per se*. More studies are needed to investigate such aspects, as observed during this experiment. In addition to the potential failures, the high pregnancy rates verified in this work in all groups may also be related to the use of the progesterone insert from the 4th day after the embryo transfer, which could have prevented the return to the natural estrous cycle, 'rescuing' less viable embryos that would otherwise be unable to trigger the maternal recognition of pregnancy, an event already proposed by Bertolini *et al*. (2002) for *in vitro*-derived bovine embryos. In fact, a pilot study carried out by our group using progesterone supplementation (intravaginal inserts) on day 4 after the artificial insemination of female goats as a way to increase pregnancy rates resulted in 41.7% (5/12) and 80.0% (8/10) pregnancy in the control and in the progesterone-treated groups, respectively (Feltrin & Bertolini, 2011, University of Fortaleza; unpublished data). This pilot study indicated the innocuity or even the potential benefit of a progesterone treatment to improve fertility in cyclical pubertal does. Since *in vitro*-manipulated embryos have a lower viability than normal, being smaller in size at early embryonic stages (Bertolini *et al*., 2002; Martin *et al*., 2007), it is possible that the rescue of some less viable embryos may have occurred (Bertolini *et al*., 2002), resulting in higher pregnancy rates than that reported in the literature for cloned goat embryos (Chavatte-Palmer *et al*., 2013).

Despite the low overall efficiency of cloning by SCNT observed in this study, especially regarding birth rates, we report the birth of a cloned goat female in August 2012, from control non-transgenic cells, using the micromanipulation of *in vitro*-matured oocytes with zona pellucida, membrane fusion, conventional activation, and transfer at the 1-cell stage embryo to the oviduct of a recipient female receiving progesterone supplementation throughout pregnancy. The important information generated in this study may serve as a basis for subsequent studies, which may contribute in the future to a greater efficiency in the production of transgenic cloned animal models in arid regions of the world, and to models that can assist in improving the quality of life of the population, such as milk-containing human lysozyme. In this sense, studies that take into account the physiology, nutrition, health, and reproductive aspects, among others (Bertolini, 2009), are required to uncover factors associated with lower reproductive performance in goat herds in the Brazilian Semi-Arid region.

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