



## Reproductive parameters and the use of MOET in transgenic founder goat carrying the human granulocyte colony-stimulating factor (hG-CSF) gene

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### Abstract

This study aimed to monitor estrous cycle parameters of a human granulocyte colony-stimulating factor (hG-CSF)-transgenic founder female goat and to perform superovulation and embryo recovery (surgical or transcervical method) for further transfer to recipients to quickly obtain offspring. Two experiments were performed using a transgenic (TF) and a non-transgenic (NTF) female. In experiment 1, three estrous cycles were monitored for the following parameters: estrus behavior, progesterone concentration and ovarian activity. In experiment 2, two superovulation/embryo recovery sessions were performed and the recovered embryos were transferred to previously prepared recipients. Data were compared by either *t* test or Fisher's exact test. The mean interval between natural estrus was  $20.7 \pm 0.6$  and  $19.7 \pm 0.6$  ( $P > 0.05$ ) days for the TF and NTF, respectively. Progesterone concentrations and ovarian activity were normal and similar between goats. The ovulation rate was similar between TF and NTF ( $12.0 \pm 1.4$  vs.  $18.0 \pm 4.2$  CL;  $P > 0.05$ ). No significant differences in embryo recovery rate ( $P > 0.05$ ) were observed between the surgical and transcervical methods for TF (69.2 vs. 72.7%) or NTF (100.0 vs. 86.7%). Sixteen embryos from the TF were transferred to recipients, and eight kids were born. Among these kids, the transgene was identified in three (two males and one female), resulting in a transgenesis rate of 37.5%. In summary, the TF is a true founder, since she proved fertility and capacity of transmitting the hG-CSF transgene to progeny, suggesting that the analyzed reproductive traits were not compromised by the presence of the transgene.

**Keywords:** embryo transfer, goat, progesterone, superovulation, transgenesis.

### Introduction

Gene pharming entails the production of recombinant, pharmaceutically active human proteins, mainly in the mammary gland of transgenic animals. Mammalian bioreactors have become an alternative to

conventional systems due to the possibility of producing recombinant proteins on a large scale, the ability to obtain correct post-translational modifications and the established methods for extracting and purifying the respective protein (Kues and Niemann, 2004). In this context, goats represent an excellent transgenesis model when considering factors such as volume of milk produced per lactation, reproductive traits and the lower investment and maintenance costs compared to cattle (Baldassarre and Karatzas, 2004).

Several recombinant proteins, mainly human, have been produced, purified and characterized from transgenic goats (reviewed in Moura *et al.*, 2011). Currently, human antithrombin III produced by transgenic goats is the only recombinant protein from an animal bioreactor that has been approved for clinical use in Europe (Schmidt, 2006) and in the USA (Kling, 2009). Thus, the transgenic technology was validated using a goat model as a viable alternative method for the production of recombinant pharmaceutical proteins.

The recombinant proteins produced for pharmaceutical use include human granulocyte colony-stimulating factor (hG-CSF), a hematopoietic cytokine that promotes the proliferation and differentiation of neutrophil precursors and the activation of mature neutrophils. This protein is widely used in different forms of neutropenia, chemotherapeutically induced leukopenia and mobilization of progenitor cells for autologous or allogenic transplants (Welte *et al.*, 1996). In addition, hG-CSF has been investigated as a neuroprotective agent in cases of stroke (Solaroglu *et al.*, 2006) and myocardial infarction (Harada *et al.*, 2005).

Due to the importance of this recombinant protein in human medicine, our group began a program to produce hG-CSF-transgenic goats using pronuclear microinjection. Two transgenic founders were obtained (a male and a female), and the female expressed hG-CSF in quantities sufficient for commercial use (Freitas *et al.*, 2012). After obtaining a transgenic founder goat, it is imperative to investigate and characterize its feasibility as a potential bioreactor for commercial purposes (Jackson *et al.*, 2010). Furthermore, the rapid transmission of the transgenic goat characteristics to

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offspring is indispensable for obtaining a transgenic herd to increase the availability of this recombinant protein and meet market demand. Thus, this study was divided into two experiments with one general aim each: i) to monitor the estrous cycles of the transgenic female by progesteronemia and ovarian activity evaluation and ii) to perform the Multiple Ovulation and Embryo Transfer (MOET) to quickly obtain the offspring of the transgenic founder female.

## Materials and Methods

### *Animal ethics and biosafety*

The Committees of Animal Ethics (09144595-7/50) and Biosafety (003/2010) of the State University of Ceará approved all protocols used in this study. Furthermore, goats were treated according to the guidelines for the ethical use of animals in research (Association for the Study of Animal Behaviour - ASAB, 2006).

### *Animals, experimental area and management*

Two Caniné female goats were used in this experiment: a hG-CSF-transgenic (TF) and another, non-transgenic (NTF), used as a control. The two females were obtained from the same experiment to produce transgenic goats using pronuclear microinjection, (Freitas *et al.*, 2012). At the beginning of the experiment the females were two years old and nulliparous and presented a body weight of 25.5 kg. The experiment was carried out in the Laboratory of Physiology and Control of Reproduction (LFCR), located in Fortaleza, Brazil, at 3°47'41''S and 38°33'26''W, from January to March (Summer) and then from August to October (Spring). Female goats reared at this latitude are usually non-seasonal breeders, exhibiting recurrent estrous cycles throughout the year. One month before the experiment started, estrus was monitored daily using teaser bucks to verify the females' cyclicity. All animals were raised under a semi-intensive system with daily morning access to a pasture of Tifton (*Cynodon dactylon*) and receiving hay of this grass in stalls. Water and mineralized salt were given *ad libitum*, and the diet was supplemented with commercial concentrate (18% crude protein).

### *Experiment 1*

#### *Estrous cycle monitoring*

Three consecutive and spontaneous estrous cycles were evaluated to monitor estrus and ovarian activity. Estrus was observed daily. The ovarian activity was verified by ultrasound, which was performed every three days beginning on estrus onset (day 0). Ovaries were observed using transrectal real-time ultrasonography

(Falco 100, Pie-Medical, Maastricht, Netherlands) coupled to a 6/8 MHz linear probe. At the same time blood was collected by jugular venipuncture. Serum samples were separated by centrifugation (3,000 x g, 15 min) and stored at -20°C until assaying for progesterone concentration, which was carried out using commercially available chemiluminescence immunoassays with an automated Elecsys immunoanalyzer (Roche-Boehringer, Mannheim, Germany). The sensitivity of the assay was 0.03 ng/ml.

### *Experiment 2*

#### *Estrus synchronization and superovulation*

The two embryo donor goats (TF and NTF) and nine undefined breed recipients were subjected to estrus synchronization with the use of intravaginal sponges (Progespon, Syntex, Buenos Aires, Argentina) impregnated with 60 mg medroxyprogesterone acetate for 10 days. On day 8 of the progesterone treatment an im injection of 75 µg D-cloprostenol (Prolise, Arsa SRL, Buenos Aires, Argentina) was administered. Donors received 120 mg NIH-FSH P1 (Folltropin-V, Bioniche, Ontario, Canada) im divided into six doses at 12 h intervals (30/30; 15/15; 15/15 mg) starting 48 h before the sponge removal. This dose was determined in a previous study carried out in our laboratory (Moura *et al.*, 2010). Also 48 h prior to the end of progesterone treatment, recipients received im injections of 300 IU of eCG (Novormon, Buenos Aires, Argentina). The sponges were removed at the time of the fifth FSH injection. To prevent the premature regression of corpora lutea (CL), flunixin meglumine (1.1 mg/kg; Flumedin, Jofadel, Varginha, Brazil) was administered twice daily for four days beginning on the third day after sponge removal. Estrus was detected from 12 h after sponge removal at 4 h intervals, and the donors were hand-mated at estrus onset and 24 h later.

#### *Progesterone and white blood cell analyses*

Blood samples were collected in intervals of 3 or 4 days (twice weekly) by jugular veinpuncture, into vacuum tubes (BD Vacutainer, New Jersey, USA) with or without EDTA, always at the same time of the day (morning), from the sponge insertion (day 0) until the subsequent natural estrus. Blood samples without EDTA were processed as previously described for the analysis of serum progesterone. Blood samples with EDTA were used to determine the total white blood cell (WBC) count, which was performed with an automatic analyzer CELL-Dyn 3700 (Abbott Park, Illinois, USA).

#### *Embryo recovery and transfer*

Two *in vivo* embryo production sessions were performed within a ~7 month interval; one by surgery



(laparotomy) and the other through a nonsurgical (transcervical) method. For both conditions embryo recovery was performed seven days after estrus. The ovulatory response of donors and recipients was verified by laparoscopy to assess the number of CL prior to embryo recovery and transfer, respectively. Donors and recipients were deprived of food and water for 24 h before embryo recovery and transfer. In the laparotomy procedure, the donors were anesthetized using intravenous administration of 20 mg/kg body weight of sodium thiopental (Thiopentax, Cristália, São Paulo, Brazil); anesthesia was maintained with isoflurane (Isoforine, Cristália, São Paulo, Brazil) via endotracheal intubation.

The surgical embryo recovery was performed by a medial ventral incision to expose the genital tract, and at this moment, the CL number was recorded. A catheter connected to a sterile 20 ml syringe containing flushing medium (DMPBS Flush; Nutricell, São Paulo, Brazil) was inserted near the uterus bifurcation. Another catheter was inserted in the utero-tubal junction, where the flushing medium was collected in 50 ml plastic tubes.

For the transcervical embryo recovery donors received an im injection of 37.5 µg D-cloprostenol 12 h before the procedure. The females received acepromazine maleate im (1 mg/kg; Acepran 1%, Vetnil, São Paulo, Brazil). Immediately before the introduction of the vaginal speculum, an epidural block without epinephrine (2 ml/female; Lidocaine hydrochloride 2%, Anestésico L Pearson, Eurofarma, Brazil) was performed. Embryo recovery was performed with a circuit and catheter for small ruminants (Circuit/catheter to collect embryos for sheep and goats, Embrapa, Brasília, Brazil). A number 8 catheter was used, with no balloon and equipped with a stylet to pass through the cervix. The stylet was removed, and the catheter was attached to the end of circuit. After flushing the first uterine horn with a total volume of 120 ml of DMPBS, the catheter was gently pulled back and guided to another horn with the help of a finger inserted into the female rectum. The second horn was then washed. The process was repeated until a total volume of 240 ml per uterine horn was collected. The recovered medium was examined under a stereomicroscope (SMZ-800, Nikon, Kawasaki, Japan) for embryo identification and evaluation regarding the stage of development and quality of the embryos according to the morphological criteria of the International Embryo Transfer Society (Stringfellow and Givens, 2009).

The embryo transfer was performed by semi-laparoscopy as described by Green *et al.* (2009).

Briefly, for each recipient, 1-2 embryos recovered from the TF were transferred into the top of the uterine ipsilateral horn by semi-laparoscopy to an ovary bearing at least one functional CL.

#### *Pregnancy detection and offspring transgene analysis*

Pregnancy was detected by ultrasound 30 days following embryo transfer and weekly during pregnancy to confirm progressive fetal development. The hG-CSF transgene was detected in skin biopsies from the ears of two-week-old kids using PCR amplification according to the method reported by Freitas *et al.* (2007).

#### *Analysis of data*

Data are presented as the mean  $\pm$  SD. Due to the small amount of animals it was not possible to draw comparisons for some variables. When possible, the means were compared by *t* test and the values in percentages by Fisher's exact test. The significance level was 5%.

## **Results**

### *Experiment 1*

After checking females' cyclicity, the first natural estrus was defined as day 0 for monitoring estrous cycles. Both females showed clinical signs of estrus in three cycles. The mean interval between estrus was  $20.7 \pm 0.6$  and  $19.7 \pm 0.6$  days ( $P > 0.05$ ) for the TF and NTF, respectively.

When ultrasounds were performed, ovaries appeared as well-defined structures of oval shape and were slightly hypoechoic compared to the surrounding tissues. For the four observed estrus, the TF had only one ovulation at each estrus, whereas the NTF presented one, two, one and one ovulation, respectively.

Serum progesterone concentrations during the three consecutive cycles are shown in Fig. 1. During the follicular phase (days 0 to 3 of the estrous cycle), progesterone concentrations were  $0.5 \pm 0.4$  and  $0.6 \pm 0.5$  ng/ml ( $P > 0.05$ ) for the TF and NTF, respectively. During the following days the progesteronemia remained high from day 6 to approximately day 18 and ranged from 5.2 to 12.2 ng/ml (TF) and 3.6 to 15.1 ng/ml (NTF), showing a similar pattern during the three estrous cycles (Fig. 1). The luteal phase was characterized by an average progesterone concentration of  $8.1 \pm 2.4$  and  $7.7 \pm 3.7$  ng/ml ( $P > 0.05$ ) for the TF and NTF, respectively.

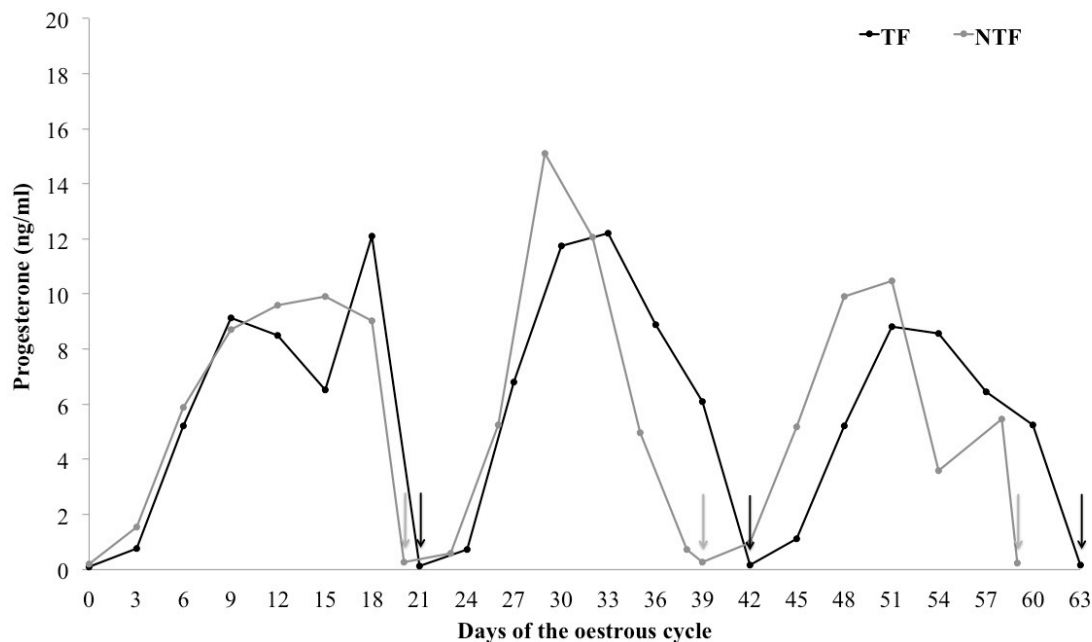


Figure 1. Serum progesterone concentrations during three consecutive estrous cycles in the transgenic (TF) and the non-transgenic female (NTF). The day of the first estrus for both females is considered day 0. Black or gray arrows indicate the days of estrus in TF or NTF, respectively.

### Experiment 2

After the end of superovulation treatments, both females responded to hormonal stimulation, displaying signs of estrus. The ovulation rate and embryo recovery data are presented in Table 1. The ovulation rate was similar between the TF and NTF, averaging  $12.0 \pm 1.4$  and  $18.0 \pm 4.2$  CL, respectively ( $P > 0.05$ ). There were no cases of premature regression of CL in either superovulation session.

After the first treatment for superovulation, embryos were collected by the surgical method (Table 1). This method resulted in a recovery rate of 69.2% (TF) or 100.0% (NTF). After recovery, the TF produced nine embryos (eight blastocysts and one morula), whereas

the NTF produced 21 embryos (six blastocysts, nine morulae and six degenerated embryos). The second superovulation treatment was followed by transcervical recovery (Table 1). The recovery rate for this method was 72.7% (TF) or 86.7% (NTF). Although the females were nulliparous at the time of recovery, the transcervical collection was carried out without difficulty and recovered almost all medium at the end of the procedure. Thus, the TF yielded eight embryos (six blastocysts, one morula and one degenerated embryo), and the NTF yielded 13 embryos (one blastocyst, eleven morulae and one degenerated embryo). There were no significant differences between embryo recovery methods. In both superovulation treatments the fertilization rate was 100% for T and NT females.

Table 1. Parameters for surgical and transcervical embryo recovery in the transgenic (TF) and the non-transgenic female (NTF).

Parameter	Surgical		Transcervical	
	TF	NTF	TF	NTF
Number of flushings	1	1	1	1
Number of ovulations	13	21	11	15
Embryos recovered	9	21	8	13
Recovery rate (%)	69.2 <sup>a</sup>	100.0 <sup>a</sup>	72.7 <sup>a</sup>	86.7 <sup>a</sup>

<sup>a</sup>The comparisons were made between recovery methods for each experimental female ( $P > 0.05$ ).

Regarding serum progesterone concentrations, an expected profile before and after hormonal treatment was observed (Fig. 2). In the first and second hormonal treatments, no functional CL was present upon the insertion of the sponge, as verified by the concentrations of progesterone. Just before embryo recovery, for

surgical or transcervical, the progesterone concentrations were elevated for TF (13.5 and 16.0 ng/ml) and NTF (18.9 and 37.0 ng/ml), respectively. These progesterone values were followed by a sharp decrease in response to the luteolytic injection.

The leukocyte profile was similar for both

MOET, and thus the results are presented as an average. The NTF remained within the normal range for goat species ( $4$  to  $13 \times 10^6/\text{ml}$ ) before, during and after MOET, averaging  $9.1 \pm 2.4 \times 10^6/\text{ml}$  from day 0 up to day 44. Conversely, the opposite was observed for the TF, which demonstrated a permanent leukocytosis, averaging  $45.5 \pm 13.9 \times 10^6/\text{ml}$  throughout the experimental period. Regarding each step, the TF had  $\sim 37.2 \times 10^6/\text{ml}$  on days 0, 2 and 5 (before FSH stimulation),  $\sim 42.8 \times 10^6/\text{ml}$  on days 9, 12 and 16 (during FSH stimulation),  $\sim 49.6 \times 10^6/\text{ml}$  on days 19 up to 44 (after MOET was performed). It is important to highlight that the leukocytosis was due to a neutrophilia whereas the other cell counts were within the normal range for goats. The NTF had  $\sim 44.2\%$  (surgical MOET) and  $51.0\%$  (transcervical MOET) of relative neutrophil counts from days 0 to 44 whereas the TF had  $80.7\%$  (surgical MOET) and

$80.0\%$  (transcervical MOET) of these cells in the same period.

Approximately eight days after both embryo recoveries the TF had at least one functional CL, indicated by progesterone higher than  $1 \text{ ng/ml}$ . Interestingly the NTF neither showed estrus nor ovulation after both embryo recoveries.

Hormonal treatment synchronized estrus and induced ovulation in all recipients, producing an average ovulation rate of  $2.4 \pm 1.5 \text{ CL}$ . All embryos collected from the NTF were vitrified whereas the ones collected from the TF were transferred to recipient goats. The pregnancy rate at 30 days after embryo transfer was  $77.8\%$  (7/9). However, the kidding rate was  $55.5\%$  (5/9), with the birth of eight kids (four males and four females). These offspring included three with the hG-CSF transgene (two males and one female), leading to a transgenic rate of  $37.5\%$ .

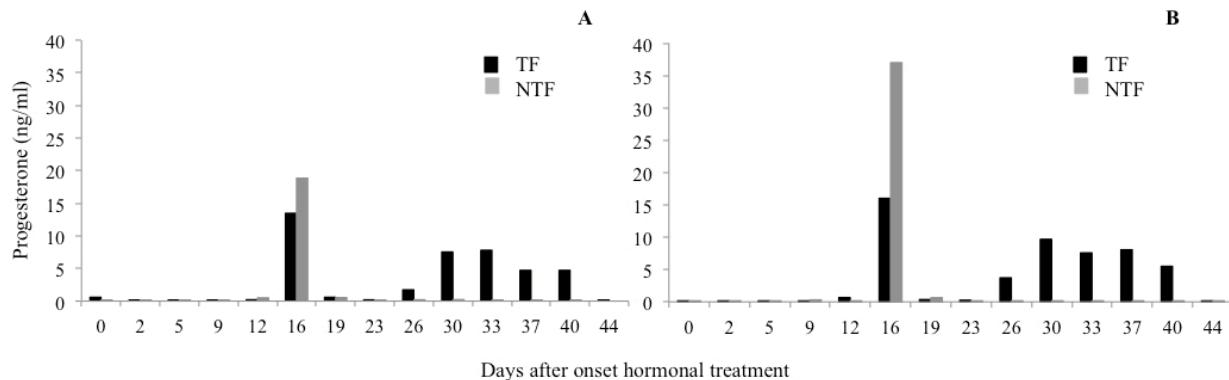


Figure 2. Serum progesterone concentrations before and after surgical (A) and transcervical (B) embryo recovery in the transgenic (TF) and the non-transgenic female (NTF). The day of sponge insertion was considered day 0.

### Discussion

Due to latitude, both females showed the cyclic activity expected for goats raised near the equator. Whereas the average duration of the goat estrous cycle is 21 days, its length is highly variable. A study with Alpine goats during the breeding season recorded 77% cycles of normal duration, 14% short and 9% of long cycles (Baril *et al.*, 1993). However, in the present work, at the end of three successive cycles, this variability was not detected, with goats showing estrus from 19 to 21 days.

The follicular phase of the estrous cycle corresponds to the wave of follicle development that culminates in the selection of the ovulatory follicle, and it involves the maturation of gonadotropin-dependent follicles until ovulation. Approximately five days after the onset of estrus, cells from the ovulating follicle turn into luteal cells and form the CL. They secrete progesterone, inducing its increase and remaining at high concentrations ( $>1 \text{ ng/ml}$ ) for 16 days (reviewed by Fatet *et al.*, 2011). In our experimental animals, the pattern and levels of increase in progesterone

concentration and then its decline after the luteal phase were similar to those found in other goat breeds (Leyva-Ocaris *et al.*, 1995; Khanum *et al.*, 2008). Ko *et al.* (2000) also obtained a hG-CSF-transgenic goat, which grew without any health problems, became sexually mature and exhibited a normal estrous cycle.

Concerning the second experiment, the ovulation rate verified after the superovulatory treatment is in agreement with previous results by our group in Canindé goats (Moura *et al.*, 2010). In goats, the use of hormonal superovulatory treatments has been correlated with early regression of the CL (Pintado *et al.*, 1998; Saharrea *et al.*, 1998). However, this was not observed in the present study, probably due to the use of an anti-prostaglandin agent. In both hormonal treatments an increase in serum progesterone on day 16 after the start of the hormonal treatment was noticed, consistent with the superovulatory response. The level of progesterone is correlated with the number of CL (Jarrell and Diziuk, 1991). One day after embryo recovery, progesterone decreased ( $< 1 \text{ ng/ml}$ ) due to the luteolytic action.

Regarding the white blood cell analysis, the TF



demonstrated a similar leukocyte profile since when she aged 1.5 months (Freitas *et al.*, 2012). A leukocytosis was reported due to a neutrophilia although the other granulocytic and non-granulocytic cell counts were within the normal range for goat species (Pugh, 2002). This is expectable since the TF has a transgene associated to increase of neutrophils. It is noteworthy that the pattern was not affected before, during or after MOET and that the doe remained healthy throughout the whole experimental period.

Quickly generating transgenic progeny from a transgenic founder goat can be achieved by several techniques, including MOET, *in vitro* fertilization (IVF), and somatic cell nuclear transfer (SCNT). The use of MOET may be more practical than IVF or SCNT. However, animal care organizations around the world are enforcing regulations that limit the number of surgical procedures that can be performed per animal ranging between one and four (Baldassarre *et al.*, 2003). Therefore, the use of less invasive recovery techniques is preferential. Additionally, they avoid causing adhesions (such as those induced by the surgical method), may allow the use of successive embryo recoveries in donors (Suyadi and Holtz, 2000). In this study, the methods of embryo recovery did not affect the recovery rate in both females. Souza *et al.* (2008), also working with a naturalized breed from northeastern Brazil, observed a recovery rate of 80% after surgical embryo recovery. Considering the inestimable value of the TF, we decided to use a less invasive approach for embryo collection. Both females were nulliparous and of a small size breed. The transcervical method minimized the trauma to the female, making it possible to recover a high number of embryos per donor, considering the potential of its use in several collections.

It was confirmed that our transgenic goat is a true founder since she was able to produce transgenic offspring. As our TF was obtained by pronuclear microinjection, we expected the transgene to be inherited by 50% of the offspring. The production of the F1 generation of this line apparently transmitted the hG-CSF transgene in a Mendelian fashion, presuming they were hemizygous for the transgene. Melican and Gavin (2008) also used superovulation coupled with embryo transfer to accelerate the production of transgenic progeny from a founder dairy goat. Those authors observed a transgenesis rate of 26%, which was slightly lower than that observed in our study.

In conclusion, the hG-CSF-transgenic female founder presented estrous cycles and a progesterone profile which were normal and regular. Additionally, this goat is a true founder, since it is fertile and capable of transmitting the hG-CSF transgene to progeny by MOET. Therefore, the analyzed reproductive traits were not compromised due to the presence of the transgene.

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