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ORIGINAL RESEARCH PAPER

Phenotypic features of first-generation transgenic goats for human granulocyte-colony stimulation factor production in milk

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Abstract Human granulocyte-colony stimulating factor (hG-CSF) is a hematopoietic growth factor used in neutropenic patients. It is produced in transgenic bacteria or cultured mammalian cells. As an alternative, we now show that hG-CSF can be expressed in the mammary gland of first-generation (F1) transgenic goats during induced lactation. Despite lower milk production, transgenic females presented a similar milk composition (fat, protein and lactose) when compared to non-transgenic ($p > 0.05$) ones. The mean concentration (±SD) of recombinant hG-CSF in milk during lactation was 360 ± 178 μg ml^{-1}. All clinical parameters, as well as kidney and liver function, indicated that F1 transgenic goats were healthy. Additionally, no ectopic hG-CSF expression was detected in studied tissues of F1 transgenic males. Thus, F1 hG-CSF-transgenic goats can express the recombinant protein in milk at quantities compatible with their use as bioreactors in a commercial-scale protein-production program.

Keywords Bioreactor · Goats · Human granulocyte-colony stimulating factor · Induced lactation · Neutrophilia · Transgenesis · Transgenic goats

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Introduction

Recombinant proteins of high economic value have been expressed in genetically-modified mammalian cells, which are better prepared for the synthesis of complex proteins. However, the need of large capital investment, high operating costs and relatively low production levels result in the inability to produce more than a few kilograms of protein per year (Houdebine 2009). Given these limitations, the transgenic animal platform in which the recombinant protein is expressed usually in their mammary gland and thus purified from their milk, appeared as a promising method due to some features, such as low operating costs and virtually unlimited capacity to scale-up by simply breeding transgenic animals (Kues and Niemann 2011).

Human granulocyte-colony stimulating factor (hG-CSF) is a cytokine of high economic value currently produced in bacterial and Chinese hamster ovary (CHO) cells for clinical use. This cytokine is a glycoprotein that influences the proliferation, survival, maturation and functional activation of cells from the neutrophilic granulocyte lineage. Its main clinical application is to reduce the time of neutropenia (Crea et al. 2009).

Our group reported the production of two transgenic goats (10M and 12F lineages) containing hG-CSF fused to goat α-S1 casein (CSN1S1) promoter (Freitas et al. 2012). Later, we have demonstrated that the female founder (12F) successfully expressed the

recombinant protein in her milk at a concentration that is compatible with commercially viable investments (Moura et al. 2013). However, it was not reported if this phenotypic characteristic is also present in 12F and 10M first-generation (F1) progenies. Additionally, it is essential to check the health of transgenic specimens, regarding any potential effect of hG-CSF expression.

In the present study, besides the hG-CSF expression in milk during hormonally-induced lactation of F1 specimens, we investigated the ectopic expression by enzyme-linked immunosorbent assay (ELISA) of blood serum and by real-time RT-PCR (qRT-PCR) of lymphocytes and some organs as liver, spleen and lymph nodes. Finally, potential general health disorders were assessed by serum biochemistry, hematological and clinical evaluations.

Materials and methods

Animal ethics and biosecurity

All protocols used in this study were approved by the Committee of Animal Ethics of the State University of Ceará (09144595-7/50) and Brazil's Biosafety Technical National Committee (CQB 228/06) to work with genetically modified animals.

Experimental animals

Twelve 10 month old Canindé goats (six transgenic and six non-transgenic) were used. Six nulliparous females (three transgenic and three non-transgenic) were used for induction of lactation. Six males (three transgenic and three non-transgenic) were used for organ biopsies aiming the investigation of ectopic expression by qRT-PCR. Transgenic (T) specimens were F1 generated by breeding of founders (10M or 12F lineage) with non-transgenic animals according to described by Freitas et al. (2012). Non-transgenic (NT) specimens were used as controls.

Induced lactation and milk composition

Experimental females (T and NT) were hormonally induced to lactate using the protocol earlier described by Cammuso et al. (2000). The females were hand-milked daily until D30 (start of milk production is day 0 = D0) and thereafter at crescent intervals until the lactation stopped. Three milk samples were collected from each animal in the third week of lactation and sent to a laboratory (Embrapa Dairy Cattle) to analyze milk composition percentages of fat, protein, lactose, dry extract, dry defatted extract and somatic cell count (SCC). Analysis of total protein, fat, lactose and solids were conducted in automated electronic device (Bentley Combi 2300; Bentley Instruments, Inc.) via infrared spectroscopy medium. The same equipment was used to evaluate SCC by flow cytometry method.

hG-CSF quantifications

Human G-CSF quantifications were determined in both milk and blood samples of T and NT females by solid-phase sandwich ELISA. Whey samples from D0, D4, D8, D12, D16, D20, D24, D28, D32 and D48 were obtained by milk centrifugation at $3000\times g$ for 10 min. Blood samples were collected by jugular vein puncture at D-18, D-10, D-2, D0, D8, D16, D24, D32 and D48 and blood serum was prepared by centrifugation at $4400\times g$ for 15 min. For whey samples, hG-CSF was measured using a low-sensitivity (<20 pg ml^{-1}) kit (Invitrogen), whereas for blood serum a high-sensitivity (<1 pg ml^{-1}) kit (RayBiotech) was used. Milk and blood samples were diluted to 1:100,000 and 1:1 (v:v) respectively, in buffered standard solutions provided by the manufacturer. The absorbance was measured at 450 nm in a plate reader.

Serum biochemistry, hematological and clinical evaluations

Eight days before and during lactation, once per week, blood samples were collected by jugular vein puncture. Serum samples were used to determine urea, creatinine, glucose, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations using a BT 3000 plus (Winer Lab). After D30, these analyses were performed every 14 days. For hematological evaluations, blood samples were collected 8 days before and during the induced lactation, every 2 days by jugular venipuncture. During the drying period, samples were collected only at milking day. Blood samples were used to determine both total and differential white blood cell (WBC) counts, which were performed by an automatic analyzer (CELL-Dyn 3700, Abbott Laboratories). Physiological parameters

such as respiratory rate, heart rate and rectal temperature were measured before, during and after lactation, every 2 days always in the morning. During this period, ocular mucosa, lymph nodes and mammary gland inspections were also conducted.

Sample collections, RNA extraction and qRT-PCR

Ectopic transgene expression was investigated by qRT-PCR in some cell and tissue samples of F1 specimens. Thus, lymphocytes were isolated from peripheral blood samples collected by jugular vein puncture of T and NT females in two moments: D-18 and D16. Lymphocyte isolation was performed using a density gradient cell separation medium (Histopaque 1083; Sigma). Liver and spleen samples were collected by laparoscopy whereas lymph nodes samples were surgically recovered in all T and NT males. All cell and tissue samples were stored at −80 °C until RNA extraction. Total RNA was extracted from cell and tissue samples of F1 specimens using the RNeasy Mini Kit (Qiagen). Reverse transcription and qRT-PCR were performed as described by Pereira et al. (2012).

Data analysis

Data are presented as mean ± standard deviation (SD) and compared using the Prism 6.0 software (GraphPad, San Diego, USA) at a confidence level of 95 %. Due to lack of normality of the data, either Mann–Whitney–Wilcoxon or Kruskal–Wallis nonparametric test was used as appropriate.

Results

Lactation and milk composition

All T and NT females responded to the hormonal treatment for induction of lactation in the first attempt and individual milk productions are presented in the Fig. 1a. One goat had premature shutdown of milk production at D8 of lactation and was not used for average calculations purposes. During 30 days of lactation, the daily average was 26 ± 23 ml per goat for T and 55.4 ± 24.4 ml per goat for NT animals ($p < 0.05$). At the lactation peak, the daily milk production average was 40.7 ± 32.4 ml per goat for T and 86.7 ± 18.4 ml per goat for NT females ($p > 0.05$). The milk composition was similar ($p > 0.05$) between T versus NT goats: fat (7.3 ± 3.2 vs. 6.2 ± 1.4 %), protein (6.9 ± 1.0 vs. 6.2 ± 0.6 %), lactose (4 ± 0.8 vs. 4.9 ± 1 %), dry extract (11.8 ± 2 vs. 12.3 ± 1 %) and dry defatted extract (19.1 ± 3.5 vs. 18.5 ± 1.5 %). However, SCC was significantly greater ($p < 0.05$) in T (7.3 ± 3.4 × 10^6 cells ml^{-1}) when compared to NT females (1.3 ± 1.4 × 10^6 cells ml^{-1}).

hG-CSF quantification

Human G-CSF was detected and quantified in milk samples of all T animals and recombinant protein production are presented in Fig. 1b. The mean concentration (±SD) of protein in milk during the 30 days of lactation was 360 ± 178 μg ml^{-1}. Human G-CSF concentration in the milk of T goats ranged from 25.1 to 1,099 μg ml^{-1}. In milk samples of NT goats, hG-CSF concentration was null at any time (Fig. 1b). Concerning the blood serum analysis (Fig. 1c), hG-CSF was first detected, for two T goats, 2 days before the beginning of milk production at 0.073 and 0.265 ng ml^{-1}. The greatest value was observed on D0 for two females (0.298 and 0.764 ng ml^{-1}) and D8 for the other goat (1.041 ng ml^{-1}).

Serum biochemistry, hematological and clinical evaluations

Total WBC counts for T females before, during and after lactation are shown in Fig. 1d. Before, during and after lactation the mean WBC of T females differed from NT ones (Table 1). Although other cell counts were occasionally higher in T females, differential counts showed that this difference was mainly due to an increased number of neutrophils. The serum biochemical parameters of T and NT females throughout the experimental period are presented in Table 2. In comparison with NT females, the results show no changes in serum concentrations of glucose, urea, creatinine, AST and ALT in T females. Additionally, all values were within the normal range reported in the literature for goats. Concerning the clinical parameters, all values remained within the normal range throughout the experimental period and with no significant difference between T and NT goats (Table 3). Finally, no alteration was observed in ocular mucosa, lymph nodes, and mammary gland inspection.

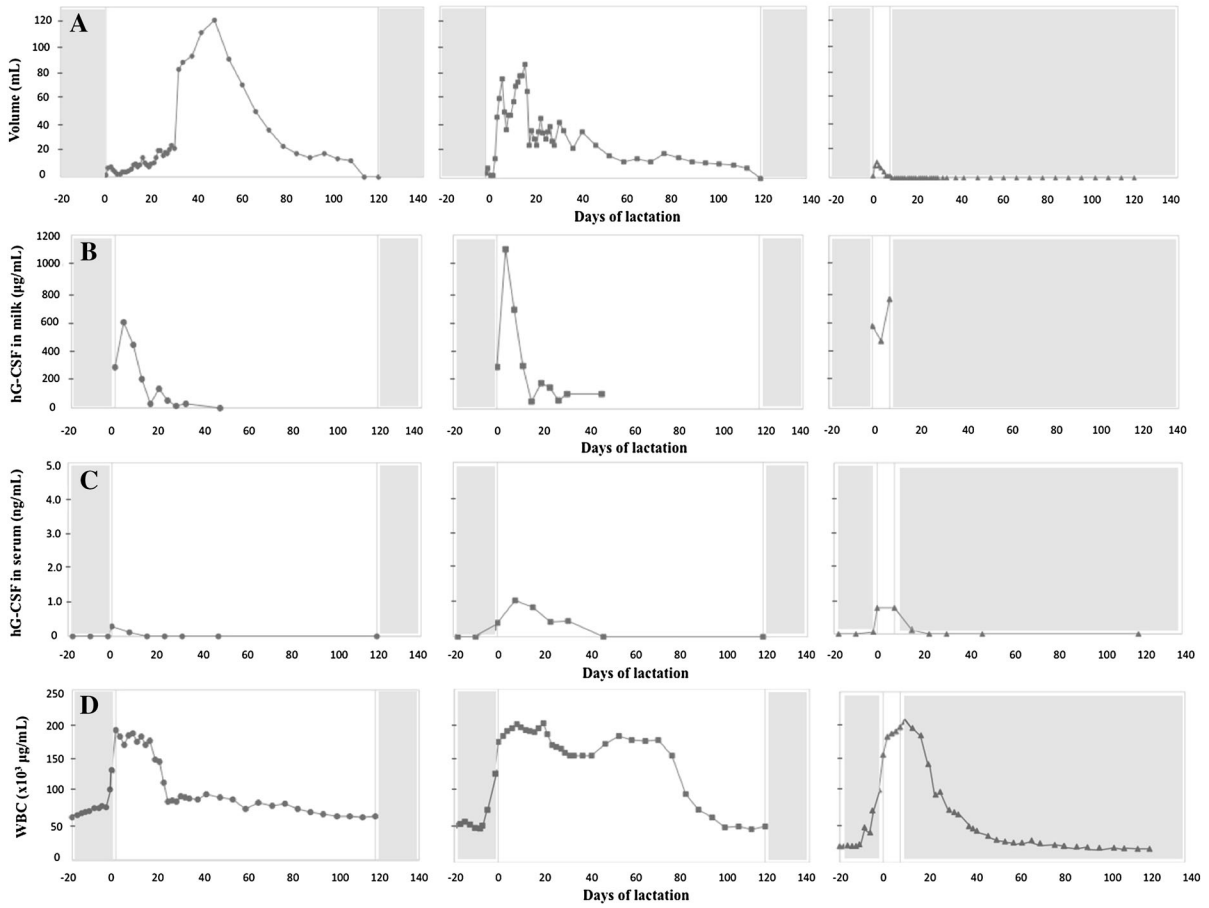


Fig. 1 Hormonally induced lactation of F1-generation hG-CSF-transgenic goats. Milk production (**a**) hG-CSF concentration in both milk (**b**) and blood serum (**c**) and total leukocyte counts (**d**) of three transgenic females (*filled circle, filled square* and *filled triangle*) were plotted versus lactation period. Periods before (negative days) and after milk secretion were presented in *shaded area*

Ectopic transgene expression investigation

No hG-CSF transcripts were detected in lymphocyte samples of T and NT females before (Figs. 2a, b) or during (Figs. 2c, d) lactation. Male tissues (liver, spleen and lymph nodes) also showed no hG-CSF transcripts (data not shown) and the emitted fluorescent signals were very similar to those presented by female lymphocytes.

Discussion

Overall, concerning hG-CSF expression, the milk of F1 transgenic females had similar orders of magnitude of protein concentration (µg ml^{-1}) as previously reported in the founder female (Moura et al. 2013). Thus, during the first 15 days of induced lactation, F1 produced in milk a daily average of 334 µg ml^{-1} of hG-CSF, whereas the female founder averaged 628 µg ml^{-1} at the same lactation period. This relatively similar average is not surprising since the expression of a transgene is known to remain essentially at a constant level in the different individuals of a line over number of generations (Colman 1996). The amount described in the present work was also greater than that achieved for transgenic mouse lines (40 µg ml^{-1}; Serova et al. 2012) generated with the same DNA construction used in our transgenic goats. Additionally, the values of the present study were much greater than 50 µg ml^{-1}, previously reported for transgenic goats with a β-casein promoter driving hG-

Table 1 Leukocyte count in peripheral blood of F1-generation hG-CSF-transgenic (T) and non-transgenic (NT) goats before, during and after hormonally induced lactation

Period	Group	Cells $\times 10^3$ μl^{-1} (mean ± SD)					
		WBC	Neutrophils	Lymphocytes	Eosinophils	Basophils	Monocytes
Before	T	$48.5 \pm 18.6^{A,a}$	$39.7 \pm 23.9^{A,a}$	$15.6 \pm 5.1^{A,a}$	$3.3 \pm 2.0^{A,a}$	$0 \pm 0^{A,a}$	$0.9 \pm 1.8^{A,a}$
	NT	$13.8 \pm 3.9^{A,B,b}$	$6.4 \pm 2.7^{A,b}$	$7.1 \pm 2.4^{A,B,b}$	$0.3 \pm 0.4^{A,b}$	$0 \pm 0^{A,a}$	$0.1 \pm 0.2^{A,b}$
During	T	$139.6 \pm 49.4^{B,a}$	$118.3 \pm 46.5^{B,a}$	$20.4 \pm 9.8^{B,a}$	$2.2 \pm 2.2^{B,a}$	$0 \pm 0^{A,a}$	$1.1 \pm 1.6^{A,a}$
	NT	$16.6 \pm 2.8^{B,b}$	$8.8 \pm 2.6^{B,b}$	$7.2 \pm 2.^{A,b}$	$1.8 \pm 5.2^{B,b}$	$0 \pm 0^{A,a}$	$0.2 \pm 0.2^{A,a}$
After	T	$63.2 \pm 50.4^{A,a}$	$46.4 \pm 20^{A,a}$	$15.8 \pm 7.5^{A,B,a}$	$6 \pm 7.2^{A,a}$	$0 \pm 0^{A,a}$	$2.7 \pm 5.2^{A,a}$
	NT	$9.3 \pm 2.9^{A,b}$	$4 \pm 1.5^{C,b}$	$4.9 \pm 1.8^{B,b}$	$0.4 \pm 0.3^{A,B,b}$	$0 \pm 0^{A,a}$	$0.1 \pm 0.1^{A,b}$
Normal range[1]		4–13	1.2–7.2	2–9	0.05–0.65	0–0.12	0–0.55

[1] Reference value reported by Pugh (2002). WBC = total white blood cell count. Within a column values with different superscripts differ significantly ($p < 0.05$)

[a,b] Differ between T versus. NT at the same period

[A,B,C] Differ between the period (before vs. during vs. after) for the same group of animals

Table 2 Serum biochemistry of F1-generation hG-CSF-transgenic (T) and non-transgenic (NT) goats before, during and after hormonally induced lactation

Period	Group	Biochemical dosages (mean ± SD)				
		Glucose[a] (mg dL^{-1})	Urea[b] (mg dL^{-1})	Creatinine[a] (mg dL^{-1})	ALT[a] (IU L^{-1})	AST[a] (IU L^{-1})
Before	T	54.5 ± 7.1	35.1 ± 9.5	1.1 ± 0.2	15.3 ± 2	70.2 ± 21.2
	NT	58.6 ± 6.2	35.8 ± 8.5	1.1 ± 0.1	18.3 ± 2.2	78.4 ± 13.2
During	T	48.2 ± 7.6	33.1 ± 6.5	1 ± 0.2	15.2 ± 2.5	142 ± 64.7
	NT	53.7 ± 9.2	34.5 ± 4.7	1 ± 0.1	18.6 ± 4.7	88.6 ± 14.0
After	T	58.2 ± 7.5	31.7 ± 8.6	1 ± 0.1	16.4 ± 2.2	119 ± 52.6
	NT	54.7 ± 4.8	38.8 ± 7	1 ± 0.1	18.4 ± 1.5	94.2 ± 21.1
Normal range		48.2–76	21.4–42.8	0.7–1.5	15.3–52.3	66–230

Reference values reported by Boyd (1984)[a] and Kaneko et al. (1997)[b]. (AST) *aspartate aminotransferase*, (ALT) *alanine aminotransferase*

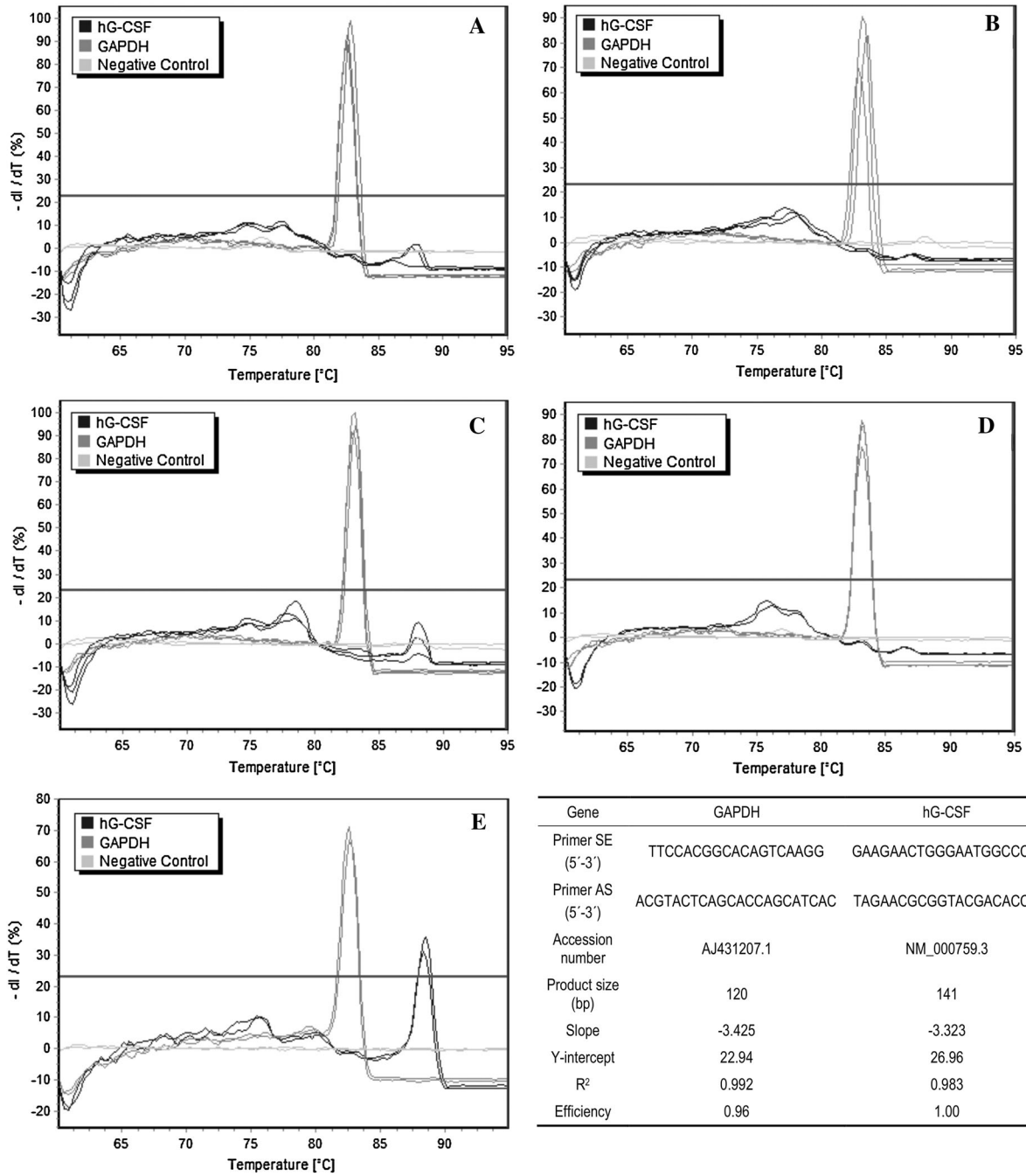
Within a column values with different superscripts differ significantly ($p < 0.05$)

Table 3 Clinical monitoring of F1-generation hG-CSF-transgenic (T) and nontransgenic (NT) goats before, during and after during hormonally induced lactation

Period	Group	Clinical parameters (mean ± SD)		
		Rectal temperature (°C)	Respiratory rate (breaths/min)	Heart rate (beats/min)
Before	T	38.3 ± 0.5	19.6 ± 2.5	86.8 ± 11.8
	NT	38.1 ± 0.5	20.6 ± 3	74.8 ± 8.4
During	T	38.4 ± 0.5	19.8 ± 2.6	84.3 ± 14.3
	NT	38.0 ± 0.4	20.0 ± 2.8	73.2 ± 7.9
After	T	38.1 ± 0.4	18.0 ± 1.9	80.4 ± 9.8
	NT	38.1 ± 0.4	20.0 ± 3	73.2 ± 7.6
Normal range		37.5–39.7	12–25	70–110

Reference value reported by Pugh, 2002

Within a column values with different superscripts differ significantly ($p < 0.05$)



Gene	GAPDH	hG-CSF
Primer SE (5′-3′)	TTCCACGGCACAGTCAAGG	GAAGAACTGGGAATGGCCCC
Primer AS (5′-3′)	ACGTACTCAGCACCAGCATCAC	TAGAACGCGGTACGACACCT
Accession number	AJ431207.1	NM_000759.3
Product size (bp)	120	141
Slope	-3.425	-3.323
Y-intercept	22.94	26.96
R^2	0.992	0.983
Efficiency	0.96	1.00

Fig. 2 Analysis of ectopic hG-CSF expression in blood lymphocytes of F1-generation hG-CSF-transgenic goats. Representative derivative melting curves of hG-CSF or GAPDH (reference gene) amplicons produced by real-time qRT-PCR reactions. Templates were achieved before (**a, b**) or during (**c, d**) lactation period from transgenic (**a, c**) or non-transgenic (**b, d**) goats. The plasmid (pGoatcasGCSF) previously used to generate 10M and 12F transgenic lineages were used as template for positive-control qRT-PCR reactions (**e**). Negative controls were constituted of mRNA templates without reverse transcriptase (**a–d**) or water instead of DNA (**e**). The primers and standard curve parameters (slope, Y-intercept, linearity and efficacy) were also presented in the *bottom right of the figure*

CSF expression during normal lactation (Lee et al. 2000). In this context, it is reasonable to assume that the protein expression profile observed in our transgenic goat lines (10 M and 12F) is compatible with investments for recombinant production in milk on a commercial scale.

Some studies have shown that the production of large quantities of recombinant protein in the mammary gland of transgenic goats compromises their physiology of lactation (Baldassarre et al. 2008). Likewise, in the present work, the total milk volume produced by T was lower than NT females. Moreover, a transgenic goat had premature shutdown in milk production. A similar event was earlier described for transgenic goats hormonally induced to lactate (Cammuso et al. 2000) or even to non-transgenic goats submitted to induced lactation (Mellado et al. 1996). Studies in transgenic goats expressing human butyrylcholinesterase demonstrated that their lactation was characterized by a slow/delayed start of milk production, a relatively normal milk volume at peak and a premature shutdown of milk production compared to control animals (Baldassarre et al. 2008). One hypothesis for this alteration was suggested by the same authors who described an impaired fat secretion at the level of secretory epithelium and a dramatic increase in the number of phagocytes in milk (not associated with mammary infection). In the present study, a similar proposition could explain the lower volume and premature shutdown in transgenic milk production due to an increase in SCC. In addition, it is important to highlight that analysis of clinical mastitis indicators remained within the normal range for the T females throughout lactation period. On the other hand, the percentage of milk fat in T (7.3 %) was similar to NT (6.2 %). Likewise, both protein and lactose amounts did not differ between groups.

The hG-CSF protein specifically regulates the in vivo proliferation and differentiation of neutrophilic granulocyte precursor cells from the bone marrow (Crea et al. 2009). Taking into account that this recombinant protein is produced in the mammary gland of female goats, the potential for systemic in vivo biological activity in the transgenic animal is a possibility and it has been previously reported (Ko et al. 2000). Thus, as performed in founders (Freitas et al. 2012; Moura et al. 2013), F1 specimens were also monitored for serum hG-CSF levels and leukocyte count profile, besides other health indicators, as serum biochemistry and clinical parameters. As expected, F1 T goats presented higher total (WBC) and neutrophil counts than NT, with few changes in other cell counts. Similarly to the founder, serum hG-CSF levels increased shortly before the start of milk production. Additionally, after lactation, the blood cellularity was restored and serum hG-CSF returned to null level. Aiming to explain these findings, transgene ectopic expression was investigated by qRT-PCR. In light of the report that describes the physiologic presence of CSN1S1 transcripts in peripheral blood mononuclear cells of goats (Tokarska et al. 2001), we investigated if CSN1S1 promoter of DNA vector used could drive ectopic (also referred as "illegitimate") expression of hG-CSF in transgenic goats. However, no hG-CSF transcript was detected in lymphocytes in this study. Ectopic transgene expression was evaluated in F1 males submitted to a noninvasive collection method to access liver, spleen and lymph nodes samples. Even using a high sensitivity method, a similar result was observed and no hG-CSF transcript was detected.

Despite the possibility of leukocyte dynamics observed in lactating T females be associated with serum hG-CSF levels, another mechanism may explain the presence of the recombinant protein in serum with no apparent ectopic expression. Thus, according to Mao et al. (1991), the tight junctions between adjacent mammary secretory cells do not develop until shortly before delivery. Hence, proteins constitutively synthesized by developing epithelial secretory cells are secreted into the interstitial fluid and ultimately finding their way to whey. In this context, Salamone et al. (2006), working with bovine transgenic for growth hormone, suggested that the presence of circulating recombinant protein may be the result of leakage from the mammary gland and thus not exactly an ectopic expression.

Finally, despite the alteration in blood leukocytes, the goats remained healthy throughout the experimental period as indicated by the absence of changes in clinical and serum biochemistry measurements. Then, neither chronic nor acute kidney disease was detected, as demonstrated by urea (primary metabolite derived from dietary protein and tissue protein turnover) and creatinine (product of muscle catabolism). Likewise, indicators of liver function (ALT and AST) suggested normal hepatic functioning, as well as glycemic level. Corroborating these health biochemical indicators, physiological monitored parameters remained within the normal range.

Conclusions

First-generation hG-CSF-transgenic goats were able to express recombinant protein in milk, after hormonal induction of lactation, at quantities compatible with their use as bioreactors in a commercial-scale protein-production program. Some possibilities of transgene ectopic expression were ruled out, such as in blood lymphocytes, liver, spleen and lymph nodes. Despite the marked transient neutrophilia, the transgenic females remained clinically healthy throughout the experimental period until now. Further investigations involving hormonal regulation of the promoter will help to clarify the increase in leukocyte counts and the peak of serum hG-CSF at the early lactation period.

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