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RIBRIO IVAN TAVARES PEREIRA BATISTA

CARACTERÍSTICAS GENOTÍPICAS E FENOTÍPICAS DE CAPRINOS TRANSGÊNICOS EXPRESSANDO O FATOR ESTIMULANTE DE COLÔNIAS DE GRANULÓCITOS HUMANO (hG-CSF) NA GLÂNDULA MAMÁRIA

FORTALEZA
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Tese apresentada ao Programa de Pós-Graduação em Ciências Veterinárias da Faculdade de Veterinária da Universidade Estadual do Ceará, como requisito parcial para a obtenção do título de Doutor em Ciências Veterinárias.
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Dedico este trabalho

a toda minha família!!!

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“Eu sei de muito pouco. Mas tenho a meu favor tudo o que não sei e – por ser um campo virgem – está livre de preconceitos. Tudo o que não sei é a minha parte maior e melhor: é minha largueza. É com ela que eu compreenderia tudo. Tudo o que eu não sei é que constitui a minha verdade” (Clarice Lispector)

RESUMO

A expressão de proteínas recombinantes de valor farmacêutico no leite de animais transgênicos é uma solução emergente para obtenção de moléculas que não são expressas corretamente nas plataformas padrões de biorreatores. No entanto, a modificação induzida pela inserção do gene exógeno, associada ao controle inadequado da expressão do transgene, pode resultar na alteração dos processos fisiológicos normais. Assim, para garantir que a saúde dos animais da linhagem transgênica recém-produzida não foi comprometida, torna-se necessária uma avaliação genotípica e fenotípica dos indivíduos da linhagem. Dessa forma, neste estudo foi caracterizado genotípica e fenotipicamente duas linhagens de caprinos transgênicos (10M e 12F) para expressão do Fator Estimulante de Colônias de Granulócitos Humano (hG-CSF) na glândula mamaria. A caracterização genotípica dos animais transgênicos quanto ao número de cópias do transgene, realizada utilizando diferentes estratégias metodológicas de PCR em tempo real (qPCR), demonstrou que o valor de número de cópias do transgene na linhagem 10M variou de 7 a 8, enquanto que na 12F de 10 a 13. Quanto a caracterização fenotípica, demonstramos que inserção do transgene hG-CSF não teve efeito deletério sobre os parâmetros produtivos e reprodutivos da geração F1. A alteração no perfil leucocitário, observada em todos os períodos da vida dos animais transgênicos, não está ligada a uma expressão ectópica. As fêmeas F1, expressaram corretamente a proteína recombinante e numa concentração compatível para serem utilizadas como biorreatores. Porém, a análise do desempenho lactacional foi caracterizada pela menor produção leiteira, sem alteração na composição do mesmo (gordura, proteína e lactose), em comparação com as fêmeas não-transgênicas. Por outro lado, embora sem ocorrência de mastite, foi observado aumento nas células somáticas do leite nas fêmeas F1. Durante todo este estudo, os parâmetros clínicos, bem como funções renal e hepática, indicaram que a geração F1 de caprinos transgênicos são saudáveis. Adicionalmente, estudos em linfonodos, leucócitos, baço e fígado, demosntraram não existir expressão ectópica nas fêmeas estudadas. Em conclusão, cabras F1 transgênicas para hG-CSF foram capazes de expressar a proteína recombinante no leite e em quantidades compatíveis com a sua utilização como biorreatores em um programa de produção de proteína em escala industrial. Adicionalmente, todos os animais apresentaram-se normais, demosntrando que o transgene não teve efeito deletério sobre a saúde dos mesmos, quer pela presença ou como consequência do perfil de leucocitário alterado.

Palavras-chave: Reprodução. Crescimento. Lactação. Transgênese. Caprinos. hG-CSF. Bem-estar animal.

ABSTRACT

Milk expression of recombinant proteins of pharmaceutical value in transgenic dairy animals is an emerging solution to produce molecules that cannot be made efficiently using the standard bioreactor platforms. However, genetic modification induced by insertion of exogenous gene, associated with inadequate control of transgene expression, may result in the alteration of normal physiological processes. Thus, to ensure that the health of newly produced transgenic line animals was not compromised; genotypic and phenotypic assessment of individuals of the lineage is required. Therefore, in this study we characterized the genotype and phenotype of two lineages of transgenic goats (10M and 12F) for expression of human granulocyte-colony stimulating factor (hG-CSF) in the mammary gland. Genotypic characterization of transgenic animals regarding the transgene coy number (CN), performed using different methodological strategies of real-time PCR (qPCR) showed that CN values calculated for each goat line ranged from 7 to 8 for 10M and 10 to 13 for 12F. Concerning phenotypic characterization, we demonstrated that the insertion of the transgene hG-CSF had no deleterious effect on productive and reproductive parameters of the F1 generation of transgenic goats. The change in blood leukocytes, observed at all times during the life of transgenic animals was not associated with any ectopic expression. The F1 transgenic females correctly expressed recombinant protein and a concentration compatible for their use as bioreactors. However, analysis of lactational performance was characterized by a low milk production, without changes in milk composition (fat, protein and lactose), in comparison with non-transgenic females. On the other hand, an increase in somatic cells was observed in the milk of female transgenic F1, associated with mastitis was not observed. Throughout the study period, all clinical parameters, as well as renal and hepatic function indicated that the F1 generation of transgenic goats are healthy. Additionally, there was no ectopic expression of the recombinant protein in the lymph node, leukocytes, spleen and liver was not detected the presence. In conclusion, F1 transgenic goats to hG -CSF were able to express the recombinant protein in milk in a pattern compatible with their use as bioreactors in a program of protein production on a commercial scale quantity. Still, it is possible to conjecture that all animals are normal and that the transgene had no deleterious effect on the health of animals, either by presence or as a consequence of altered blood leukocytes.

Keywords: Reproduction. Growth. Lactation. Transgenesis. Goats. hG-CSF. Animal welfare.

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LISTA DE ABREVIATURAS E SIGLAS

ALT: Alanine aminotransferase (Alanina aminotransferase)

AST: Aspartate aminotransferase (Aspartato aminotransferase)

ATIII: Antithrombin III (Antitrombina III)

CHO: Chinese hamster ovary (Ovário de hamster chinês)

CN: Copy number (Número de cópias)

CQB: Certificado de Qualidade em Biossegurança

CRISPR: Clustered, regularity interspaced, short palindromic repeats

CSN1S1: α-S1 casein (α-S1 caseina)

C_T: Cycle threshold (Limiar do ciclo)

CTNBio : Comissão Técnica Nacional de Biossegurança

DNA: Deoxyribonucleic acid (Ácido desoxirribonucleico)

E: Efficiency (Eficiência)

EDTA: Ethylenediamine tetraacetic acid (Ácido etilenodiamino tetra-acético)

Eff: Efficiency correction (Correção de eficiência)

EGFR: Epidermal Growth Factor Receptor (Receptor do Fator de Crescimento Epidérmico)

ELISA: Enzyme-Linked Immunosorbent Assay (Ensaio Imunoenzimático)

EMA: European Medicines Agency (Agência Européia de Medicina)

EPO: Erythropoietin (Eritropoietina)

FDA: Food and Drug Administration

gDNA : Genomic DNA (DNA genômico)

GFP: Green Fluorescent Protein (Proteína Fluorescente Verde)

GH: Growth Hormone (Hormônio do Crescimento)

Gluc: Glucagon

GMA: Genetically Modified Animal (Animal Geneticamente Modificado)

hG-CSF: human Granulocyte Colony Stimulating Factor (Fator Estimulante de Colônias de Granulocitos humano)

ICSI: Intracitoplasmatic sperm inject (Injeção intracitoplasmática de espermatozoide)

LFCR : Laboratório de Fisiologia e Controle da Reprodução

MG: Milk gene (Gene do leite)

NT : Non-transgenic (Não transgênico)

PCR: Polymerase Chain Reaction (Reação em Cadeia da Polimerase)

pH: Potential of hydrogen (Potencial de hidrogênio)

qPCR: Quatitative PCR (PCR quantitativo)

Abs: Quantification normalized absolute (Quantificação absoluta normalizada)

RIA: Radioimmunoassay (Radioimunoensaio)

RNA: Ribonucleic acid (Ácido ribonucléico)

SCC: Somatic Cell Count (Contagem de Células Somáticas)

SCNT: Somatic Cell Nuclear Transfer (Transferência Nuclear de Células Somáticas)

SDS: Sodium dodecyl sulfate (dodecilsulfato de sódio)

SMGT: Sperm-mediated gene transfer (transferência gênica mediada por espermatozóide)

SNCT : Somatic Cell Nuclear Transfer (transferência nuclear de células somáticas)

StC : Standard curve (curva padrão)

T: Transgenic (transgênico)

TAL: Transcription activator-like (efetoras ativadores de transcrição)

TALENs: Transcription activator-like effector nucleases (nucleases efetoras como ativador de transcrição)

Tm: Melting temperature (temperatura de fusão)

TNF: Tumor Necrosis Factor (fator de necrose tumoral)

tPA: tissue Plasminogen Activator (ativador do plasminogênio tecidual)

WBC: White blood cells (glóbulos brancos)

ZFN: Zinc-finger nucleases (nucleases dedos-de-zinco)

ΔC_T: Delta cycle threshold (Variação do valor de C_t)

SUMÁRIO

1 INTRODUÇÃO

O sector da biotecnologia tem crescido intensamente em todo o mundo nas últimas quatro décadas, após o desenvolvimento da tecnologia de DNA recombinante na década de 70 pelo ganhador do Prêmio Nobel da química, Paul Berg. Posteriormente a esta descoberta, as primeiras proteínas recombinantes terapêuticas foram produzidos no início dos anos 1980, a começar com a insulina humana recombinante (WILLIAMS *et al.,* 1982). Desde então, possibilidades praticamente ilimitadas de produção das proteínas recombinantes, tem sido propostas. Com mercado global superior a 120 biliões de dólares por ano, a indústria farmacêutica e grupos de pesquisa acadêmicos continuam a desenvolver métodos eficazes para a produção de proteínas de mamíferos capazes de superar avaliações pré-clínicas e clínicas de potenciais fármacos terapêuticos (BANDARANAYAKE e ALMO, 2014).

Bactérias geneticamente modificadas foram usadas na produção das primeiras proteínas recombinantes, contudo logo apareceram vários obstáculos devido à capacidade limitada de reproduzirem modificações pós-transcricionais (tais como glicosilação, γ-carboxilação, fosforilação, etc) necessárias para a atividade e estabilidade de algumas proteínas (SWARTZ, 2001; DYCK *et al.,* 2003; HOUDEBINE, 2009). Alternativamente, as leveduras e células de insetos, apesar de apresentarem vantagens sobre as bactérias, também demonstram dificuldades no correto dobragem e glicosilação de proteínas humanas recombinantes (CONDREAY e KOST, 2007). Assim, as células de mamíferos, principalmente, células de ovário de hamster chinês (CHO), têm sido uzadas com sucesso a aproximadamente três décadas na produção de proteína recombinante (JAYAPAL *et al.*, 2007). No entanto, a necessidade de grandes investimentos de capital, altos custos operacionais e níveis relativamente baixos de produção resultam na incapacidade de produzirem não mais que alguns quilos de proteína por ano (JAYAPAL *et al,* 2007; HOUDEBINE, 2009; BALDASSARE, 2012). Dadas estas limitações, a plataforma de

animais transgênicos no qual a proteína recombinante é expressa na glândula mamaria e assim purificada a partir de seu leite, aparece nos anos 90, como um método promissor (HOUDEBINE, 2009). O sistema oferece excelente capacidade de síntese de proteína na glândula mamária, baixo investimento de capital, baixo custo operacionais e capacidade praticamente ilimitada de expansão, por intermédio da produção de mais animais transgênicos (BALDASSARRE, 2012; KUES e NIEMANN, 2011; PARK *et al.*, 2006).

Sendo assim, nos últimos 30 anos, um número substancial de proteínas recombinantes vem sendo expresso no leite de animais transgênicos, incluindo camundongos, coelhos, ovelhas, vacas e cabras. Esta ultima, eleita atualmente como modelo da transgenese animal para produção de recombinates, vem sendo utilizada com sucesso na produção diversas proteínas como antitrombina (comercializada como *ATryn* pela GTC *Biotherapeutics*, EUA), o primeiro produto derivado de animais transgênicos e aprovado pela EMEA (*European Medicines Agency*) e subsequentemente pela FDA (*Food and Drug Administration*) (Lavine, 2009), lisozima (MAGA *et al*., 2006), butirilcolinesterase (HUANG *et al*., 2007), e lactoferrina (ZHANG *et al.*, 2008). Contudo, num certo número de casos, distúrbios fisiológicos, produtivo (PURSEL *et al*., 2004), reprodutivo (MALESZEWSKI *et al*., 1998; BRYLA *et al.*, 2010) e lactacionais (BALDASSARE *et al.*, 2008) decorrentes da inserção aleatória do transgene, controle inadequado da expressão do transgene e alta expressão do transgene tem sido relatados. Certamente, a saúde e o bem-estar dos animais transgênicos criados para fins de produção é de importância fundamental para aceitação dos consumidores (Jackson et al., 2010). Apesar disso, informações referente a esses aspectos ainda não são bem estudadas. O objetivo deste trabalho é avaliar os aspectos produtivo, reprodutivo e lactacional de animal transgênicos, utilizando como modelo caprinos transgênicos para Fator Estimulante de colônias de Granulocitos Humano (hG-CSG).

2 REVISÃO DE LITERATURA

PARTE I: CAPRINOS (*Capra hircus*) COMO BIORREATORES PARA PRODUÇÃO DE PROTEÍNAS RECOMBINANTES DE INTERESSE PARA INDÚSTRIA FARMACÊUTICA

Resumo

Cabras são particularmente eficientes na produção de proteínas recombinante, uma vez que produzem quantidades consideráveis de leite associado a menores custos de manutenção e investimento em relação as vacas. Assim, o objetivo desta revisão é apresentar o estado-da-arte para a obtenção de cabras transgênicas que produzem proteínas recombinantes para posterior utilização na indústria farmacêutica. Além disso, serão discutidas as abordagens para a integração dirigida local-específico de transgene, bem como o interesse econômico desta atividade.

Palavras-chave: cabra; proteína recombinante; farmacêutica; transgênese

Esta parte da revisão de literatura foi submetido ao periódico **Cloning & Transgenesis** (ISSN: 2168-9849)

Goats (*Capra hircus*) as Bioreactors for Production of Recombinant Proteins Interesting to Pharmaceutical Industry

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Abstract

Goats are a particularly efficient mean of producing recombinant proteins since they produce considerable amounts of milk, and incur lower investment and maintenance costs than cows. Thus, the aim of this review is to present the state-of-the-art for obtaining transgenic goats producing recombinant proteins for further utilization in the pharmaceutical industry. Additionally, the approaches to directed site-specific integration of transgene as well as the economic interest for this activity will be discussed.

Keywords: Goat; Recombinant protein; Pharmaceuticals; Transgenesis

Abbreviations: AT: antithrombin; CHO: chinese hamster ovary; CRISPR: clustered, regularity interspaced, short palindromic repeats; Cas: CRISPR-associated; EPO:

erythropoietin; GMA: genetically modified animal; G-CSF: granulocyte-colony stimulating factor; GH: growth hormone; EGFR: epidermal growth factor receptor; ICSI: intra citoplasmatic sperm inject; MG: milk gene; SCNT: somatic cell nuclear transfer; SMGT: sperm-mediated gene transfer; TAL: transcription activator-like; TALENs: transcription activator-like effector nucleases; TNF: tumor necrosis factor; tPA: tissue plasminogen activator; ZFN: zinc-finger nucleases.

Introduction

Recombinant DNA technology has revolutionized the production of therapeutic proteins. Thus, genes of a great number of human proteins have already been identified and cloned, including clotting factors, growth hormone (GH), insulin, erythropoietin (EPO), among others. The first attempts to produce therapeutic proteins from cloned genes were made in yeast and bacteria. However, for many proteins this is not viable, because microorganisms are not capable to make the posttranslational modifications necessary for protein activity [1]. Although mammalian cell culture provides the posttranslational modifications, they are very expensive. Thus, the use of farm animals as bioreactors may be the better choice to produce recombinant therapeutic proteins in their mammary gland. For this activity, the term "pharming" (portmanteau of farming and pharmaceutical) was created referring to the use of genetic engineering to obtain a transgenic or genetically modified animal (GMA).

The generation of transgenic large ruminants (cattle) is, however, very expensive due to the long gestation period, small litter size and high maintenance costs when compared to other species, as for example, sheep and goats. In contrast, goats are very interesting considering their high yield of purified product, relatively short generation interval and production of multiple offspring [2]. In addition, The European Agency for the Evaluation of Medicinal

Products [3] and the Food and Drug Administration [4] approved the commercialization of the first human biological drug (antithrombin - AT) produced by transgenic goat.

Generating Transgenic Goats

Production of transgenic livestock was demonstrated to be feasible almost three decades ago [5]. It became apparent almost immediately that the method used to produce the transgenic livestock had substantial limitations that would impede its use both for research and commercial applications. Nevertheless, transgenic goats have been obtained to date by two methods: pronuclear microinjection and somatic cell nuclear transfer (Figure 1).

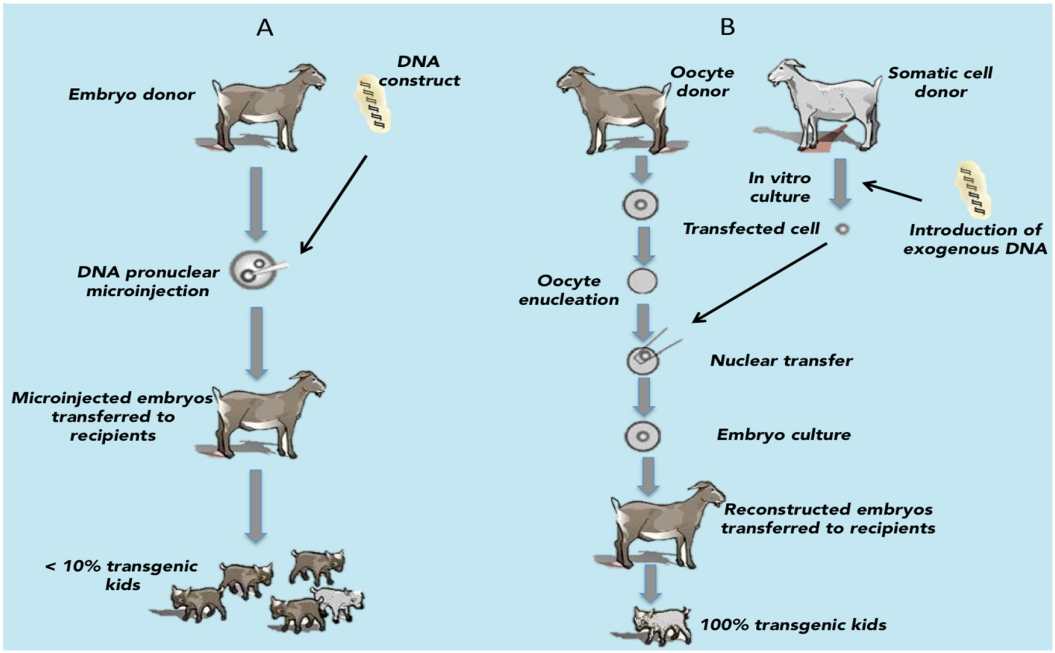

Figure 1: Traditional methods used to obtain transgenic goats: (A) pronuclear microinjection of DNA and (B) somatic cell nuclear transfer (SCNT).

Pronuclear microinjection

The aim of the first report on genetically manipulated goat embryos was to obtain transgenic animals that secreted pharmaceuticals, and in particular the human tissue plasminogen

activator (tPA), in their milk [6]. After this first success, several other human proteins have been produced in goats using pronuclear microinjection. However, the overall efficiency of this technique is poor, especially when compared to that obtained in mice. In goats, around 1% or less of the injected zygotes gives birth to a transgenic kid [7].

Pronuclear microinjection has a simple concept: to inject a small volume containing the gene of interest into a pronucleus of a zygote, and then transfer the zygotes to the oviduct of a recipient. However, the microinjection is a procedure that requires a certain amount of dexterity and a significant amount of patience. In mouse, pronuclei are clearly visible during the latter phase of the zygotic stage of development. In other species (cattle, pigs and goats), it is necessary to centrifuge embryos.

During the experiments performed in our laboratory, goat zygotes showed non-transparent cytoplasm and approximately 125-130 μm in diameter (Figure 2A, B). Non-transparent cytoplasm is due to the presence of a large amount of lipid granules that hinder the visualization of pronuclei. In addition, pronuclei seem to be visible sometimes, but this impression can, however, appear false after attentive examination with an inverted microscope equipped with interferential contrast optics and using variable lighting. The presence of the second polar body is a rather marked indication that the egg has been fertilized. The presence of the second polar body is typical for all zygote stages. It can be located in contact with the first polar body or not far away from it. The first polar body most often stays at the degradation stage; sometimes zygotes with three polar bodies occur if by that moment the division of the first polar body has already occurred.

In goat embryos, the pronuclei can be visualized without centrifugation in approximately 30% of times [6] (Figure 2C). To facilitate visualization of pronuclei, all the fertilized eggs were subjected to centrifugation, which contributes to precipitation of the lipid granules. The pronuclei of the late zygotes were located closely to one another and mainly in the middle

zone of the cytoplasm though closer to the dark pole with dark granules, one of them (male) was somewhat larger than the other (female) (Figure 2D, F). Their pronucleolars, unlike those in mouse, rabbit or swine pronuclei, were not visualized and morphologically rather resembled sheep and cow pronuclei. Both pronuclei could not be always simultaneously observed, and one of them could be located in the lipid granules. However, even after very careful examination of the centrifuged zygotes in a microscope with Nomarsky optics, the pronuclei could not always be clearly observed. Zygotes of different goat breeds differ in a degree of visualization of pronuclei. For instance, in Canindé goats the visualization was in almost 100% of examined zygotes, whereas in Saanen this rate was only slightly higher than 70% [8].

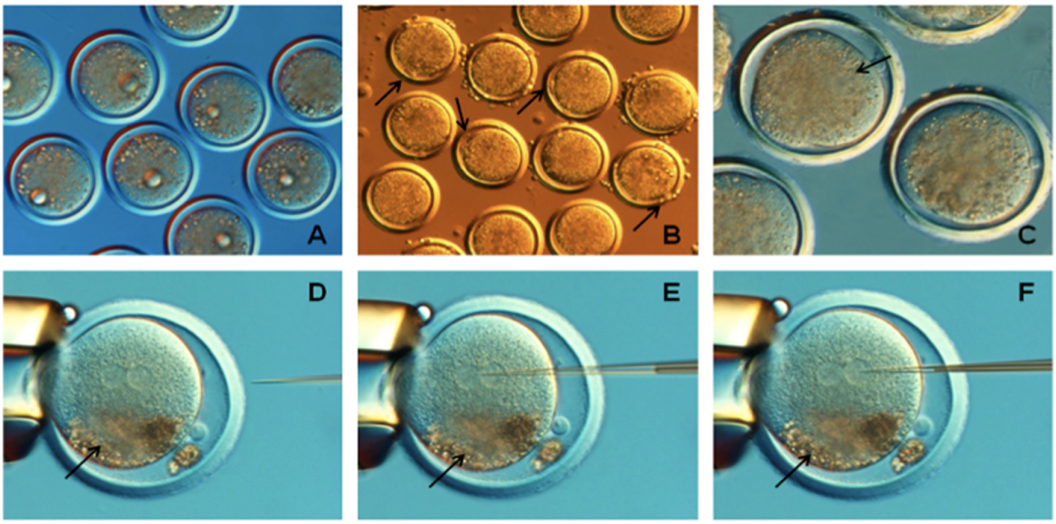

Figure 2: Morphology of goat oocytes and zygotes and details of pronuclear microinjection. A: unfertilized oocytes; secondary polar body is absent (×200). B: Saanen goat zygotes; arrows indicate secondary polar bodies and pronuclei are not visible (×200). C: Canindé goat zygotes; arrow indicates pronuclei visible without centrifugation (×300). D, E, F: DNA microinjection into centrifuged pronuclei of Canindé goat zygotes; arrow indicates pole with lipid granules. Microinjection was performed with an inverted microscope (×300) equipped with DIC (TE2000; Nikon, Japan) and a pair of micromanipulators (Narishige, Japan).

Somatic Cell Nuclear Transfer (SCNT)

SCNT, combined with molecular biology and cell culture methods, shows a variety of applications. Among the different areas, transgenesis is possibly the one that has benefited the most with the advances in this biotechnique in the sense of increasing the efficiency and reducing costs. Since the birth of Dolly sheep [9], the basic SCNT technology remains the same. It consists on the transfer of the donor cell nuclei to enucleated oocytes with later reconstruction of the embryo through the cell fusion. By the use of SCNT technique it is possible to produce transgenic animals through the transfection of nuclei with vectors of DNA expression or by cloning transgenic founder animals [7].

In the SCNT method utilizing nuclei transfection, exogenous DNA is randomly incorporated into the genome using selective pressure. Moreover, transgenic cells can be completely characterized with respect to the integration region, integrated number of copies and integrity of the transgene before the nuclear transfer step. Although the capacity for development of the reconstructed embryos is low, the majority of the offspring are transgenic, making this technique more efficient than pronuclear microinjection [10]. However, the use of SCNT still has some limitations, as for example: reprogramming may be incomplete, resulting in embryonic loss, abortion, or abnormal development [11].

Alternative methods

Figure 3 shows the current state of transgenic technology development in goats showing the pioneer studies at each stage of success. While some problems inherent in traditional techniques still persist, some groups are working on alternative methods. Among these methods, some have proven to be feasible in other species, as for example, the sperm-mediated gene transfer (SMGT). Recently, it was shown that although goat spermatozoa can uptake DNA, the presence of seminal fluid partially inhibits it [12]. Before this study, other

group [13] verified the possibility of using SMGT to produce transgenic goat embryos. In this work, the authors enhanced the technique by the use of intracytoplasmic sperm injection (ICSI) procedure. This study showed that the technique (*in vitro* fertilization *vs.* ICSI), sperm status (motile *vs.* live-immotile *vs.* dead) and to some extent DNA concentration affect embryo development, transgene transmission and expression.

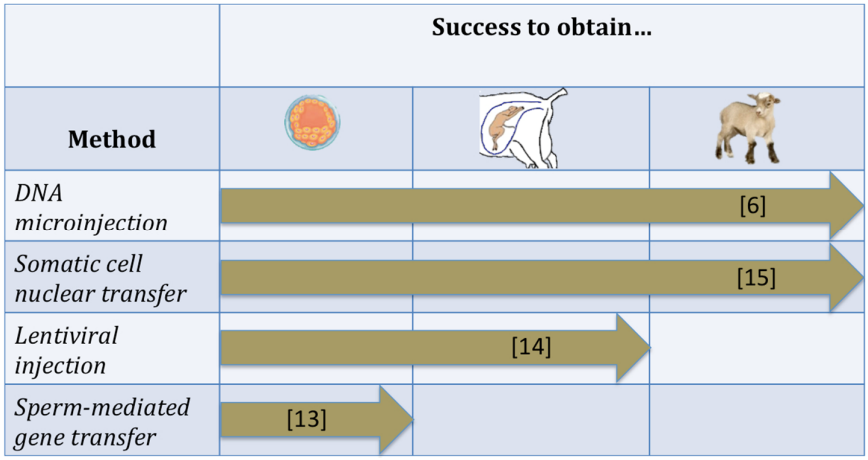

Figure 3: Success to obtain transgenic embryos, fetuses or offspring in goats according to the different methods used.

The results obtained by these two groups suggested that SMGT is applicable to goats. However, the genetic characterization of the resulting transgenic kids, such as mosaicism and transgene copy number, is required. An alternative to gene inactivation by homologous recombination is gene knockdown by RNA interference. Thus, lentiviral vectors were used to deliver short-hairpin RNA expression cassettes targeting the prion protein mRNA in goat fibroblasts. These cells were posteriorly subsequently used for nuclear transplantation. The analysis of the transgenic fetuses (brain) confirmed the knockdown of the targeted mRNA and of the encoded PrP protein [14].

Transgene design and approaches to directed site-specific integration of transgene into animal genome

Typical design of transgenes used for generation of animals as bioreactors includes three basic elements: 5'-flanking sequences of the milk gene (MG), often together with non-translated exon 1 and intron 1; genomic region or cDNA coding protein of interest, and 3'-genomic flanking sequence of the MG including non-translated exon(s) and intron(s), and 3'-UTR or rare other genes, for example, growth hormone [16,17].

Variability in expression of transgenes directed by the promoter of various MGs was reported by many investigators [16,18,19]. Most researchers believe that incorrect transgene expression, in particularity ectopic and high variability occurs mainly due to its random insertion into the recipient genome [16-18,20]. It is presumed that transgene expression depends on the chromatin environment in which it is located, a phenomenon known as the position-effect. Meanwhile, for successful creation of transgenic goats effective as bioreactors it is required the expression of transgene exclusively in mammary gland.

The criteria for correct transgene expression under control of a MG promoter include: i) the expression must be restricted to the lactating mammary gland, without ectopic expression; ii) the expression must take place in all of the epithelial cells of the mammary gland, without cell mosaicism; and iii) there must be low variability in transgenic expression between animals originated from different founders.

Since almost 30 years passed from the first generation of farm animals [5], many efforts were applied to reach the criteria. For instance, in the first researches on transgenic animals including goat promoters of the MGs fused with cDNA coding human protein were used [15]. However, genomic sequences of the gene of interest provide higher levels than cDNA sequences presumably due to regulatory elements located within introns [21]. Also, during the search for optimal transgene designs diverse variants of the MGs promoters were tested. As a

rule, an increase of the promoter size prompt increase transgene expression [16]. The most difficult problem is to overcome the ectopic transgene expression. Progress in identification of the regulatory sites within the promoters of milk genes allowed partially resolving the problem [22].

Here is pertinent to note that parameters of expression of the transgene tested in transgenic mice may be different when the same transgene is introduced in genome of another species. For instance, we did not observe expression of the human Granulocyte-Colony Stimulating Factor (G-CSF) after birth of transgenic mice in other tissue except the mammary gland [23] whereas goats carrying the same transgene showed leukocytosis (due to elevated number of neutrophils) at birth and persisted throughout their life [24]. It is obvious that the neutrophilia in the transgenic goats is a result of expression of the human G-CSF in fetuses before birth. Despite conservatism in organization of the MGs in mammals one cannot exclude that they may have distinct functions. This is supported by the finding that hG-CSF secretion into milk of transgenic mice was at a lower concentration when compared to transgenic goats [23,25].

A long genomic DNA fragment expressed in bacterial artificial chromosomes or yeast artificial chromosomes often provides correct expression of the transgene [26]. However, long DNA fragments can be fragmented during the microinjection procedure.

Although not yet used in goats, three new approaches were developed allowing creating site-specific endogenous gene modifications in cell cultures and animals. The first is zinc-finger nucleases (ZFN) technology basing on joint of the transcriptional zinc-finger factor and the nuclease domain of Fok I. The hybrid protein links a DNA binding domain of the zinc-finger type to the Fok I nuclease and, hence, induces double-strand breaks at preselected genomic sites [27]. Combining the ZFN-technology with nuclear transfer, Whyte et al. [28] have generated piglets with the targeted GFP transgene. It was the first communication on farm animals carrying the site-specific knock out mutations.

Similarly to ZFNs, the transcription activator-like (TAL) effector nucleases (TALENs) are able to create double-strand breaks site-specific manner and then are either sealed by homologous recombination with mutant, synthetic oligodeoxynucleotides or by non-homologous end-joining repair giving rise often deletions and insertions [29].

The third approach to genome editing is based on the use of clustered, regularity interspaced, short palindromic repeats (CRISPR) together with CRISPR-associated (Cas) which provide an adaptive microbial immune response against viruses and plasmids [30,31]. The first communications on generation of knockout mice by CRISPR/Cas-mediated gene editing have practically appeared simultaneously [32,33].

In general, the ZFN, TALEN and C CRISPR/Cas technologies open perspectives for site-specific genome editing and potentially can be applied to many other species including goats.

Market for Recombinant Proteins from Goats

The global protein therapeutics market reached US$ 138.3 billion in 2012 and this market is expected to increase to nearly US$ 179.1 billion in 2018 [34]. This can be an interesting point for companies who want to use the “goat model” as bioreactor. However, even though it was always considered as a highly-perspective approach, the number of new commercial recombinant proteins that have successfully reached the market still counts only two products [35]. The first reason to this is the level of technology of the production of transgenic animals. Even though it has reached significant developments and lots of improvements were made, it still takes a long time to find out and test which design of the mammary expression system should be used in order to produce a particular protein. Some of the attempts towards this direction are still being unsuccessful, especially in the case of highly-active human proteins or those having very complex posttranslational modifications [36].

The second reason is caused by the existence of patents on all “blockbusters” of the recombinant protein market, which are still being produced in other systems, such as bacteria or chinese hamster ovary (CHO) cells. Presumably because of this fact, pioneers of the industry had to concentrate on orphan drugs. Efforts taken to bring an orphan drug produced by a transgenic animal to the market were higher than those for a blockbuster produced in conventional expression systems, but its commercial benefit was usually less, which makes such business quite risky. A number of companies went bankrupt since the beginning of the era of transgenic animal bioreactors, which serves as a good proof of it. Even the most notable of them had to struggle. Just recently, a key-player of the industry, GTC-Biotherapeutics (USA), which goat-derived recombinant human AT (ATryn®) was the first one to reach the market, had to cut about 50 of their employees and make two loans from its French partner company “LFB Biotechnologies”. On January 2013, GTC-Biotherapeutics announced that it was acquired by LFB and changed its company name to “rEvo”.

So far the only goat-derived recombinant human protein on the market is still the above-described human ATIII. However, the available data indicate that there are few dozens of various recombinant proteins produced in goats to date. Despite the fate of some of these projects is unknown, several of these proteins were reported to reach the stage of clinical trials.

Human blood serum proteins are of significant medical importance and the need of their use is constantly growing. This made them a desirable aim for bioreactor industry [18]. Thus, since the 90’s, blood clotting factors were produced by transgenic goats. Most of the mentioned recombinant blood proteins were developed in USA by GTC-Biotherapeutics. This is quite unsurprising, since GTC-Biotherapeutics is the biggest company on the market and it has a U.S. patent, issued in 2006 and expiring in 2021, that covers the production of all therapeutic proteins in the milk of transgenic mammals.

However, activities in this field are also carried out in other countries, such as Iran, where goats expressing human factor IX were produced [37]. Another important and rapidly growing segment is the production of therapeutic human monoclonal antibodies. Many of them are successfully expressed in other recombinant production systems, but levels of their production can be higher with the use of transgenic goats. Despite the fact that in published articles there are almost no data on transgenic goats producing monoclonal antibodies, some sources, such as GTC-Biotherapeutics 2010 annual report [38], evidences that goats expressing human monoclonal antibodies against CD20 receptor, CD137 receptor, tumor necrosis factor (TNF) and epidermal growth factor receptor (EGFR) already exists. Moreover, methods for the purification of recombinant antibodies from goat milk have been described [39].

Recombinant hormones, growth factors and cytokines is a more complex task, since they have high biological activity and may have adverse effect on the health of transgenic animal [23]. Nevertheless, transgenic goats expressing G-CSF [24,40] and EPO [36] were already produced.

Developments of other commercially important proteins expressed in transgenic goats are also the subject of interest. Thus, several groups have reported the creation of transgenic goats expressing high levels of human lactoferrin [41,42]. Another interesting project supported by the US Ministry of Defense was conducted by PharmAthene Inc., who has created a herd of goats expressing human butyrylcholinesterase [43]. It is assumed, that this enzyme will be successfully used as an antidote against organophosphorus poisons.

It seems that after all these years the industry is close to accumulate its critical mass of developments, which as we hope will finally turn into a number of new goat-derived proteins on the market.

Conclusions

Even though new different strategies are being developed for laboratory animals, in goats, the pronuclear microinjection and SCNT remain the most used tools for obtaining transgenic animals. It can be concluded that currently there is no perfect transgene design that could be reliable to provide correct tissue-specific expression of transgene at high level.

Concerning the use of transgenic goats in pharmaceutical industry, the level of technology is growing and guidelines regulating the use of these animals and quality assessment for milk-derived recombinant proteins are getting more established, which makes the whole way easier than it was before. Furthermore, since patents on many of the pharma "blockbusters" will soon expire, one can expect the appearance of their biosimilars. Since then the cost of recombinant protein production will become the main criteria, making transgenic goats a very competitive production system.

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PARTE II: ANÁLISE DA INTEGRAÇÃO DO TRANSGENE EM ANIMAL POR PCR EM TEMPO REAL

Resumo

Caracterização de animais geneticamente modificados em relação a zigosidade e número de cópias do transgene é importante fator para a criação de uma linhagem e a utilização do animal no propósito para qual foi obtida. Southern-blot, hibridização *in situ* por fluorescência ou acasalamento são técnicas tradicionalmente utilizadas na caracterização de animais transgênicos. Contudo, são laboriosos, demorados e geram resultados ambíguos com exceção de acasalamento. Com o surgimento da tecnologia de PCR em tempo real, diferentes estratégias têm sido descritas para a análise de animais transgênicos. No entanto, a precisão na quantificação por este método pode ser influenciado, em todas as etapas da metodologia aplicada. Esta revisão aborda os aspectos que influenciam as etapas pré-analíticos (métodos extração e quantificação de DNA) e pós-PCR (estratégias de quantificação e análise dos dados) na quantificação de número de cópias e análise da zigosidade em animais transgênicos.

Palavras-chave: Número de cópias; Integração do transgene; Zigosidade; Animal transgênico

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Transgene Integration Analysis in Animal by Real-Time Quantitative PCR

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Running title: Genotype study in transgenic animal

Abstract

Characterization of genetically modified animals in relation to zygosity and transgene copy is important factor for breeding a transgenic line and the use of transgenic animal in the purpose for which it was obtained. Southern-blot, fluorescence in situ hybridization or mating is techniques traditionally used in the characterization of transgenic animals. However, are laborious, time-consuming and with the exception of mating, give ambiguous results. With the emergence of the real-time quantitative PCR technology, different applications have been described for the analysis of transgenic organisms by determination of several paraments to transgenic analysis. However, the accuracy in quantitation by this method can be influenced in all steps of the applied methodology. This review focuses on aspects that influence pre-analytical steps (DNA extraction and DNA quantification methods), and post-PCR (quantification strategies and data analysis) in copy number quantification and zygosity analysis in transgenic animals.

Key words: Copy number; Transgene integration; Zygosity; Transgenic animal

INTRODUCTION

After obtaining transgenic animal, the molecular characterization of events, is an essential step in identifying promising transgenic animal to construct of a lineage. A part of these analyses is the transgene copy number quantification and zygosity analysis. Both transgene copy number and zygosity are important in the genetic analysis of gene function. Multiple transgene copies could lead to extremely high expression of the gene, and sometimes result in transgene silencing (Tang et al., 2007). For insertional mutation studies where transposons are usually inserted into the endogenous gene to abolish the gene function, heterozygous transgene allele often leaves one functional copy of the gene, and homozygous transgene alleles is preferred for functional studies. For these reasons, transgene copy number and zygosity determination are usually an essential part of trasngene studies.

Transgene copy number is a key issue for transgenic studies since it is directly relevant to the effectiveness of transgenic event and data interpretation (Yuan et al., 2007). Transgene copy number is defined as the number of exogenous DNA insert(s) in the genome. For example, if the exogenous DNA fragment inserts only once at a single locus of the genome, it is a single copy transgenic event. The copy number is closely relevant to another concept, zygosity. If a single exogenous DNA insert exists as two identical alleles on the homologous chromosomes in the organism, it is a homozygous transgenic line with one copy of transgene. If the single exogenous DNA insert exists in only one of the homologous chromosomes, it is a hemizygous transgenic line with one copy of transgene. Based on the Mendelian inheritance of the transgene transmission to F1 is 100% when the animal is homozygous and 50% when hemizygote. However, animals in hemizygotes with multiple insertions position different allelic can produce the rate of 75% transmission of the transgene (Figure 1).

The conventional method for trangene copy number determination is Southern blot hybridization (Tinkle et al., 1994). However, Southern blot hybridization based transgene

copy number determination is both costly, time consuming, and requires tens of microgram quantities of high-quality DNA. A robust alternative to Southern blot analysis would be helpful for gene function analysis and large scale mutant library based on transgenic techniques requiring a high-throughput determination of transgene copy number.

Real-time PCR (qPCR) has emerged the method of choice for fast, affordable, and efficient estimation of copy number. Various platforms and efforts have been proposed to improve the accuracy of qPCR for this application using both the standard curve based method and the ΔCt method involving an internal reference gene (Joshi et al., 2008; Ballester et al., 2004). However, despite the obvious advantages of qPCR, the accuracy and detection limit of qPCR based transgene determination has been controversial (Mason et al., 2002). In this review we discuss the factors (pre-analytical, quantification strategies and data analysis) that affect the accuracy and reproducibility of qPCR quantification of the number of copies and analysis of zygosity.

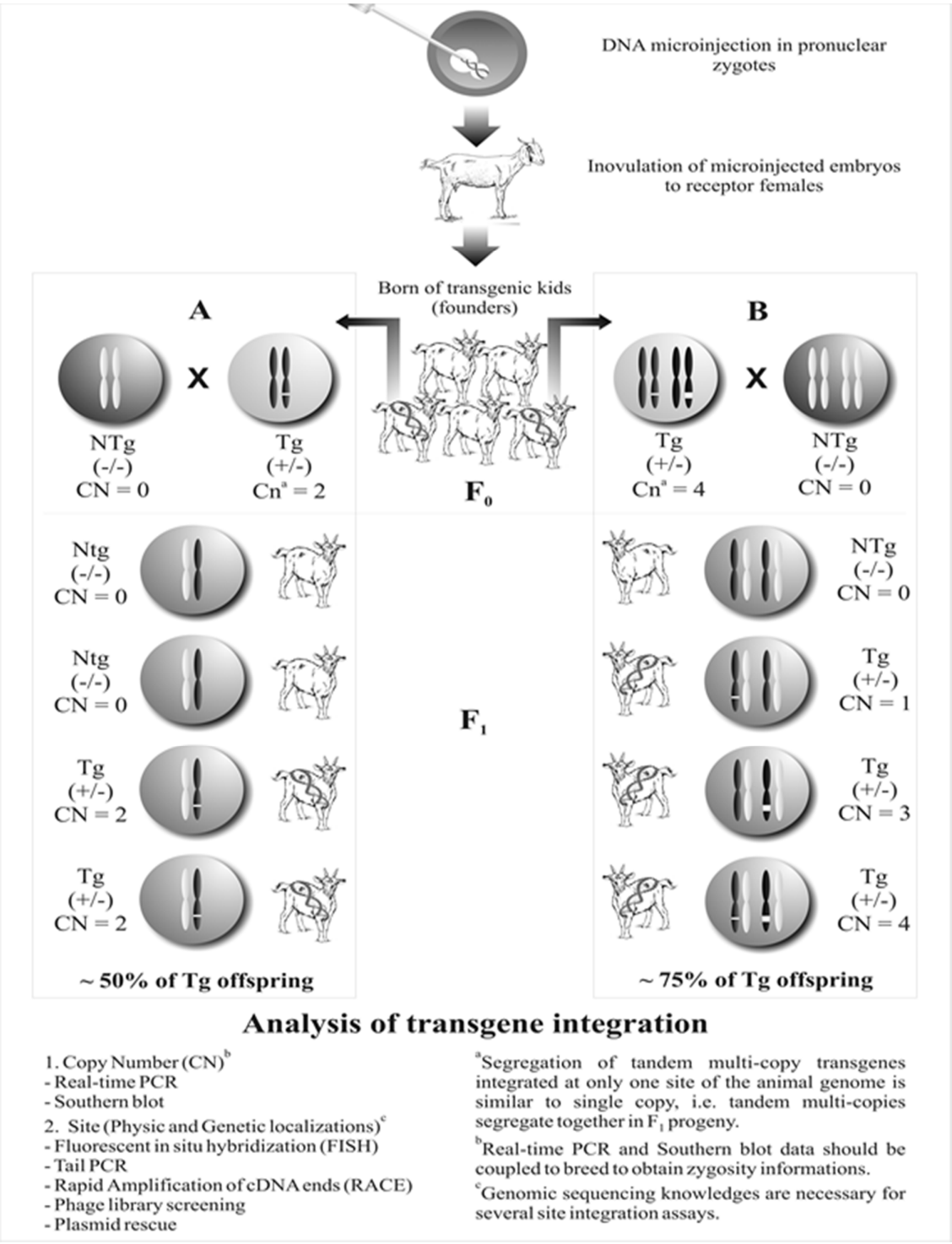

Figure 1. Schematic representation of transgenic animal production by pronuclear microinjection, segregation of the transgene in F1 lineage and methods to analysis of transgenic integration.

REAL TIME QUANTITATIVE PCR (qPCR)

qPCR analysis has been widely used for its ability to detect very low quantities of a nucleic acid sequence with very high specificity, due to the high temperature used when annealing the specific primers to the target sequence during the PCR, in comparison with the lower stringency applied for probe hybridization in Southern blotting (Guénin et al., 2009). The high sensitivity is conferred by the exponential nature of the PCR reactions, which enable specific sequences to be detected in samples even if only a few copies are present.

Didactically qPCR technique can be divided into three steps: pre-qPCR procedures (such as DNA extraction and quantification), qPCR amplifications and post-qPCR procedures (mathematical and statistical data analysis) (Burkardt, 2000). The following topics describe these steps and the effects in final qPCR accuracy.

Pre-qPCR procedures – extraction and quantification of DNA

The aim of a nucleic acid extraction method is to isolate DNA of suitable integrity, purity and of sufficient quantity for diagnostic applications by qPCR (Terry et al., 2002). This is because obtaining DNA of high quality is paramount for ensuring confidence in all subsequent steps in the process of generating analytical measurements. However, the quality of the template DNA obtained can vary according to the extraction method used, thereby influencing the accuracy of the quantification.

The basic phenol/chloroform extraction buffer consists of 100 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0, 200 mM NaCl, 0.2% sodium dodecyl sulfate (SDS) and 0.1 mg/mL proteinase K (molecular biology grade). The phenol/chloroform/isoamyl alcohol (25:24:1) mix is used to remove proteins and polysaccharides and ammonium acetate and ethanol to DNA precipitation (Tesson et al., 2002). For real-time PCR use, RNA is removed from the nucleic acid preparation with enzymes, as RNase A and RNase T1. According to published reports by

Demeke et al. (2009) and Corbisier et al. (2007), phenol/chloroform extracted DNA needs further purification to be used for real-time PCR. The purification of phenol/chloroform extracted DNA with a Genomic Tip 20 column results in a linear calibration curve and a leads to better reproducibility of results (Corbisier et al., 2007). Nevertheless, some studies have succeeded to perform the copy number quantifications and zygosity analysis using this method without purification provided for amplification by real-time PCR (Ballester et al., 2004; Tesson et al., 2002). However, according to our experiments, gDNA obtained using methods based on a silica matrix are more efficient for amplification by qPCR.

Inefficient PCR amplification may occur if an insufficient quantity of DNA is used or may be due to the presence of a series of inhibitors (Table 1). Inhibitors in the DNA can reduce the efficiency and/or reproducibility of PCR and thus may contribute to inaccurate qPCR results. The inhibitory mode of action of some of these compounds may be linked with DNA precipitation and denaturation or the ability of the polymerase enzyme to bind to magnesium ions (Rossen et al., 1992). Inhibitors may kinetically modify PCR amplification by chelating Mg^{2+}, a cofactor for all DNA polymerases, including Taq polymerase, or by binding to target DNA or the DNA polymerase (Wilson, 1997). Enzyme inhibitors may originate from either the animal tissue or the reagents used for DNA isolation (Burkhart et al., 2002). An inhibition test using either internal controls or evaluation of the linearity of the calibration curves should be performed to determine the suitability of the extracted DNA for real-time PCR amplification (Lipp et al., 1999; Corbisier et al., 2007).

Prior to qPCR, stock DNA extracts are commonly quantified and diluted so that all reference and test samples contain identical amounts of this nucleic acid. An accurate determination of DNA concentration in a sample is a critical component for analysis of copy number and zygosity by qPCR. In general, DNA quantification prior to qPCR increases confidence in negative PCR results, where insufficient target DNA could otherwise be interpreted as a false

negative. Two principal methods used for DNA quantification are UV spectrophotometry (absorbance 260 nm – A260) and fluorometry. According to Shokere et al., (2009) A260 and fluorescent–dye methods of quantifying intact genomic DNA provide relatively concordant DNA quantification values. However, the quantification values differ significantly for an identical DNA extract that has been degraded with its non-degraded counterpart. This study revealed that A260 values overestimate by an average of 20.3% (± 6.1) and fluorescent–dye methods underestimate by an average of 145.8% (± 6.0) the DNA concentration of PCR-amplifiable intact DNA extracts. Furthermore, when fluorescent–dye methods of DNA quantification were compared with A260 methods, an average percent difference of 10.1% (± 6.3) was reported for intact genomic DNA, but a much more significant percent difference of 152% (± 10.3) was reported in degraded genomic DNA.

qPCR amplifications – Quantitative Strategies

Target nucleic acids can be quantified using two-strategy absolute or relative quantification. The absolute quantification determines the absolute amount of target, whereas relative quantification determines the ratio between the gene target amounts in two samples (an unknown and another previous quantified, named calibrator) (Wong and Medrano, 2005). All strategies demand to plot standard curves for mathematical validation of gene amplifications. A standard curve is generated using a dilution series of at least five different concentrations of the DNA template (Livak and Schmittgen, 2001). The most important parameters calculated with the plots are: 1) linearity (Pearson correlation coefficient, R^2) – must to be greater than 0.96 and it is also important to make PCR reactions with DNA amounts that are within the linear range of amplification. 2) Efficiency (E) – must to be close or equal to 1.0 (ideal values are between 0.9 and 1.1) (Smith and Osborn, 2009).

Table 1. Examples of PCR inhibitors reported and methods to minimize inhibition.

Inhibitors	Description and inhibitory concentration	Methods to minimize inhibition
EDTA	≥ 0.5 mM 1 mM (Rossen et al., 1992)	Reduce the concentration of EDTA to 0.1 mM in the TE buffer or simply use Tris-HCl (10 mM) to bring DNA in solution. DNA can also be brought in pure water (but the DNA cannot be stored for long-term use)
Ethanol	>1% (v/v) (Weyant et al., 1990)	Dry pellet and resuspend
Isopropanol		Dry pellet and resuspend (Terry et al., 2002)
Protein	1% casein hydrolysate in PCR mixture caused inhibition (Rossen et al., 1992)	Use SDS or guanidinium buffers, proteinase K
	Proteinase K (Burkhart et al., 2002)	Non proteinase K based genomic DNA isolation method (Burkhart et al., 2002)
Detergents	SDS (Weyant et al., 1990)	Wash with 70% ethanol
Sodium acetate	≥ 5 mM (Peist et al., 2001)	Wash with 70% ethanol
Sodium chloride	≥ 25 mM (Peist et al., 2001)	Wash with 70% ethanol or use silica-based purification (Terry et al., 2002)

Absolute quantification

Absolute quantification (Figure 2) can be achieved by a relation of the C_T measurement to a standard curve that can be obtained by diluting a standard DNA sample (as a plasmid) with the transgene sequence for which the exact DNA concentration and molecular weight is known (Pfaffl and Hageleit, 2001). The C_T values can thereby be related to a distinct number of plasmids and with the knowledge of the molecular weight of the haploid animal genome, the number of molecules represented by a certain amount of animal DNA can be estimated (Bubner and Baldwin, 2004).

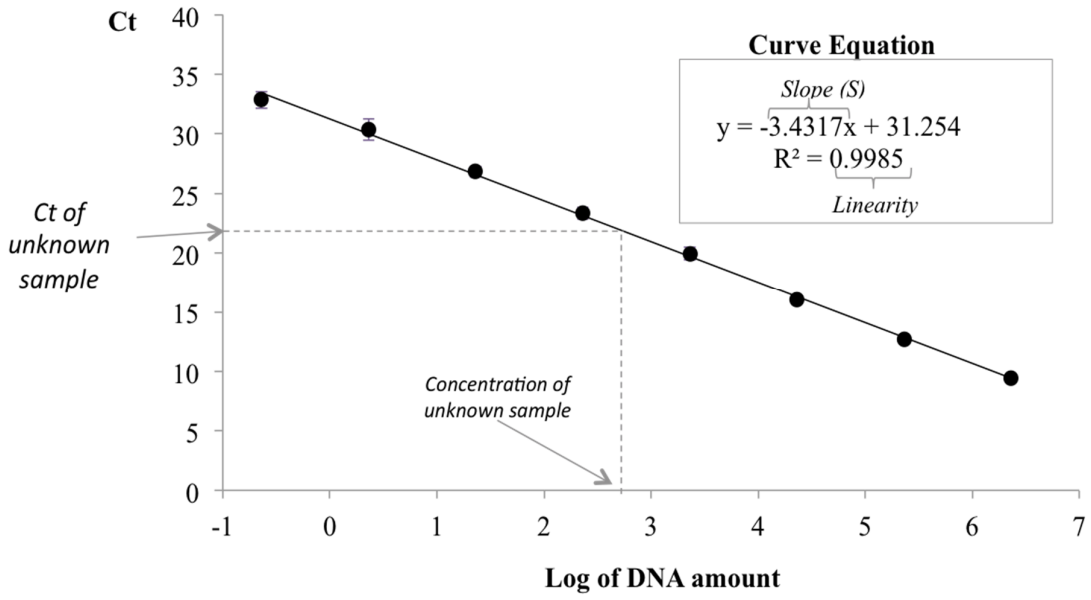

Figure 2. Typical standard curve and principles of absolute quantification strategy. The CT values were plotted versus DNA amounts used for qPCR amplifications. The slope of the tendency curve achieved by linear regression is used to determine the efficiency of qPCR. The Pearson correlation coefficient (R2) is the linearity and should be close near to the unit (or 100%). Determination of a sample of interest (unknown sample) is performed by extrapolating the CT value on the standard curve.

The amount of unknown target should fall within the range tested. Amplification of the standard dilution series and of the target sequence is carried out in separate wells. The C_T values of the standard samples are determined. Then, the C_T value of the unknown sample is compared with the standard curve to determine the amount of target in the unknown sample. A given number of animal DNA molecules yield the same C_T value as the same number of plasmids, if all molecules contain one copy of the transgene (i.e., if the animal is homozygous). For heterozygous animal with only half of the molecules containing the expression cassette, the C_T value will count for half of the number of plasmids. This method was validated by Schmidt and Parrott (2001).

The advantages of this method are that large amounts of standard that can be produced, its identity can be verified by sequencing and DNA can easily be quantified by spectrophotometry or fluorometry. Plasmid standards should be linearized since the amplification efficiency of a linearized plasmid often differs from that supercoiled conformation and more closely simulates the amplification efficiency of genomic DNA. Additionally, due to variations in inhibitor levels of qPCR between tissues is recommended also the use of plasmids mixed with genomic DNA samples of non-transgenic animals to delineate the curves in order to simulate possible interferences of each tissue (Joshi et al., 2008).

The main advantage of this approach to quantification is that there is no need to use a calibrator (a sample for which the copy number is exactly known, typically from a Southern blot). However, the accuracy of this strategy is directly associated with the precision in DNA quantification. As discussed above, currently, the main DNA quantification methods are spectrometry (A260 nm) and fluorometry, which can, respectively, overestimate and underestimate the real amounts of DNA. Consequently, these matters will reflect in the C_T values, compromising the final result (Demeke and Jenkins, 2010). This imprecision in the

final result can be even more pronounced when the plasmids are used to construct the standard curve, due to the small mass of this deoxyribonucleic acid (small errors in the quantification reflect in large variations in the C_T).

Several groups have used this method successfully for the characterization of transgenic animals (Table 2). With this method, Kong et al. (2009) determined the correlation between copy number and transgene expression using transgenic pigs for green fluorescent protein (GFP) as model. Interestingly, there was a reduction in the transgene copy number of these animals over the life. This result was further confirmed with the technical Southern bloth.

Relative quantification by standard curves

For copy number and zygosity analyses, absolute quantification of the physical amount of transgene copies is not necessary; relating the CT signals to relative standard curves of serial dilutions of the target gene and an endogenous control gene is sufficient to determine if a sample contains as much target as a certain dilution step of the standard (Pfaffl, 2001). Normalization is performed by dividing the dilution equivalent of the transgene by the dilution equivalent of the endogenous control (Pfaffl et al., 2004). The unit-less normalized dilution equivalents require a sample that serves as calibrator. A good calibrator for quantifying copy number is a homozygous animal with one copy of the transgene. Samples with half the normalized dilution equivalent used as the calibrator are heterozygous; samples with the same normalized dilution equivalent are homozygous for one copy, and so on. The quantification procedure differs depending on whether the target and the endogenous reference gene are amplified with comparable or different efficiencies. This strategy has been used mainly to determine the zygosity of transgenic animals (Shitara et al., 2004; Ji et al., 2005; Haurogné et al., 2007).

Table 2. Technical details in DNA extraction, quantification and real-time PCR for copy number and zygosity analysis of transgenic animals.

	Copy number				**Zygosity**			
	Joshi *et al.* (2008)	Ballester *et al.* (2004)	Chandler *et al.* (2007)	Kong *et al.* (2009)	Haurogné *et al.* (2007)	Shitara *et al.* (2004)	Tesson *et al.* (2002)	Ji *et al.* (2005)
Species	*Mus musculus*	*Mus musculus*	*Mus musculus*	*Sus domesticus*	*Mus musculus*	*Mus musculus*	*Mus musculus*	*Danio rerio*
Tissue for DNAg extraction	liver and lung	liver	tail	Fibroblast cells	tail	tail	tail	Tail
DNAg extraction method	P.C.	P.C.	P.C.	Universal Genomic DNA Extraction Kit Ver.3.0	phenol–chloroform protocol	phenol–chloroform protocol	phenol–chloroform protocol	DNeasy Tissue Kit (Qiagen)
Quantification method	UV spectrophotometry at 260 nm	N.E.	UV spectrophotometry at 260 nm	--	UV spectrophotometry at 260 nm	UV spectrophotometry at 260 nm	UV spectrophotometry at 260 nm	UV spectrophotometry at 260 nm
Reference gene	β-actin	Glucagon	*Jun* gene	TFRC	Mouse gap junction channel protein alpha 5	--	Rat hypoxanthine phosphoribosyltransferase (rHPRT)	Gene bank AC087105
qPCR chemistry	SYBR Green	TaqMan	TaqMan	SYBR Green	GeneAmp 5700 SYBR Green	SDS 7900 CYBR Green	SDS7700 TaqMan	SDS7700 TaqMan
Normalization	DNA quantification	Endogenous control	DNA quantification	DNA quantification	Endogenous control	Endogenous control	Endogenous control	Endogenous control
Quantification	Absolute	$2^{-\Delta\Delta Ct}$	Absolute	Absolute	$2^{-\Delta\Delta Ct}$	$2^{-\Delta\Delta Ct}$	$2^{-\Delta\Delta Ct}$	Relative standard curve
Calibrator	N.A.	*Mus musculus*	N.A.	N.A.	Animal of known zygosity	Animal of known zygosity	Animal of known zygosity	Animal of known zygosity
Quantification method of calibrator	N.A.	Southern blot	N.A	N.A	--	FISH	Southern blot	FISH

N.A. = Not applicable. P.C.= Protocol based on phenol–chloroform method. P. DNAg extraction kit based on spin technology (TaKaRa).

Relative quantification by comparative Ct method

The comparative CT is the most used relative quantification method for several purposes, including transgene analysis (Ballester et al., 2004; Bubner and Baldwin, 2004). However, the validation of comparative CT for transgene copy quantification requires a previous comparison between standard curves plotted for the transgene (target) and the reference gene. Thus, the efficiency ($E = 10^{(-1/Slope)}$) of both transgene and reference gene amplifications must be high similar. A simple way to determine what relative quantification method can be applied is to plot ΔCT values (calculated as the difference between target and reference gene CTs) versus log of DNA amounts. The comparative CT method can be used if the slope of this plot is between -0.1 and 01 (Figure 3). However, if the slope is out of this range, the indicated method should be the relative quantification by standard curves.

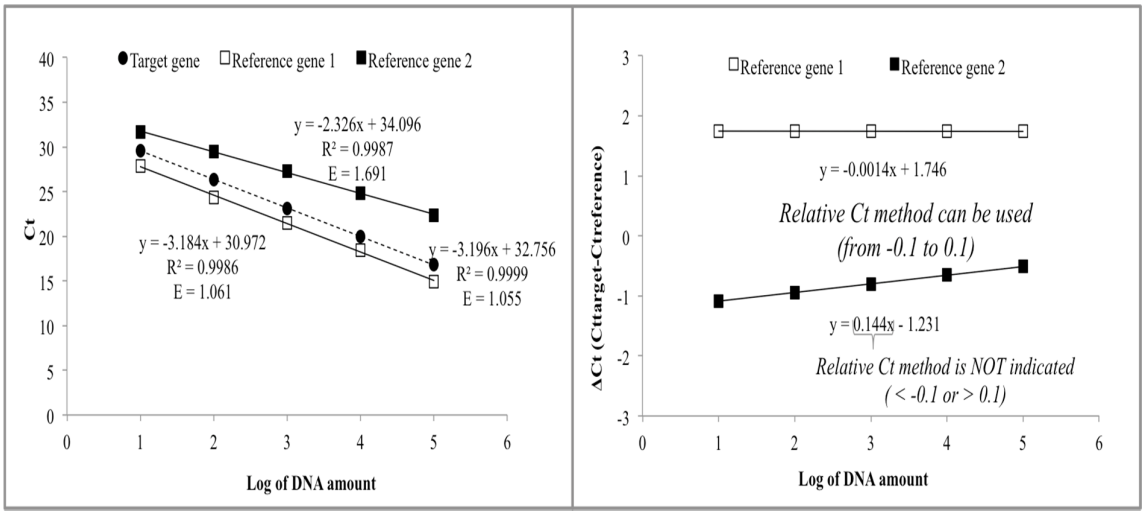

Figure 3. Determination of real-time PCR efficiencies from the slopes of the calibration curve. To compare the amplification efficiencies of the 2 target sequences, the CT values of reference gene 1are subtracted from the CT values of target gene. The difference in CT values is then plotted against the logarithm of the template amount. If the slope of the resulting straight line is < 0.1, amplification efficiencies are comparable.

While requiring an endogenous control and a calibrator, differs from the relative standard method by relying on equal PCR efficiencies with the transgene and the endogenous control genes. The preparation of standard curves is only required to determine the amplification efficiencies of the transgene and endogenous control genes in an initial experiment. In all subsequent experiments, no standard curve is required for quantification of the target sequence. According to Livak and Schmittgen (2001), if all amplicons amplify with the same efficiency, the difference ΔCT between the CT for the transgene (CTt) and the CT for the endogenous control (CTe) is constant, independent of the amount of chromosomal DNA (ΔCT = CTt – CTe). As for quantification with relative standards, a calibrator is a homozygous one-copy animal. Thus, all samples with the same ΔCT as the calibrator contain one copy of the transgene. More generally, the ratio of the initial amount of transgene in the sample (Xs) to the initial amount of transgene in the calibrator (Xcal) can be calculated as follows ($Xs/Xcal = (1+E)^{-\Delta\Delta CT}$), where: ΔΔCt = ΔCTs – ΔCTcal. Whereas for copy number calculation ΔΔCTs will be zero (one-copy animals) or negative (multi-copy animals), zygosity analysis should yield ΔΔCTs of zero (homozygous) or one (heterozygous). As long as the efficiencies for transgene and endogenous control are the same, calculations with E<1 are also possible. The $2^{\Delta\Delta CT}$ method is simple to apply, because DNA concentrations do not have to be measured. Its utility has been demonstrated for animal copy number determination (Ballester et al., 2004) and zygosity analysis in animals (Tesson et al., 2002).

Post-qPCR procedures – Mathematical and Statistical Considerations

Some studies have reported that two-fold differences are the detection limit for determining transgene copy numbers in genetically modified organisms by qPCR, due to high variation in the values of CT (Bubner and Baldwin, 2004; Bubner et al., 2004) and use of no appropriate statistical models for analyzing the results (Yuan et al., 2007).

According to Bubner and Baldwin (2004), when the standard deviation of CT values for all samples and amplicons is below 0.3, precise copy numbers will be possible; if it is higher, the interpretation of copy numbers will be difficult. Normally, small fluctuations in the starting conditions of a PCR assay will lead to large fluctuation of the product amount, which is expressed in CT values (or equivalents). Thus, seemingly small standard deviations of CT values (between 0.3 and 1) are amplified in the analysis because a CT difference of one represents a two-fold difference in starting amount. In addition to the variability of the transgene measurements, the variability of the endogenous control measurement must also be considered (Bubner et al., 2004).

On the other hand, some authors have suggested that the qPCR based transgene copy number quantification tends to be ambiguous and subjective stemming from the lack of proper statistical analysis and data quality control to render a reliable quantification of copy number with a prediction value. Despite the recent progresses in statistical analysis of real-time PCR, few publications have integrated these advancements in qPCR based transgene copy number quantification. Explicit hypothesis testing has been seldom invoked for transgene copy number, and a predetermined and clear P value and confidence levels of estimation have not been specified; i.e. copy number = 1; $P < 0.05$ (Yuan et al., 2007).

Confidence levels are especially important for qPCR based transgene copy number determination, since it defines the precision and sensitivity of the assay. In case that confidence interval spans two integers, say, 1 and 2, it will be difficult to determine the actual copy number. Because of the limitations on statistical procedures, the results of analysis are often ambiguous and without clear confidence intervals. Moreover, robust quality control associated with real-time PCR analysis is also seldomly presented and has even been discouraged (Prior et al., 2006).

Recent advances in qPCR statistical analysis have provided an opportunity for improving qPCR based transgene copy number estimation with rigorous quality control and robust hypothesis testing. In this context, Yuan et al. 2007 proposed statistical methods allow the qPCR-based transgene copy number quantification to be more reliable and precise. According to the authors proper confidence intervals are necessary for unambiguous prediction of trangene copy number.

CONCLUSIONS

Therefore, this review allows us to conclude that real-time PCR is a powerful tool for the characterization of transgenic animals, especially for copy number determination and zygosity analysis. This quantitative technique has the potential to become a widespread tool in animal transformation research, because it helps to characterize the lines, to infer or to explain transgene expression levels and to drive the reproductive managements for livestock establishment. However, the choice of the quantitative PCR method must be accompanied by appropriate validations, ensuring that the measurements are correct and adjusted to the experimental conditions (genes, primers, templates, temperatures). Finally, specific transgenic animal lines that are considered for research or commercial release probably will require the confirmation of both copy number and zygosity data by independent methods, such as Southern blot and mating, respectively. Hence, real-time PCR is a tool that complements rather than replaces traditional procedures.

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3 JUSTIFICATIVA

A neutropenia é o termo utilizado para caracterizar a redução na contagem absoluta de neutrófilos. Essa redução pode ser causada por distúrbios tais como: doenças auto-imunes sistêmicas (artrite reumatóide e lupus eritematoso), doenças infecciosas (SIDA e tuberculose), neutropenia devido à leucemia (DALE *et al.*, 2003) e a quiomioterapia (CREA *et al.*, 2009). Essa redução no número de neutrófilos circulantes é uma das complicações mais frequentes em pacientes submetidas à quimioterapia, tornando-as mais suscetíveis as infecções hospitalares potencialmente letais. Segundo Crea *et al.* (2009), a sepse é a causa mais comum de morte prematura entre pacientes com câncer, sendo que o risco de infecção é estritamente correlacionado com o grau e a duração da neutropenia.

Durante várias décadas, o único tratamento para neutropenia induzida por quimioterapia é antibioticoterapia geralmente limitada à pacientes com febre, empregado no sentido de combate e não de prevenção. Posteriormente, vários estudos demonstraram que a administração de G-CSF recombinante reduz a duração e o grau de neutropenia após a quimioterapia intensiva em pacientes com câncer, atuando de forma profilática, na prevenção de neutropenia febril (revisado por CREA *et al.*, 2009). O G-CSF atua estimulando a neutropoiese e a função de neutrófilos circulantes, aumentando assim a resposta antimicrobiana dos pacientes (DEMETRI e GRIFFIN, 1991). Baseado nisso, as diretrizes atuais recomenda o uso de G-CSF em pacientes, com risco substancial de neutropenia febril (CREA *et al.*, 2009).

Atualmente, o hG-CSF recombinante é produzida no sistema bacteriano e células de ovário de hamster chinês (células CHO) e comercializados com o nome de *filgrastim* e *lenograstim*, respectivamente. A proteína obtida neste ultimo, como se trata de um sistema de expressão de célula de mamífero, é indistinguível da proteína natural (humana G-CSF). No entanto, a necessidade de grandes investimentos de capital, altos custos operacionais e níveis

relativamente baixos de produção resulta na incapacidade de sistemas de células CHO produzirem mais que alguns quilos de proteína por ano (JAYAPAL *et al,* 2007; HOUDEBINE, 2009; BALDASSARE, 2012), o que elava o significativamente o custo desse fármaco no mercado. Como alternativa, a plataforma de animais transgênicos para a expressão de hG-CSF na glândula mamaria foi proposto como um método viável para sua produção em escala comercial (DVORYANCHIKOV *et al*., 2005; KO *et al*., 2000; SEROVA *et al*., 2012).

Assim, em 2012 nosso grupo relatou a produção de duas linhagens (10M e 12F) de caprinos transgênicos para expressão de hG-CSF na glândula mamaria, com o objetivo de produzir essa proteína recombinante em escala comercial (FREITAS *et al*., 2012). Contudo, previamente a está etapa a caracterização das linhagens quanto a capacidade de transmitir o transgene a seus descendentes, a expressão correta da molécula de interesse e numa concentração compatível para que o investimento de tempo e dinheiro seja comercialmente viável (WALL *et al*., 1997), é um passo fundamental. Assim, no presente estudo propomos caracterizar genotípica (número de cópias do transgene) e fenotípicamente (parâmetros produtivos e reprodutivos, desempenho lactacional e expressão do transgene) as duas linhagem relatada por Freitas et al. (2012). Adicionalmente os aspectos associado a saúde desses animais também foram avaliados em diferentes momentos de vida como sugerido por Van Reenen (2009).

4 HIPÓTESES CIENTÍFICAS

- A presença do transgene hG-CSF no genoma de caprinos, obtidos por microinjeção pronuclear, não compromete os parâmetros produtivos, sanitários ou reprodutivos desses animais;
- A geração F1 de caprinos transgênicos para hG-CSF, expressam a proteína recombinante na glândula mamaria;
- A expressão do transgene hG-CSF na glândula mamaria da geração F1 não compromete a fisiologia da lactação, composição do leite e perfil leucocitário;
- A geração F1 de caprinos transgênicos para hG-CSF, não apresentam expressão ectópica nos linfócitos, linfonodo, fígado e baço.

5 OBJETIVOS

5.1 OBJETIVO GERAL

- Caracterizar genotípica e fenotipicamente caprinos transgênicos expressando o fator estimulante de colônias de granulócitos humano (hG-CSF) na glândula mamária.

5.2 OBJETIVOS ESPECÍFICOS

- Determinar o número de cópias do transgene (hG-CSF) no genoma do caprino fundador e na geração F1, através de qPCR em tempo real;
- Avaliar o efeito da inserção do transgene nos parâmetros produtivos, reprodutivos e na proliferação e diferenciação das células granulocítica na linhagem F1;
- Avaliar o desempenho da lactação induzida hormonalmente e a composição de leite das fêmeas F1 transgênicas para expressão de hG-CSF no leite;
- Avaliar a presença e o perfil de expressão de hG-CSF no leite das fêmeas F1 transgênica;
- Analisar o perfil leucocitário das fêmeas F1 transgênica durante a expressão a lactação induzida;
- Avaliar expressão ectópica no linfonodo, baço e fígado de machos transgênicos por PCR em tempo real.

6 CAPÍTULO 1

Estratégias Metodológicas para Quantificação de Número de Cópias do Transgene em Caprinos (*Capra hircus*), utilizando PCR em Tempo Real

Resumo

Considerando a importância de cabras como modelos transgênicos, bem como a raridade de estudos de número de cópia (CN) em animais de produção, o presente trabalho teve como objetivo avaliar as estratégias metodológicas utilizando qPCR para a precisa e exata quantificação de CN de transgene em caprinos. Foram utilizados linhagens camundongos e caprinos transgênicas para factor estimulante colônias de granulócitos humanos. Após selecionar o melhor método de extração de DNA genômico a ser aplicada em amostras de camundongos e caprinos, as variações intra-ensaio, exatidão e precisão de quantificações CN foram avaliadas. As condições otimizadas foram submetidas a estratégias matemáticas e usadas para quantificar CN nas linhas caprina. Os resultados foram os seguintes: validação de condições qPCR é necessário, e a eficiência de amplificação é o mais importante. Quantificações absolutas e relativas são capazes de produzir resultados semelhantes. Para quantificação absoluta normalizada, o mesmo fragmento de plasmídeo usado para gerar linhas de caprinos deve ser misturado com o DNA genômico de animais selvagem, permitindo a escolha de um gene endógeno de referência para a normalização dos dados. Para quantificações relativas, um método de extração de DNA genômico à base de resina é fortemente recomendado ao usar cauda de rato como calibradores para evitar inibidores de tecidos específicos. Eficiências de amplificações de qPCR (≥95%) permitem medições confiáveis de CN com tecnologia SYBR. TaqMan deve ser utilizada com precaução em caprinos, se a sequência nucleotídica do gene de referência endógena ainda não é bem

conhecido. Aderindo a estas orientações gerais podem resultar em determinação CN mais exata em caprinos. Mesmo quando se trabalha em circunstâncias não ideais, se ensaios são realizados respeitando os requisitos mínimos de qPCR, boas estimativas do CN do transgene podem ser alcançadas.

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Methodological Strategies for Transgene Copy Number Quantification in Goats (*Capra hircus*) Using Real-Time PCR

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Abstract

Taking into account the importance of goats as transgenic models, as well as the rarity of copy number (CN) studies in farm animals, the present work aimed to evaluate methodological strategies for accurate and precise transgene CN quantification in goats using qPCR. Mouse and goat lines transgenic for human Granulocyte-Colony Stimulating Factor were used. After selecting the best genomic DNA extraction method to be applied in mouse and goat samples, intra-assay variations, accuracy and precision of CN quantifications were assessed. The optimized conditions were submitted to mathematical strategies and used to quantify CN in goat lines. The findings were as follows: Validation of qPCR conditions is required, and amplification efficiency is the most important. Absolute and relative quantifications are able to produce similar results. For normalized absolute quantification, the same plasmid fragment used to generate goat lines must be mixed with wild-type goat genomic DNA, allowing the choice of an endogenous reference gene for data normalization. For relative quantifications, a resin-based genomic DNA extraction method is strongly recommended when using mouse tail tips as calibrators to avoid tissue-specific inhibitors. Efficient qPCR amplifications (≥95%) allow reliable CN measurements with SYBR technology. TaqMan must be used with caution in goats if the nucleotide sequence of the endogenous reference gene is not yet well understood. Adhering to these general guidelines can result in more exact CN determination in goats. Even when working under non-optimal circumstances, if assays are performed that respect the minimum qPCR requirements, good estimations of transgene CN can be achieved.

Keywords: SYBR Green, TaqMan, Real-Time PCR, Transgenic mice, Copy number

Introduction

The transgenic animal platform in which a recombinant protein is expressed in the mammary gland of a genetically modified farm animal emerged in the late twentieth century as a promising approach for obtaining valuable and therapeutically interesting drugs.[1] In this context, due to the equilibrium between the maintenance cost, milk production and precocity, goats (*Capra hircus*) became one of the first choices of a model for animal transgenesis,[2] as indicated by the variety of human recombinant proteins produced by the mammary gland and secreted in the milk of transgenic goats, such as antithrombin,[3] lysozyme,[4] lactoferrin[5] and granulocyte-colony stimulating factor (hG-CSF).[6] In addition, the European Medicines Agency (EMA) announced approval of the first drug produced in an animal bioreactor: GTC Biotherapeutics' ATryn® (recombinant human antithrombin).[7]

Traditional techniques used to produce transgenic goats randomly integrate the foreign gene into a chromosome of the founder, usually in multiple copies.[8] Thus, genotypic characterization of the line becomes a critical step in a transgenesis program because parameters such as the site and number of exogenous DNA insertions may affect the expression and stability of the transgene.[9] Traditionally, Southern blotting is the method used to estimate the transgene copy number (CN)[10]; however, this requires large quantities of high-quality DNA.[11,12]

Real-time quantitative PCR (qPCR) has been presented as a rapid, sensitive and accurate method for the quantification of transgene CN in both plants and animals.[13,14] To realize the enormous potential of this technique, it is extremely important that the results be accurate and reproducible, which requires the standardization of all qPCR steps, including the performance of appropriate pre-analytical analysis, optimized reactions and accurate post-qPCR data processing.[15,16]

Estimation of transgene CN by qPCR is performed using either absolute or relative quantification strategies.[12] The absolute quantification approach measures CN based on a calibration curve constructed by serial dilution of plasmids containing the transgene,[14] while relative quantifications require a calibrator sample, usually genomic DNA (gDNA) of a transgenic specimen whose CN is known.[11] When working with a transgenic farm model, a mouse line harboring the same DNA construct is usually available[17] and can be used for calibration purposes in the qPCR reactions. In this context, it is important to determine whether additional methodological standardizations are required, i.e., to ensure that reaction efficiencies are similar for both the target and control genes in the studied species. Thus, the present work aimed to map methodological strategies for accurate and precise transgene CN quantification in goats by qPCR.

Material and Methods

Ethics Statement

This study was carried out in strict accordance with the guidelines for the ethical use of animals in research.[18] The protocol was approved by the Animal Ethics Committee of the State University of Ceará (CEUA/UECE 12/05) and Brazil's Biosafety Technical National Committee (CTNBio 228/06).

Animals and Tissue Samples

Two goat (10M, n = 7 and 12F, n = 3) and one mouse (#78, n = 9) hG-CSF-transgenic lines were used to obtain gDNA samples. As previously reported by our group,[6] transgenic founder goats (a male for 10M line and a female for 12F line) were bred with non-transgenic goats to obtain first generation specimens (5 males and 4 females). Goat and mouse tissue samples were obtained from ear biopsies and tail tips, respectively. Transgenic goat samples were

collected from both the founders and first generation. The mouse line (5 males and 4 females) was obtained as described previously.[19] Non-transgenic mouse (3 males and 3 females) and goat (3 males and 3 females) samples were also used.

Experimental Design

To establish the best qPCR strategy (or strategies) to quantify transgene CN in the goat genome, the present investigation was divided into three parts. The first experiment aimed to select the best method for gDNA extraction to be applied to both mouse and goat tissue samples. At this step, the level of interference generated by gDNA samples on qPCR amplification was evaluated. Thus, both mouse and goat samples of non-transgenic specimens were obtained by three different gDNA extraction methods (K1, K2 or P). Plasmid DNA (43 fg) containing the same hG-CSF construction as that present in both the mouse and goat genomes was mixed with 40 ng gDNA and submitted to transgene (TgCD region) and reference gene (glucagon, Gluc) qPCR amplifications. Both exogenous (hG-CSF) and endogenous (Gluc) genes were evaluated based on the derivative melting curve of the amplicons. In the second experiment, intra-assay variations as well as the accuracy and precision of CN quantifications were assessed by qPCR reactions with two template amounts achieved from the #78 line specimens using the gDNA extraction method chosen in the first step. Additionally, the PCR conditions were quantitatively analyzed through the construction of standard curves with two ranges of template concentrations using both SYBR Green and TaqMan technologies. Finally, in the last part, different mathematical strategies were used to quantify CN in the goat genome using the conditions selected in the second assay and applied to 10M and 12F specimens. The normalized absolute quantification was performed using plasmid DNA, while all relative quantifications (comparative Ct, efficiency correction or standard curve) used line #78 as a calibrator.

gDNA Extraction

The gDNA samples were extracted from 20-30 mg mouse tips or goat ear biopsies by both resin (K1) and protein precipitation (K2) -based commercial kits or by the phenol-chloroform method (P). The K1 and K2 extractions were performed with the Wizard SV Genomic DNA Purification System Kit and Wizard Genomic DNA Purification (Promega), respectively, following the manufacturer's instructions. The phenol-chloroform gDNA extraction was performed as reported previously.[20] DNA samples were eluted or resuspended in 100 μL nuclease-free water and stored at -80°C until qPCR. Plasmid DNA was obtained as described previously.[6] DNA concentrations were accurately accessed by fluorimetry using the Qubit fluorimeter (Invitrogen) and Quant-iT dsDNA BR Assay Kit (Invitrogen) for gDNA or Quant-iT dsDNA HS Assay Kit (Invitrogen) for plasmid DNA following the manufacturer's instructions. Derivative melting curves for both TgCD and Gluc amplicons were plotted. Additionally, amplifications were analyzed for the cycle threshold (Ct) values and for the existence of a melting temperature (Tm) for the produced amplicons.

qPCR Reactions

The qPCR reactions consisted of 20 μL total volume containing 10 μL 2× FastStart Universal SYBR-Green Master Mix (Roche) or 10 μL 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 500 nM of each primer (Table 1), 250 nM probe (only for TaqMan technology) and 1 μL gDNA sample (at a final concentration that varied according to the experiment). The qPCR amplifications were performed in a Mastercycler ep Realplex 4S (Eppendorf) set for the following parameters when using SYBR-Green technology: 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s, 55ºC for 15 s and 60ºC for 30 s. TaqMan amplifications were performed under the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95ºC and 1 min at 60°C. Specificity was ascertained after completion of the amplification

in the presence of SYBR Green by a melting procedure (55-95°C, starting fluorescence acquisition at 55°C and taking measurements at 10 s intervals until the temperature reached 95°C). Amplifications of two different transgene regions (TgAB or TgCD) and the reference gene (Gluc) were performed in separate and independent reactions using primers (and probes for TaqMan reactions) synthesized by Integrated DNA Technologies. In the first experiment, only TgCD transgene regions were amplified.

Intra-assay Variation, Accuracy and Precision of CN Quantifications

Two standard curves (low and high template concentrations; LC and HC, respectively) for the transgene (TgCD or TgAB) and reference gene (Gluc) were constructed through triplicate qPCR amplifications with serial dilutions (factor of 5) of the DNA template constituted by non-transgenic mouse or goat gDNA (pool of six samples, 3 males and 3 females) mixed with the hG-CSF plasmid. The final template amount in the LC curve ranged from $5.1\times10^{+1}$ to 3.2×10^{-3} ng for gDNA and from 1.8×10^{-4} to 5.6×10^{-8} ng for plasmids. In the HC curve this variation was $7.6\times10^{+1}$ to 8.70×10^{-4} and 4.33×10^{-2} to 5.54×10^{-7} ng gDNA and to plasmids, respectively. Assay sensitivity was assessed only for LC curves and was expressed as the template copy number detected in triplicate reactions. Standard curves were also used to determine the linearity (R^2) and the efficiency (Equation 1)[21] of the qPCR amplifications. Linearity was expressed as the square of the Pearson correlation coefficient. Both LC and HC standard curves were plotted when using SYBR Green technology, while only LC curves were drawn when using TaqMan probes.

Equation 1: $E = 10^{\left(\frac{-1}{\text{slope}}\right)}$, where E is the qPCR efficiency.

Table 1. Oligonucleotides used to amplify two distinct regions of the transgene (TgAB and TgCD) and the reference glucagon (Gluc) gene in qPCR assays.

Target gene	Primers and probes	Sequence (5´- 3´)	Product size (pb)	Accession number
Gluc	gmGluc-SE	AACATTGCCAAACGTCATGATG	130	AY588290.1[a] NM_008100.3[b]
	gmGluc-AS	GCCTTCCTCGGCCTTTCA		
	Probe	5HEX/CATGCTGAA/ZEN/GGGACCTTTACCAGTGATG/3IABkFQ		
TgAB	TgAB-SE	GAGCTTCCTGGAGGTGTCGTA	96	X03656.1[c] X59856.2[d]
	TgAB-AS	CAAAGACCATAACTGTGGAGTCC		
	Probe	6-FAM/TCTTTCAGG/ZEN/GCTGGGCAAGGT/3IABkFQ		
TgCD	TgCD-SE	TCTTGACAACCATGGCTGGAC	65	AJ504710.2[e] X03656.1[c]
	TgCD-AS	GCCAAGACACTCACCCATCAG		
	Probe	6-FAM/TGCCACCACCCA/ZEN/GAGCCCCATGA/3IABkFQ		

[a]Goat glucagon gene, [b]mouse glucagon gene, [c]hG-CSF gene, [d]bovine casein gene, [e]goat casein gene. Transgene construction includes the hG-CSF gene and both bovine and goat casein genes.

The parameters of the standard curves obtained were used for quantification of the normalized absolute value (Abs) of CN (Equation 2)[21] and the number of the expressed transgene copies (an integer value) per haploid genome. In Equation 2, the superscripts *transgene* and *reference* refer to the transgene (TgAB or TgCD) and reference gene (Gluc) amplifications, respectively

Equation 2: $CN = 10^{\left[\left(\frac{Ct - Y_{intercept}}{slope}\right)^{transgene} - \left(\frac{Ct - Y_{intercept}}{slope}\right)^{reference}\right]}$

To determine the accuracy and precision of the CN quantifications, the transgene (TgCD or TgAB) and reference gene (Gluc) were amplified in 10 or 40 ng gDNA samples obtained from # 78 line mouse specimens. Additionally, Ct values were used to assess intra-assay variation. Finally, the gDNA concentration that produced the lowest Ct variations and the most accurate and precise CN measurements with SYBR Green technology was also used in TaqMan reactions.

The precision of CN quantifications was indicated by the population standard deviation (σ) and by the CN range. Accuracy was denoted by the average relative error (Equation 3) and trueness frequency (Equation 4), which were both calculated by comparison of the measured CN with the true CN value (the known CN is two copies per haploid genome for the mouse line #78).

Equation 3: $\text{Average relative error} = \left(\frac{\text{measured CN} - \text{true CN}}{\text{true CN}}\right) \times 100$

Equation 4: $\text{Trueness frequency} = \left(\frac{\text{number of observations equal to true CN value}}{\text{total number of observations}}\right) \times 100$

CN Quantifications in Goats

The CN in transgenic goat lines was calculated from triplicate qPCR reactions performed with 10 ng gDNA. Ct values and standard curve parameters ($Y_{intercept}$ and slope) for both transgene and Gluc amplifications were used for normalized absolute quantification (Abs), comparative Ct (DDCt), efficiency correction (Eff) or standard curve (StC) relative quantifications. Goat CN values are expressed as the number of transgene copies (an integer value) per diploid genome. The Abs quantification was performed as described for mice and calculated with Equation 2, but the result was multiplied by two (correction factor to obtain the CN per diploid genome in heterozygous specimens). For all relative quantifications, a single mouse specimen of line #78 was arbitrarily chosen as the calibrator. The DDCt method was calculated using Equation 5 for gene expression analysis.[21] To validate the DDCt calculations, plots of log input template amount versus ΔCt (difference between transgene and reference gene Ct value) for each animal species were also analyzed.

Equation 5*:* $CN = 2^{-(\Delta Ct_{goat} - \Delta Ct_{mouse})}$, where for each species $\Delta Ct = Ct_{transgene} - Ct_{reference\ gene}$

Equations 6 and 7 calculated the relative quantifications by the Eff [22] and StC[13, 23] methods, respectively.

Equation 6: $CN = \left[\frac{(E^{-Ct})^{transgene}}{(E^{-Ct})^{reference}}\right]^{goat} \div \left[\frac{(E^{-Ct})^{transgene}}{(E^{-Ct})^{reference}}\right]^{mouse}$, where the E and Ct values were calculated for both the transgene and reference gene in each animal species, and the superscript “mouse” refers to the calibrator.

Equation 7: $CN = \frac{10^{\left[\left(\frac{Ct-Y_{intercept}}{slope}\right)^{transgene} - \left(\frac{Ct-Y_{intercept}}{slope}\right)^{reference}\right]^{goat}}}{10^{\left[\left(\frac{Ct-Y_{intercept}}{slope}\right)^{transgene} - \left(\frac{Ct-Y_{intercept}}{slope}\right)^{reference}\right]^{mouse}}}$, where Ct, $Y_{intercept}$ and slope were calculated for both the transgene and reference gene in each animal species, and the superscript "mouse" refers to the calibrator. The precision of goat CN quantifications was indicated by σ and by the CN range as described for mice.

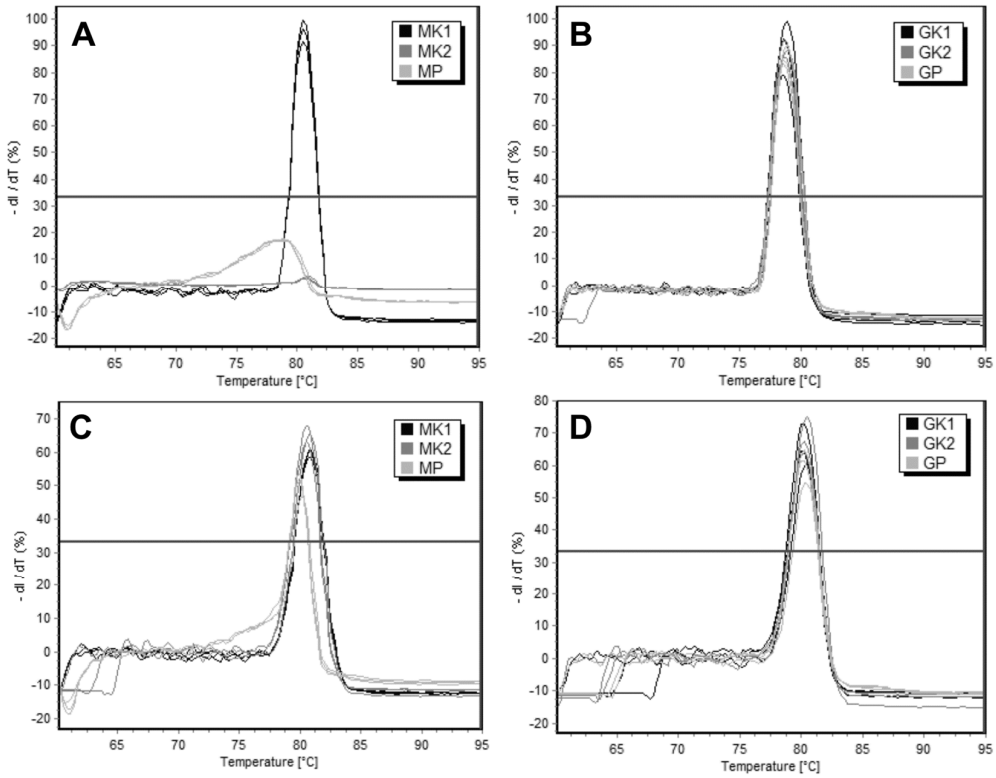
Statistical Analysis

Data are expressed as the mean ± *s* (sample standard deviation) for Ct values and mean ± σ (population standard deviation) for CN values. GraphPad Instat 3.06 software (GraphPad Software) was used for all statistical analyses. CN values were compared using one-way ANOVA followed by Tukey's test. A *P* value less than 0.05 was considered statistically significant.

Results

gDNA Extraction

The preliminary aim of the present investigation was to choose the best gDNA extraction method to be applied to mouse and goat tissue samples to quantify transgene CN by qPCR. Thus, the derivative melting curve and Tm of the TgCD and Gluc amplicons and the threshold cycle of the reactions performed with mouse or goat gDNA samples achieved by different methods (K1, K2 and P) are shown in Figure 1. All goat gDNA samples were able to produce qPCR amplicons (Figure 1B and Figure 1D). However, only the resin-based commercial kit (K1) produced mouse gDNA that was suitable for reference gene amplification (Figure 1A).



Species	gDNA extraction method	Threshold Cycle (mean±*s*)		Melting temperature (°C, mean±*s*)	
		Gluc	TgCD	Gluc	TgCD
Mouse	K1	18.71±0.20	15.40±0.21	80.5±0.06	80.8±0.06
	K2	NA	18.23±0.09	NA	80.7±0.11
	P	NA	34.87±0.53	NA	80.0±0.17
Goat	K1	19.35±0.15	15.94±0.04	78.7±0.11	80.2±0.15
	K2	19.75±0.02	16.12±0.06	78.7±0.06	80.3±0.17
	P	22.19±0.35	18.05±0.19	78.7±0.10	80.2±0.11

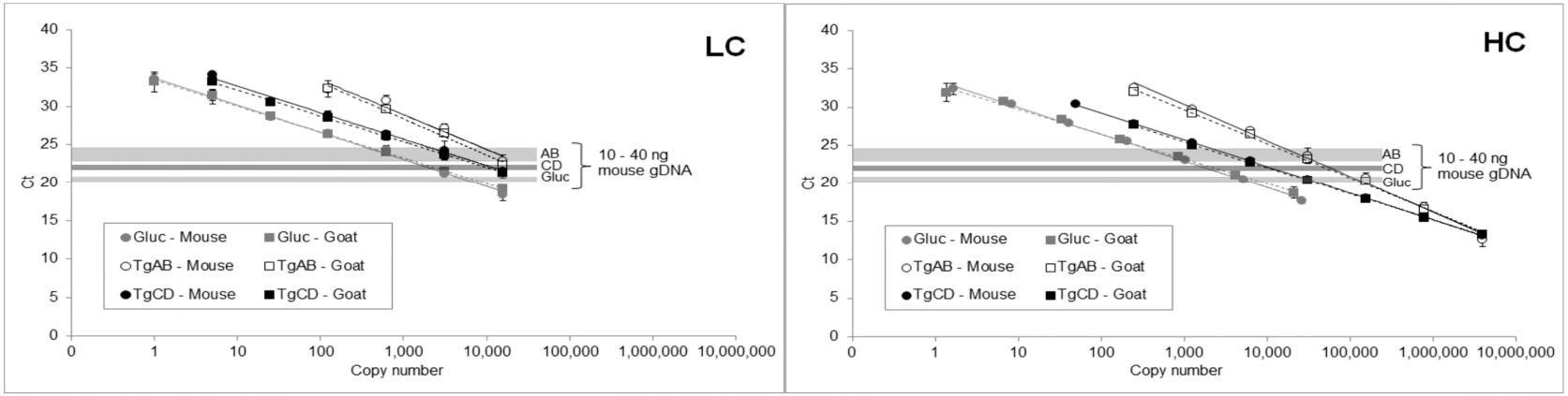
Figure 1. qPCR amplifications in mouse and goat gDNA extracted by different methods.

Derivative melting curve of amplicons of Gluc reference gene (A and B) and TgCD transgene (C and D) were plotted. Reactions were performed with mouse (A and C) or goat (B and D) gDNA obtained by the K1, K2 or P methods. The threshold cycle of reactions and melting temperature of amplicons are presented at the bottom of the figure. NA: No amplification product was detected. Commercial resin-based kit (K1), commercial protein precipitation-based kit (K2) and phenol-chloroform method (P).

Intra-assay Variation, Accuracy and Precision of CN Quantifications

Before CN quantifications, the K1 method was used to obtain mouse and goat gDNA samples that were used to validate qPCR conditions such as PCR product detection technology and range of template concentration. Thus, for each qPCR condition, standard curve parameters such as efficiency (E), linearity (R^2) and sensitivity (expressed as the lowest template CN detected in triplicate reactions) were analyzed.

In standard curves constructed with SYBR green technology and serial dilutions of mouse gDNA (Figure 2), the amplifications of the Gluc occurred with efficiency of 0.95 in low template concentrations (LC curves in Figure 2LC) and 0.98 in high template concentrations (HC curves in Figure 2HC). The TgAB and TgCD transgene regions, amplified with the efficiency of 0.66 and 0.95 in LC curve, and 0.66 and 0.92 in HC curve, respectively. The final template amount ranged from 1 to 15,525 copies for both the transgene and the reference gene per reaction (LC curves) or from 50 to 3,873,505 copies for the transgene and 1 to 25,730 copies for the reference gene per reaction (HC curves). Thus, the TgCD and Gluc had greater sensitivities (detecting as little as 1 or 5 template copies per reaction) than TgAB amplifications (sensitivity of 125 copies per reaction), despite having almost the same linearity (from 0.93 to 1.00).



Gene	Species	Template amount range	Slope	Y-intercept	R^2	Efficiency	Sensitivity (CN)
Gluc	Goat	LC	– 3.41	33.47	0.99	0.97	1
		HC	– 3.29	33.08	0.99	1.01	-
	Mouse	LC	– 3.46	33.44	0.99	0.95	1
		HC	– 3.37	33.06	0.99	0.98	-
TgAB	Goat	LC	– 4.75	42.62	0.99	0.62	125
		HC	– 4.47	43.09	1.00	0.67	-
	Mouse	LC	– 4.54	42.54	0.93	0.66	125
		HC	– 4.52	43.48	0.99	0.66	-
TgCD	Goat	LC	– 3.37	35.47	0.99	0.98	5
		HC	– 3.43	35.75	1.00	0.96	-
	Mouse	LC	– 3.45	36.08	0.98	0.95	5
		HC	– 3.52	36.32	1.00	0.92	-

Figure 2. Standard curves of SYBR green qPCR amplifications to assess transgene copy number (CN) in mouse or goats.

Amplifications of two transgene regions (TgAB or TgCD) and the reference gene (Gluc) were performed with mouse or goat gDNA. The final template amount ranged from 1 to 15,525 copies for both the transgene and the reference gene per reaction (LC) or from 50 to 3,873,505 copies for the transgene and 1 to 25,730 copies for the reference gene per reaction (HC). Assay sensitivity was assessed only for LC curves and is expressed as template CN detected in triplicate reactions.

Table 2. Intra-assay variation of qPCR amplifications for transgene CN quantification using SYBR Green and TaqMan technologies.

Transgenic specimen	Ct (Mean±*s*)								
	SYBR Green						TaqMan		
	10 ng			40 ng			10 ng		
	Gluc	TgAB	TgCD	Gluc	TgAB	TgCD	Gluc	TgAB	TgCD
I	20.67 ± 0.31	24.28 ± 0.26	22.18 ± 0.49	19.83 ± 0.18	22.44 ± 0.41	21.45 ± 0.15	25.75 ± 0.10	25.28 ± 0.22	25.65 ± 0.07
II	21.72 ± 0.04	25.99 ± 0.33	23.29 ± 0.03	20.70 ± 0.04	24.32 ± 0.11	22.24 ± 0.17	27.41 ± 0.14	26.36 ± 0.17	26.32 ± 0.25
III	20.36 ± 0.06	23.85 ± 0.17	21.70 ± 0.17	19.47 ± 0.48	21.81 ± 0.69	20.80 ± 0.50	26.24 ± 0.17	25.14 ± 0.14	24.85 ± 0.13
IV	21.04 ± 0.13	25.18 ± 0.04	22.44 ± 0.13	20.19 ± 0.29	23.10 ± 0.42	21.60 ± 0.22	26.96 ± 0.08	25.81 ± 0.16	25.40 ± 0.05
V	20.98 ± 0.16	24.87 ± 0.17	22.26 ± 0.18	19.65 ± 0.48	23.33 ± 0.57	21.57 ± 0.08	26.63 ± 0.12	25.63 ± 0.16	25.28 ± 0.11
VI	20.90 ± 0.19	24.24 ± 0.24	22.43 ± 0.18	20.91 ± 0.18	22.99 ± 0.28	22.45 ± 0.21	25.35 ± 0.11	24.46 ± 0.06	24.47 ± 0.11
VII	20.57 ± 0.33	24.12 ± 0.20	21.99 ± 0.04	20.09 ± 0.09	22.40 ± 0.61	21.59 ± 0.08	25.58 ± 0.09	24.94 ± 0.11	25.09 ± 0.12
VIII	20.87 ± 0.44	24.07 ± 1.34	22.43 ± 0.30	20.19 ± 0.27	22.66 ± 0.26	21.84 ± 0.12	25.43 ± 0.02	25.02 ± 0.08	25.76 ± 0.04
IX	20.73 ± 0.22	24.45 ± 0.31	22.21 ± 0.22	20.24 ± 0.24	22.45 ± 0.42	21.76 ± 0.31	26.49 ± 0.13	25.15 ± 0.28	24.43 ± 0.06
Mean of line	20.87	24.56	22.33	20.14	22.83	21.70	26.21	25.31	25.25
σ	0.36	0.64	0.41	0.44	0.68	0.45	0.68	0.52	0.58
Ct range[a]	1.36	2.14	1.59	1.44	2.51	1.65	2.06	1.90	1.89

Mean Ct values for the reference gene (Gluc) and the transgene (TgAB and TgCD) were calculated from qPCR reactions performed with 10 ng or 40 ng gDNA from mouse transgenic specimens (line #78). The individual variability is indicated by *s* (sample standard deviation) values measured in triplicate for each transgenic specimen. The Ct variability for the transgenic line (n=9) is denoted by the Ct range and σ (population standard deviation) values. [a]Ct range was calculated by the difference between the maximum and minimum Ct values within the column.

Using these parameters for CN quantification, Ct measurements were initially made in mouse line #78 and used to assess the intra-assay variation, accuracy and precision of the qPCR. The first observation was that the Ct values achieved with both 10 and 40 ng gDNA were successfully interpolated within the template range of both the LC and HC curves for SYBR Green (Figure 2, LC and HC, respectively). The Ct values were also used to analyze intra-assay variations (Table 2) when using these two gDNA amounts (10 or 40 ng) as templates to amplify Gluc and the transgene (TgAB or TgCD) in association with SYBR Green as the PCR product detection system. Thus, the individual variability (n = 3) was indicated by the *s* values measured for each transgenic specimen. The variability for the transgenic line (n = 9) was denoted by the Ct range and the σ values. The highest values for both *s* (1.34 and 0.69 for 10 and 40 ng template, respectively) and σ (0.64 for 10 ng and 0.68 for 40 ng) were obtained with TgAB primers. TgCD and Gluc amplifications had the lowest individual variations when using 10 ng gDNA (0.44 for Gluc and 0.30 for TgCD) compared with 40 ng (0.48 for Gluc and 0.50 for TgCD). Additionally, the lowest Ct ranges were also achieved with 10 ng template (1.36 and 1.59 for Gluc and TgCD, respectively) compared with 40 ng (1.44 and 1.65 for Gluc and TgCD, respectively).

Based on these results, a standard curve was plotted based on TaqMan technology using a low sample concentration (Figure 3). With this PCR product detection system, amplifications efficiencies were 0.99, 0.91, and 1.03 for Gluc and TgAB and TgCD transgene, respectively. The sensitivities of all genes were one template copy per reaction with linearity of the standard curves ranging from 0.97 to 0.99. CN quantification, and intra-assay variation, accuracy and precision of the qPCR in CN quantification also were performed using 10 ng of mouse gDNA. Similarly the SYBR Green technology, Ct value achieved with 10 ng gDNA was successfully interpolated within the template range of the curves (Figure 3A for TgAB and Figure 3B for TgCD). The *s* values for Gluc, TgAB and TgCD amplifications with

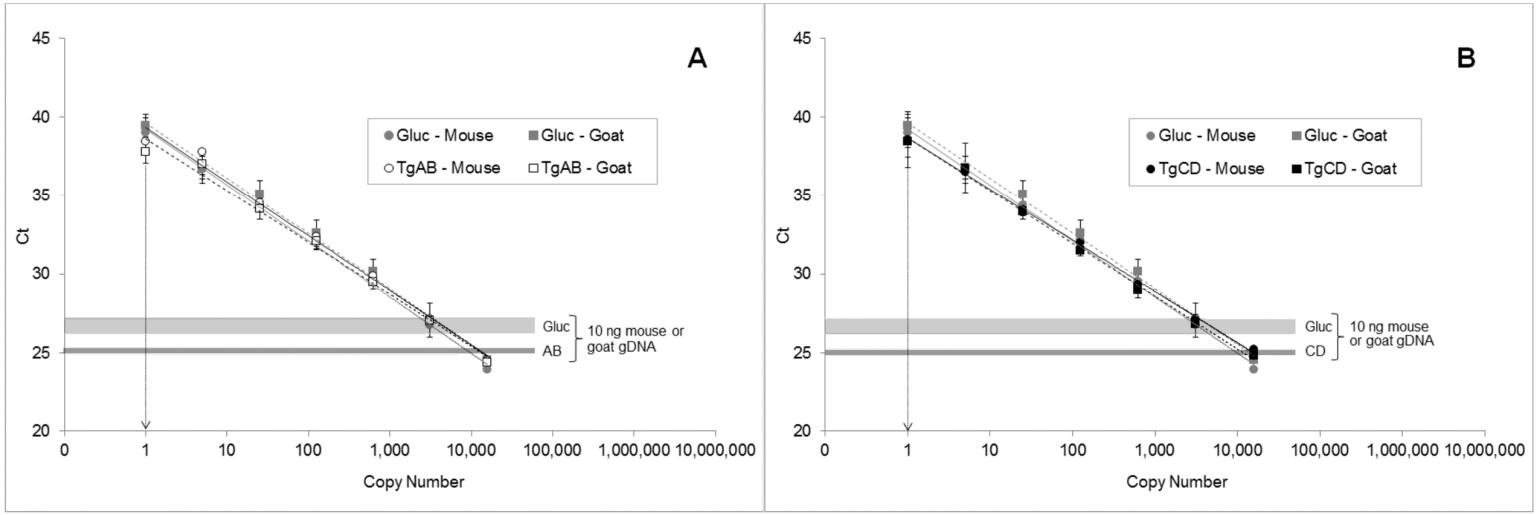
TaqMan were 0.14, 0.28 and 0.25, respectively, and 0.44, 1.34 and 0.30, respectively, with SYBR Green. The σ values and the Ct ranges were lower in TaqMan than in SYBR Green reactions. Thus, only for 10 ng gDNA reactions, TaqMan produced σ values of 0.68, 0.52 and 0.58 and Ct range values of 2.06, 1.90 and 1.89 for Gluc, TgAB and TgCD, respectively. Under the same conditions, line measurements with SYBR Green gave σ values of 0.36, 0.64 and 0.41 and Ct range values of 1.36, 2.14 and 1.59 for Gluc, TgAB and TgCD, respectively.

For line #78, the CN values themselves were calculated by absolute normalized quantification and used to infer the accuracy and precision of CN quantifications (Table 3). Amplifications of the TgCD transgene region in the presence of SYBR Green produced highly accurate and precise quantifications because only a single CN value (CN = 2, σ = 0) was achieved for all specimens of the mouse line independent of the template amount (10 or 40 ng gDNA) or curve range (LC or HC). Thus, under these conditions, the relative error was null, and the trueness frequency was 100%. However, TgAB amplifications produced CN quantifications with low accuracy (relative error from 11.1 to 177.8% and trueness frequency from 0 to 77.8%) and low precision (CN ranging from 2 to 8 and σ values from 0.42 to 1.42). Surprisingly, when using probes, TgAB amplifications produced more precise CN values than TgCD (CN ranged from 1 to 3 for TgAB and from 1 to 5 for TgCD; σ was 0.57 for TgAB and 1.25 for TgCD). Additionally, the values for TgAB with TaqMan were more accurate than for TgCD (average relative error was -5.6% for TgAB and 33.3% for TgCD and trueness frequency was 66.7% for TgAB and 22.2% for TgCD).

Table 3. Accuracy and precision of transgene CN quantifications by qPCR.

Transgenic specimen of line #78	Transgene CN*									
	SYBR								TaqMan	
	TgAB				TgCD					
	10 ng		40 ng		10 ng		40 ng		TgAB	TgCD
	LC	HC	LC	HC	LC	HC	LC	HC		
I	2	4	3	5	2	2	2	2	1	1
II	2	3	2	4	2	2	2	2	2	3
III	2	4	3	6	2	2	2	2	2	3
IV	2	3	3	5	2	2	2	2	2	4
V	2	3	2	3	2	2	2	2	2	3
VI	3	4	5	8	2	2	2	2	2	2
VII	2	4	4	6	2	2	2	2	2	2
VIII	3	5	4	6	2	2	2	2	1	1
IX	2	4	4	7	2	2	2	2	3	5
Mean of line	$2^{a,d}$	4^{b}	$3^{a,b}$	6^{c}	2^{d}	2^{d}	2^{d}	2^{d}	2^{d}	$3^{a,b,d}$
σ	0.4	0.6	0.9	1.4	0.0	0.0	0.0	0.0	0.6	1.2
CN range	2 to 3	3 to 5	2 to 4	3 to 8	2	2	2	2	1 to 3	1 to 5
Average relative error (%)	11.1	88.9	66.7	177.8	0.0	0.0	0.0	0.0	-5.6	33.3
Trueness frequency (%)	77.8	0.0	22.2	0.0	100.0	100.0	100.0	100.0	66.7	22.2

Transgene CN values were calculated from triplicate qPCR reactions performed with 10 ng or 40 ng gDNA from mouse transgenic specimens (line #78) by normalized absolute quantification. Precision of CN quantifications for the transgenic line (N=9) is indicated by σ (population standard deviation) and the CN range. Accuracy is denoted by average relative error and trueness frequency. *Transgene copies (integer values) per mouse haploid genome. Different superscripts differ significantly (p<0.05).



Gene	Species	Slope	Y-intercept	R^2	Efficiency	Sensitivity (CN)
Gluc	Goat	– 3.35	39.14	0.97	0.99	1
	Mouse	– 3.25	38.36	0.96	1.03	1
TgAB	Goat	– 3.33	38.71	0.98	1.00	1
	Mouse	– 3.56	39.64	0.99	0.91	1
TgCD	Goat	– 3.39	38.75	0.98	0.97	1
	Mouse	– 3.26	38.68	0.99	1.03	1

Figure 3. Standard curves of TaqMan qPCR amplifications to assess transgene copy number (CN) in mouse or goats.

Amplifications of two transgene regions, TgAB (A) or TgCD (B), and the reference gene (Gluc) were performed with mouse or goat gDNA at a final target amount ranging from 1 to 15,525 copies per reaction. Assay sensitivity (arrows) is expressed as template CN detected in triplicate reactions.

Similar standard curve parameters were observed between goat and murine, regardless of the curve range (LC and HC) with SYBR Green technology. Amplifications of the Gluc reference gene occurred with efficiency varying from 0.95 to 0.97 at low template concentrations (LC curves in Figure 2LC) and from 0.98 to 1.01 at high template concentrations (HC curves in Figure 2HC). The transgene amplifications of the TgCD region occurred with E values from 0.95 to 0.98 for LC curves and from 0.92 to 0.96 for HC curves. The TgAB reactions had the lowest efficiencies (from 0.62 to 0.67) in all template concentrations used for both mice and goats samples. Additionally, TgCD and Gluc had greater sensitivities than TgAB amplifications, despite having almost the same linearity (from 0.93 to 1.00) when using SYBR Green technology. In TaqMan technology, the efficiency of Gluc amplification was 1.03 for goat sample. Similar to the SYBR Green amplifications, the TaqMan efficiencies for TgCD were greater than TgAB. Additionally, the sensitivities of all genes were one template copy per reaction, regardless of the animal species (mouse or goat). The linearity of the TaqMan standard curves for TgAB, TgCD and Gluc reactions ranged from 0.97 to 0.99.

CN Quantification in Goats

The transgene CN inserted in the genomes of the 10M and 12F goat lines was quantified by absolute (using a standard curve drawn with hG-CSF plasmid and goat gDNA) and relative (using the mouse line #78 as a calibrator) methods and expressed as the CN of transgene per diploid genome (Table 4). CN values were calculated from triplicate qPCR reactions performed with 10 ng gDNA from transgenic goat specimens. Overall, as indicated by the low σ values, TgCD amplifications with SYBR Green produced highly precise CN values, independent of the mathematical strategy used. Thus, when using the Abs or Eff methods, CN ($\pm\sigma$) was 9 ($\pm$0.0) for 12F and 6 ($\pm$0.5) for 10M. When using the DDCt or StC quantifications, CN ($\pm\sigma$) was 10 ($\pm$0.0) for 12F and 7 ($\pm$0.5) for 10M. With SYBR Green, TgAB reactions

produced highly variable quantifications for both goat lines (CN ranged from 4 to 15 for 10M and from 4 to 19 for 12F; σ up to 2.9 for 10M and up to 2.1 for 12F). When experiments were performed with TaqMan technology, the measurements frequently had very low precision (Table 4). Thus, σ values were as high as 2.9 for 12F and 3.2 for 10M line. The CN values calculated for each goat line ranged from 7 to 8 for 10M and 10 to 13 for 12F. Surprisingly, the CN values generated by TgAB amplifications and the Eff method were the most discrepant among the measurements achieved for goat lines (CN±σ was 2±0.5 for both 10M and 12F lines) and did not follow the patterns described above. It is important to highlight that, before all DDCt quantifications, curves of log input DNA amount versus ΔCt were plotted for each qPCR condition and used to assess the slopes of the target and reference genes (Figure 4). The absolute values of the slopes were lower than 0.1 for TgCD amplifications and higher than 0.1 for almost all TgAB amplifications.

Table 4. Transgene CN quantifications by qPCR in goat lines.

Line	Transgenic specimen	Transgene CN*															
		SYBR Green								TaqMan							
		TgAB				TgCD				TgAB				TgCD			
		Abs	Relative quant.			Abs	Relative quant.			Abs	Relative quant.			Abs	Relative quant.		
			DDCt	Eff	StC		DDCt	Eff	StC		DDCt	Eff	StC		DDCt	Eff	StC
12F	I	5	11	19	9	9	10	9	10	10	13	2	13	7	9	9	9
	II	6	13	21	10	9	10	9	10	10	10	2	12	10	12	13	12
	III	4	9	16	7	9	10	9	10	11	14	3	14	13	15	16	16
	Mean of line	$5^{a,b}$	11^{c}	19^{d}	$9^{a,c,e}$	$9^{a,c,e}$	$10^{a,c,e}$	$9^{a,c,e}$	$10^{a,c,e}$	$10^{a,c,e}$	$12^{c,e}$	2^{b}	$13^{c,e}$	$10^{a,c,e}$	$12^{c,e}$	$13^{c,e}$	$12^{c,e}$
	σ	0.8	1.6	2.1	1.3	0.0	0.0	0.0	0.0	0.5	1.7	0.5	0.8	2.5	2.5	2.9	2.9
	CN range	4-6	9-13	16-21	7-10	9	10	9	10	10-11	10-14	2-3	12-14	7-13	9-13	9-16	9-16
10M	I	4	9	13	6	6	6	5	6	7	8	2	9	9	11	12	11
	II	4	8	13	6	6	6	5	6	8	10	2	11	10	11	12	11
	III	4	8	13	6	7	7	6	7	9	10	2	11	9	10	11	10
	IV	4	10	14	7	6	7	6	7	6	7	1	8	5	6	6	6
	V	4	11	16	7	6	7	6	7	6	7	1	7	3	3	4	4
	VI	5	12	19	9	7	7	6	7	3	4	1	4	4	6	5	5
	VII	5	12	19	9	7	7	6	7	7	9	2	9	7	8	9	8
	Mean of line	$4^{a,b}$	10^{c}	15^{d}	$7^{a,c,e}$	$6^{a,e}$	$7^{a,c,e}$	$6^{a,e}$	$7^{a,c,e}$	$7^{a,c,e}$	$8^{c,e}$	2^{b}	$8^{c,e}$	$7^{a,c,e}$	$8^{c,e}$	$8^{c,e}$	$8^{c,e}$
	σ	0.4	1.7	2.5	1.3	0.5	0.5	0.5	0.5	1.8	2.0	0.5	2.3	2.6	2.9	3.2	2.7
	CN range	4-5	8-12	13-19	6-9	6-7	7-8	5-6	6-7	3-7	4-10	1-2	4-11	3-10	3-10	4-12	4-11

Transgene CN values were calculated from triplicate qPCR reactions performed with 10 ng gDNA from transgenic goat specimens by normalized absolute quantification (Abs), comparative Ct (DDCt), efficiency correction (Eff) or standard curve (StC) relative quantifications. The precision of CN quantifications for the transgenic lines (n=3 for 12F and n=7 for 10M) is indicated by σ (population standard deviation) and the CN range. *Transgene copies (integer values) per goat diploid genome. Different superscripts within the same goat line differ significantly (p<0.05).

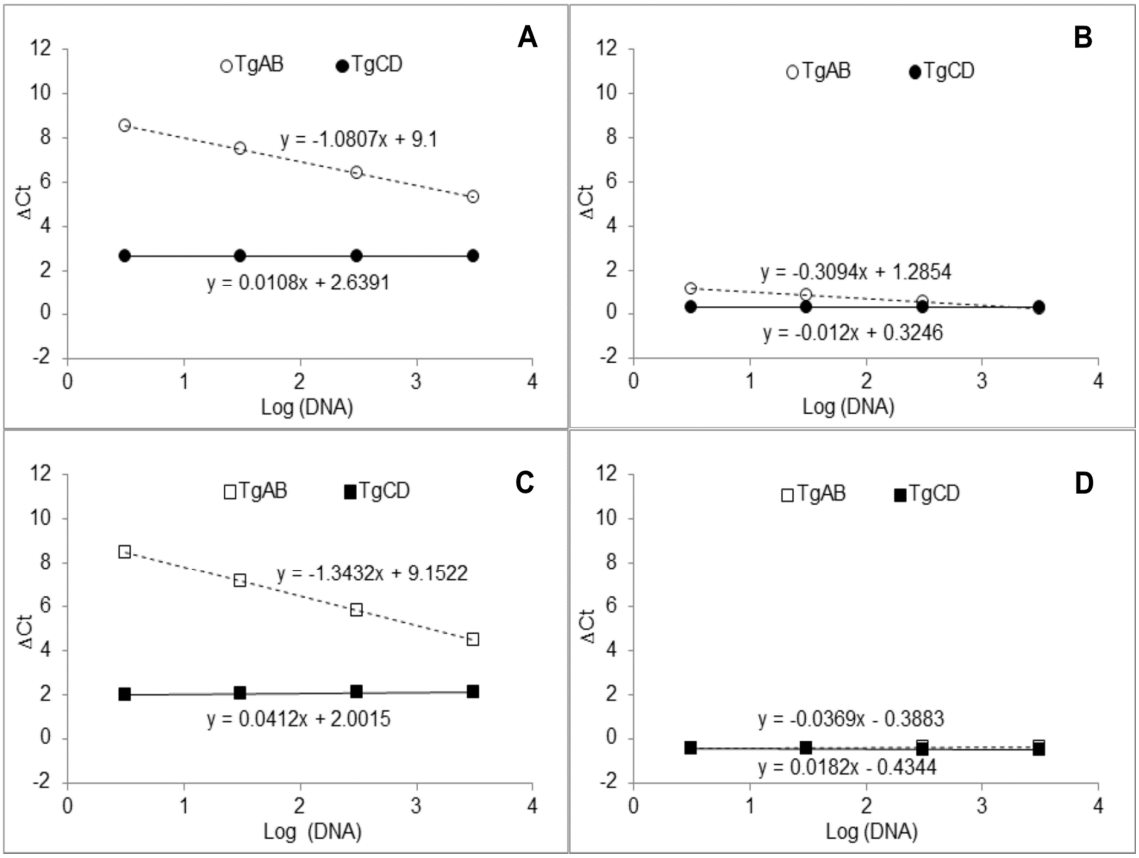


Figure 4. Comparative analysis of transgene and reference gene qPCR efficiencies to determine the DDCt relative quantification applicability. Plots of log input template amount versus ΔCt (difference between transgene and reference gene Ct values) were drawn at different concentrations of mouse (A and B) and goat (C and D) gDNA samples. Amplifications of TgAB or TgCD transgene regions and Gluc reference gene were performed using SYBR Green (A and C) or TaqMan (B and D) technologies.

Discussion

Real-time PCR amplifications are known to be highly influenced by the quality and purity of the template DNA.[24] Thus, a major caveat of real-time quantifications is that sample-specific inhibitors can compromise both the reliability and accuracy of an assay. This can be a key concern, particularly for samples derived from sources known to contain PCR inhibitors, in that any loss of amplification efficiency will generate unidentified and potentially large quantitative errors.[24, 26] The presence of PCR inhibitors in gDNA samples extracted from mouse tail tips by the phenol-chloroform method has already been reported.[27] Thus, when gDNA samples were used as the template in both conventional end-point and TaqMan qPCR reactions, unidentified contamination seemed to inhibit both the polymerase and exonuclease activities of the Taq DNA polymerase enzyme. Although a PCR-inhibitory effect was assigned to the collagenous components of mouse tissue,[28] the specific substances responsible remain unknown. In the present investigation, in addition to the possible presence of these substances, we could not explain the apparently stronger inhibition of the endogenous gene (Gluc) amplification when compared to exogenous transgene (TgCD). Regardless, the K1 method was shown to be suitable for the production of high-quality DNA suitable for qPCR applications and was used to obtain all gDNA samples used in the subsequent assays.

All commercial qPCR platforms currently rely on defining the relative position of amplification profiles. As such, they are reliant on the amplification of a serially diluted target to provide an estimate of amplification efficiency, which is essential for accurate and reliable quantifications.[22, 29] Thus, standard curves were constructed by serial dilutions of the DNA templates and used to analyze qPCR conditions in the present work. In several qPCR conditions, both the Gluc reference gene and TgCD transgene regions could be amplified with ideal efficiency ($E \geq 0.95$) when using mouse or goat gDNA as template. In contrast, amplification of the TgAB transgene region with SYBR Green presented very low efficiency

($E \leq 0.67$). Additionally, lower intra-assay variations (assessed by Ct values) and more stable CN quantifications in mouse line #78 were achieved with TgCD than with TgAB amplifications. Consequently, it appears that improved E values were strongly associated with the better accuracy and precision of transgene CN measurements achieved with TgCD when compared to TgAB.

For any quantitative measurements through real-time PCR, mathematical validation of the appropriateness of the amplification conditions is obviously necessary, including the selection of an effective linear range of detection with appropriate efficiency.[30] In the current work, amplification parameters were also assessed within two template ranges, LC and HC, starting from one to millions (LC) or to thousands (HC) of transgene copies per reaction. These conditions simulated the standard curves constructed with gDNA from animals harboring transgene copies in the genome at low (i.e., CN up to a dozen) or high (i.e., CN nearly a hundred) numbers. Overall, the linear ranges ($R^2 \geq 0.98$) of the LC and HC curves showed similar E values inferred from the exponential phase of standard curve reactions. Although this method does not account for PCR efficiencies for individual target samples, as proposed by some authors,[31] it is widely accepted that for the applied purposes E values can be extrapolated for unknown samples amplified under similar conditions.[16] Therefore, an important consideration is that the Ct of unknown samples is interpolated in the effective linear range in which the E value was calculated. For two-copy transgenic mice (line #78), the use of either 10 or 40 ng gDNA allowed accurate (relative error = 0% and trueness frequency = 100%) and precise ($\sigma = 0$) CN quantifications of TgCD and Gluc amplifications with Ct values within both the LC and HC template ranges. Inaccurate and imprecise CN values were reached with TgAB reactions that presented poor amplification efficiencies over all target concentration ranges (LC or HC) and gDNA amounts (10 or 40 ng).

Amplification efficiencies were improved at low mouse gDNA concentrations by using TaqMan instead of SYBR Green technology for Gluc (1.03 *vs* 0.95), TgAB (0.91 *vs* 0.66) and TgCD (1.03 *vs* 0.95). Although TaqMan reactions decreased intra-assay variations for each gene (as assessed by the *s* of the average of individual Ct values), this technology failed to improve accuracy and precision of CN values calculated for the mouse line # 78. Then trueness frequencies of CN determinations changed from 77.8% to 66.7% for TgAB, and from 100% to 22.2% for TgCD, with SYBR Green and TaqMan, respectively. Additionally, CN values measured with TgAB and TgCD had average relative errors of -5.6% and 33.3%, respectively, when coupled to probes, instead of 11.1% and 0%, respectively, with SYBR. Finally, TaqMan amplifications had better sensitivities (1 copy per reaction for all genes) when compared to SYBR Green. However, it is important to note that high sensitivity is not a restrictive requirement for CN quantification because qPCR reactions are often loaded with a dozen nanograms of gDNA.[11, 14, 32] Therefore, even for low-copy animals, qPCR amplifications are always performed with thousands of transgene copies per reaction.

The last step of the present investigation was to select the best strategy, including the mathematical approach, for CN quantification in goats. For simplification purposes, only the 10 ng gDNA sample and LC curves were used in assays. Additionally, despite the low amplification efficiency observed for TgAB reactions with SYBR Green in mouse samples, these reactions were kept in goat assays to compare with the TgCD quantifications. It is important to note that, of the four mathematical qPCR strategies applied for goat CN quantifications (Abs, DDCt, Eff and StC), only Abs was performed without mouse gDNA as calibrator. The most interesting finding was that the CN values for each transgenic line were almost equal regardless of calculation by relative (with mouse gDNA as calibrator) or absolute (with plasmid) methods. As observed in mouse assays, the essential requirement for reliable measurements in goats was superior amplification parameters, specifically efficiency.

Again, when amplifying TgCD with SYBR Green, the CN values calculated for each line were highly precise (σ was 0.0 and 0.5 for 12F and 10M lines, respectively). In that condition, the CN was always 9 or 10 for 12F and 6 or 7 for 10M by any mathematical method. This level of agreement is quite interesting in view of the completely different and non-interrelated nature of these strategies. As expected, quantifications performed with TgAB amplifications and SYBR have poor precision, and no predictable pattern could be established. In this situation, CN values varied absurdly from 5 to 19 for 12M and from 4 to 15 for the 10M line.

Accurate assessment of transgene CN in unknown samples relies on the use of accurate standards, i.e., plasmid or calibrator gDNA.[11, 13] According to the qPCR strategy, standards can be made with DNA extracted from a previously known specimen [33-35] or plasmid DNA.[36] When working with transgenic farm animals, the absence of a calibrator animal is a possibility that cannot be excluded. In this condition, the use of plasmid DNA provides a more flexible alternative to conventional reference material and can be used alone [37] or spiked in wild-type gDNA.[38] The advantage of mixing plasmid with gDNA is the viability of signal normalization to the endogenous gene (i.e., Gluc) present in both the unknown and standard samples. Additionally, the wild-type gDNA can be chosen from the same animal species as the unknown samples (goats in the current case). This apparently simple action allows a plethora of reference genes to be used in qPCR given that its genomic CN needs only be stable instead of quantitatively known. This situation is particularly relevant to goats, for which genomic databases are less abundant than other animal species (i.e., mouse). Here, the Gluc gene was chosen for experimental purposes because the feasibility of qPCR amplifications applied simultaneously to mice and goats has already been described.[11] Otherwise, if the aim is only to quantify a transgene CN by the Abs strategy in goats, the researcher can select another reference gene (i.e., GAPDH, actin, etc.)

Of the relative quantification strategies, DDCt is the most popular because of its quickness and ease of use.[12] Although some authors have highlighted the advantageous dispensability of efficiency calculations,[11, 12, 16, 22, 33] for DDCt quantifications to be valid, the efficiency of the target and reference genes must be approximately equal.[21] A sensitive method for assessing whether two amplicons have the same efficiency is to look at how ΔCt varies with template dilution. If the efficiencies of the two amplicons are approximately equal, the plot of log input DNA amount *versus* ΔCt has a slope of approximately zero.[21] In the present work, the slopes of ΔCt curves plotted for Gluc and TgCD were lower than 0.1 (in absolute value) for all amplification conditions. Given this background, the DDCt strategy could be adequately applied to transgene CN assessment in both mice and goats. As expected, TgAB reactions with SYBR failed to be validated for the DDCt procedure because the ΔCt slope was higher than 0.1. Again, the CN values produced in this condition were kept in results for illustrative purposes.

Relative quantifications by StC and Eff are less frequently used strategies because the calculations include efficiency corrections.[12] In fact, only StC has already been used for transgene CN quantifications.[13, 22] The Eff method was innovatively adapted in the present work from the previously described method [22] by means of adding efficiency corrections for each gene (target and reference) and animal species (mouse and goat) to quantify CN in transgenic goats. Although all relative quantifications produced similarly reliable results, the StC strategy seems to be slightly faster than DDCt because only one standard curve must be constructed (i.e., for mice). In contrast, the Eff method was difficult, laborious and tedious because four standard curves had to be built (one for each gene and animal species). Unpredictably, even though amplification efficiencies were slightly different under the various qPCR conditions, more corrections for several E values did not generate more precise results. Taking the results together, relative quantification strategies are able to produce as

reliable results as absolute strategies in goats because the essential qPCR requirements are properly respected.

When data were generated with TaqMan technology, the CN values calculated for both TgAB and TgCD were somewhat similar, especially for the 10M line, which had a larger number of specimens. The 10M CN was 7 or 8 and the 12F CN ranged from 10 to 13. However, when compared to the SYBR Green system, association with probes increased TgAB and decreased TgCD precisions for CN quantifications. Intriguingly, even after some replicates, the CN values achieved by TgAB reactions with probes calculated with the Eff method (CN = 2.0±0.5 for both 12F and 10M) remained completely discrepant from the other quantifications ($P < 0.05$). The reason for this divergence remained elusive.

Data variability produced by TaqMan technology applied to transgene CN and zygosity determination was previously reported for two mouse lines.[39] Although the authors did not explain the reason for the variability, an elaborate statistical treatment to solve the problem was presented. We hypothesized that population variability in the reference gene sequence needs be excluded before designing probes; otherwise, qPCR signals can vary even between individuals from the same transgenic line. The qPCR requirement of absence of heterogeneity in reference genes for CN determinations is an issue that has already been stated by other research groups.[30, 40, 41] In support of the above hypothesis, although goat gDNA samples were not sequenced in the present assays, it was noticed that the Ct values produced by Gluc amplifications with probes presented σ values higher than with SYBR Green. Consequently, CN calculations of TgCD and Gluc for each goat line have higher σ with TaqMan than SYBR. Finally, even CN values were variable among individuals; an acceptable estimation of CN in each transgenic goat line was still feasible with TaqMan system.

Overall, the current study allows us to enumerate some of essential aspects for transgene CN quantifications by qPCR particularly targeted to goat lines, including the following: i)

Validation of qPCR conditions is required, and amplification efficiency is the main quality control for consistent CN measurements. ii) Absolute and relative quantifications are able to produce similar, reliable CN results if a high-quality and accurately quantified standard DNA can be used. Additionally, a double-strand-DNA-selective fluorescent assay is strongly advised for standard DNA quantification. iii) For normalized absolute quantification, the same plasmid fragment used to generate goat lines must be mixed with wild-type goat gDNA, allowing the use of an endogenous reference gene for data normalization. iv) For relative quantifications, an important issue is the selection of the gDNA extraction method because tissue-specific PCR inhibitors are non-predictably distributed in animal species. A resin-based method is strongly recommended when using mouse tail tips as calibrators. v) Efficient qPCR amplifications ($E \geq 95\%$) allow reliable CN measurements with SYBR technology, independent of the chosen mathematical strategy (Abs, DDCt, Eff or StC). However, amplifications with low efficiency produce poor accuracy and imprecise CN values. vi) Because TaqMan is more susceptible to gene sequence variability than SYBR Green technology, this system must be used with caution in goats if the endogenous reference gene is not well known. Otherwise, CN quantifications with probes can present poor precision.

Conclusions

In conclusion, the present work contributes an extensive analysis of CN quantifications in transgenic goats by qPCR. Adhering to the above general guidelines will increase the quality and reliability of the determined CN. For any qPCR quantification, well-designed and properly performed procedures can deliver more exact CN determinations in goat genomes. Finally, even when working under non-optimal circumstances, if assays that respect the minimum qPCR requirements are performed, good estimations of transgene CN in goat lines can be achieved.

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5 CAPÍTULO 2

Avaliação de crescimento e caracteristicas reprodutivas da geração F1 de caprinos transgênicos para fator estimulante de colônias de granulócitos humano

Resumo

Para garantir os requisitos de bem-estar animal e que as características fenotípicas da linhagem transgênica recém-produzida não foram comprometidas, uma avaliação de todos os indivíduos é necessário. Assim, este trabalho foi desenhado para determinar o impacto da inserção do transgene Fator Estimulante de colônias de Granulócitos Humana (hG-CSF) no crescimento e características reprodutivas da primeira geração (F1) de cabras transgênicas. O peso corporal (PC), desenvolvimento (PC ao nascer, ganho médio PC até o desmame, PC ao desmame, ganho médio PC após o desmame, PC na puberdade), bem como parâmetros reprodutivos (idade à puberdade, o volume de ejaculado, concentração, total de esperma por ejaculado, motilidade massal, motilidade individual progressiva, defeitos maiores e menores) foram semelhantes ($P<0,05$) entre cabras transgênicas (T) e não-transgênicas (NT). Diferenças significativas ($P>0,05$) em média (±DP) de células brancas do sangue (WBC) foram observados entre T e NT no primeiro dia de vida ($174,6 \pm 14,7 \times 10^3$ e $15,0 \pm 4,0 \times 10^3$ células/mL), durante (66,8 ± 21,1 × 103 e 17,0 ± 4,6 × 103 células/mL) e após o período de amamentação ($36,6 \pm 4,0 \times 10^3$ e $15,5 \pm 2,2 \times 10^3$ células/mL), embora o hG-CSF não foi detectado no soro sanguíneo em todas as análises. Apesar de outras contagens de células terem sido ocasionalmente maiores nos animais T, a contagem diferencial mostrou que este aumento no WBC deve-se principalmente a um aumento do número de neutrófilos, o que representa 84,6, 67,2 e 56,8% do WBC total, respectivamente, nos três períodos. Análises

bioquímicas indicaram que rim e fígado de todas as cabras eram saudáveis. Assim, é possível conjecturar que todos os animais são normais e que o transgene não teve efeito deletério sobre parâmetros reprodutivo e crescimento, quer pela presença do transgene ou como consequência do perfil de leucócitos alteração.

Palavras-chave: *reprodução*; *crescimento*; *transgênicos*; *bem-estar*

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Evaluation of growth and reproductive traits of F1-generation transgenic goats to human Granulocyte-Colony Stimulating Factor

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Abstract

To ensure that animal welfare requirements and phenotypic characteristics of the newly produced transgenic lines are not compromised, an evaluation of all individuals is necessary. This work was designed to determine the impact of the insertion of human granulocyte-colony stimulating factor (hG-CSF) transgene on growth and reproductive characteristics in first-generation (F1) goats from two transgenic lines. Body weight (BW), development (BW at birth, mean BW gain before weaning, BW at weaning, mean BW gain after weaning, BW at puberty), as well as reproductive parameters (age at puberty, ejaculate volume, concentration, total sperm per ejaculate, massal motility, progressive individual motility, major and minor defects) were similar ($P<0.05$) between transgenic (T) and non-transgenic (NT) goats. Significant differences ($P>0.05$) in mean (±SD) white blood cell (WBC) were observed between T and NT in first day of life ($174.6\pm14.7\times10^3$ and $15.0\pm4.0\times10^3$ cells/µL), during ($66.8\pm21.1\times10^3$ and $17.0\pm4.6\times10^3$ cells/µL) and after breastfeeding ($36.6\pm4.0\times10^3$ and $15.5\pm2.2\times10^3$ cells/µL), even though hG-CSF has not been detected in blood serum in any

analysis. Although other cell counts were occasionally higher in T animals, differential counts showed that this difference was mainly due to an increased number of neutrophils, which represents 84.6, 67.2 and 56.8% of total WBC, respectively, in the three moments. Kidney and liver biochemical analysis indicated that all goats were healthy. Thus, it is possible to assume that all animals are normal and had no deleterious effects on either growth or reproductive parameters by the presence of transgene or as a consequence of leukocyte profile alteration.

Key words: *Reproduction; Growth; Transgenic; Welfare*

1. Introduction

The production of transgenic farm animals containing exogenous DNA stably incorporated into their genome, when able to be transmitted to their offspring in a Mendelian fashion, has several applications. Besides the obvious scientific interest for the study of genes and their regulation, transgenic animal technologies have been proposed as a method to accelerate livestock improvement, by means of introducing new genes or modifying the expression of endogenous genes that regulate traits of economic importance (Wheeler, 2003) and the production of recombinant proteins (Lavine, 2009).

Although there is a continuous and remarkable development in transgenic technology, the consequences of genotypic changes induced by this type of experimental mutation cannot be completely predicted. This is especially true for the majority of transgenic animals who are generated by pronuclear injection. Randomly integrated foreign DNA may increase the risk of disruption of endogenous genes (Jackson et al., 2010) and disturb normal physiological processes, resulting in possibly discomfort, which is crucial to animals' welfare (Mertens and Rulicke, 2000). Associated with this, an inappropriate control of transgene expression may

results in overexpression or expression in undesirable tissues (Pursel et al., 2004). Thus, several studies have reported physiological (Pursel et al., 1990; Pursel et al., 2004) and reproductive (Maleszewski et al., 1998; Bryla et al., 2010) disorders in transgenic animals.

Certainly, the health and welfare of transgenic animals raised for production purposes is of central importance to potential consumers and producers (Jackson et al., 2010). Animal welfare is a concept that involves both physical and subjective evaluations of the individuals (Duncan, 2005; Mertens and Rulicke, 2007). The physical aspects of general health, as standard measures of body development and reproductive fitness, such as semen quality and age at puberty can be used as indicators of transgenic animal's health (Jackson et al., 2010). Another key indicator of health in living organisms is their ability to grow from birth to adulthood in a similar way to young individuals of the same species and breed. Van Reenen (2009) suggested that the study of a transgenic line should be performed not only during the period of transgene expression, but also at different stages of life, including: pregnancy, birth, puberty and adulthood.

Our group reported the production of a couple of transgenic (T) goats (10M and 12F lineages) containing human granulocyte-colony stimulating factor (hG-CSF) fused to goat α-S1 casein (CSN1S1) promoter produced by pronuclear injection (Freitas et al., 2012). This protein acts in the prevention of febrile neutropenia and currently it is recommended for patients with substantial risks of such symptoms to stimulate the proliferation and differentiation of granulocytic lineage cells (Crea et al., 2009). Interestingly, the female founder (12F) successfully expressed the recombinant protein in her milk and at a concentration that was compatible with commercially viable investments (Moura et al., 2013). Moreover, the hG-CSF transgene was transmitted by Mendelian fashion to first-generation progenies (F1) from both lineages (Freitas et al., 2012). However, according to Mertens and Rülicke (2007), to ensure transgenic animal welfare requirements, a careful phenotype characterization and

welfare assessment has to be done routinely for each newly produced lineage, at individual and lineage level, starting by the standardized monitoring of founders and their consequent generations.

This work was designed to evaluate the impact of the hG-CSF transgene insertion in F1 goats derived from two transgenic lineages in comparison with non-transgenic (NT) controls since their birth up to 10 months-old on: body weight (BW) development, reproductive parameters such as age at puberty and semen quality, leukocyte profile, hepatic and renal function analysis, and ectopic expression for the protein in blood serum.

2. Material and Methods

2.1. Animal ethics and biosecurity

All protocols used in this study were approved by the Committee of Animal Ethics (09144595-7/50) of the State University of Ceará and Brazil's Biosafety Technical National Committee (CQB 0228/06) to work with genetically modified organisms. Additionally, all experimental procedures were conducted according to the guidelines for the ethical use of animals in research (Asab, 2006).

2.2. Location and experimental animals

The experiment was conducted between 2010 and 2012 in the Laboratory of Physiology and Reproduction Control of the State University of Ceará, Fortaleza (3º43'47" S latitude and 38º 30'37" W longitude, and approximately 15 m above sea level). For F1 generation, both T lineages (12F and 10M) were mated with NT goats. A total of nine experimental animals of Canindé breed were obtained and used; three T females, two T males, one NT female and three NT males.

2.3. Experimental conditions

After birth, all animals (T and NT) received breastfeeding up to 3 months of age when weaning was performed. Before weaning, once a day for a period of 5 h, the animals were separated from their mothers and kept in another pen, where they had free access to water, Tifton (Cynodon dactylon) hay, commercial concentrate (Fri-Borrego, Fri-Ribe, Teresina, Brazil), and mineralized salt licks. After this period, the animals' diets were supplemented with 0.2 kg/day of the concentrate mentioned above, with Tifton hay, water and mineralized salt licks ad libitum.

2.4. Assessment of body weight (BW) development

Goats were weekly monitored regarding their BW development from birth to 10 months of age by the use of an electronic scale adapted for the species (Leader, Araçatuba, Brazil). It is noteworthy that weight measurement was always performed during early morning, aiming to obtain the data before feeding the animals, maintaining a standard analysis of the values obtained.

2.5. Puberty detection in females

The determination of the onset of ovarian activity initiated at 3 months of age until puberty by plasma progesterone concentration analysis. Blood samples were collected from all female goats (T and NT) by jugular venipuncture using 4-mL tubes containing EDTA (BD Vacutainer, Becton Dickinson and Company, Holdrege, USA), always in the morning once per week. The tubes were placed on ice until centrifugation at 4,400 × *g* for 15 min. Plasma was aliquoted and immediately sent to the laboratory for hormone assay. Progesterone concentrations were measured using a commercial chemiluminescence specific kit (Immulite

Siemens, Deerfield, USA; sensitivity of 0.03 ng/mL). Progesterone concentrations greater than 1 ng/mL in two consecutive samples were considered as indicative of luteal activity, indicating the onset of puberty (Al-Hozab and Basiouni, 1999).

2.6. Puberty detection in male and sperm analysis

The detection of puberty started at 3 months of age, when individual sexual behavior was assessed once per week. Males had a limit of 5 min to mount female goats (presenting the same body size as them) induced to estrus by weekly injections of 2 mg estradiol cypionate (E.C.P., Pfizer Animal Health, New York, USA) i.m. After the mount followed by ejaculation, vaginal smear slides were obtained and observed under a phase-contrast optical microscope (Eclipse E 200, Nikon, Tokyo, Japan) for the presence of live sperm. The animals were considered pubescent when presented the following sequences of sexual behavior, penile exposure, mating, ejaculation, and presence of mobile spermatozoa in the ejaculate (Delgadillo et al., 2007).

In order to verify the morphological and physical characteristics of the semen, at least five samples were collected from each buck ageing 8 to 10 months old through the artificial vagina method direct into graduated plastic tubes. After collection, the semen was evaluated by its volume, concentration, total sperm per ejaculate, massal motility, percentage of sperm cells with progressive individual motility and spermatic morphology (minor, major and total defects) according to Souza et al. (2011). All semen analyses were assessed through phase-contrast optical microscopy (Eclipse E 200).

2.7. Hematological and biochemical analysis

Blood samples were collected from all male and female goats (T and NT) by jugular venipuncture using 4-mL tubes containing EDTA (BD Vacutainer®), always in the morning

once per week for white blood cell (WBC) and differential cell counts. To determine the total WBC an automatic analyzer (CELL-Dyn 3700, Abbott Laboratories, Abbott Park, Illinois, USA) was used whereas differential cell count was performed using a small drop of blood smeared on a clean microscope slide and then examined by Romanowsky staining (Nikon Eclipse E400, Tokyo, Japan). Conversely, blood serum biochemical analysis was performed every 15 days by the use of serum separation tubes (BD Vacutainer®) to determine urea, creatinine, glucose, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations (BT 3000 plus; Winer Lab, Rosario, Argentina). The tubes were placed on ice until centrifugation at $4{,}400 \times g$ for 15 min. Plasma and serum were aliquoted and immediately sent to the laboratory for each assay.

2.8. hG-CSF analysis in blood serum

The same process was conducted for blood collection and serum sample obtention for hG-CSF quantifications in all T and NT goats by solid-phase sandwich ELISA using high-sensitivity kit (< 1 pg/mL; RayBiotech, Norcross, GA, USA). Three serum samples were collected from all animals throughout the experiment: in their first day of life, at 1 month old (during) and at 10 months old (after breastfeeding). After collection, the samples were subsequently frozen until hormone assay. All procedures were performed according to the manufacturer. The optical density was measured at 450 nm in a plate reader (Multiskan FC; Thermo Scientific, Helsinki, Finland).

2.9. Statistical analysis

Data are presented as mean ± standard deviation (SD) and compared using the Graph Pad Instat 3.6 software (GraphPad Software, San Diego, USA) at a confidence level of 95%. BW at birth, mean BW gain before weaning, BW at weaning, mean BW gain after weaning, BW

at puberty, age at puberty, ejaculate volume, concentration, total sperm per ejaculate, massal motility, progressive individual motility, major defect, minor defect, total defect and serum biochemistry analysis of T and NT were analyzed by Student's t-test. Due to lack of normality of the WBC data, nonparametric test was used (Mann-Whitney-Wilcoxon tests).

3. Results

3.1. Body weight development

Over two years of the trial, no significant differences were detected between T and NT animals regarding their BW development ($P > 0.05$; Table 1). In addition, numerically similar values were observed in females and males, respectively, for BW at birth (T: 2.62 ± 0.38; NT: 2.20) and (T: 2.43 ± 0.11; NT: 2.38 ± 0.16), mean BW gain before weaning (T: 0.85 ± 0.10; NT: 0.74 ± 0.24) and (T: 0.88 ± 0.17; NT: 0.86 ± 0.25), mean BW gain after weaning (T: 0.28 ± 0.46; NT: 0.27 ± 0.50) and (T: 0.19 ± 0.43; NT: 0.18 ± 0.29), BW at weaning (T: 13.67 ± 1.19; NT: 11.80) and (T: 13.80 ± 1.34; NT: 13.55 ± 2.55), and BW at puberty (T: 18.50 ± 3.93; NT: 17.69) and (T: 16.70 ± 1.34; NT: 13.97 ± 1.92). The comparison between males and females were not statistically analyzed due to small group size (< 3 animals).

3.2. Puberty and physical and morphological characteristics of semen

The age at puberty (months) was similar ($P > 0.05$) between T (5.13 ± 1.00) and NT (5.06 ± 1.20). Regarding the gender, females and males, respectively, were observed to be pubertal at (T: 5.30 ± 1.40; NT: 6.77) and (T: 4.90 ± 0.00; NT: 4.50 ± 0.49) months. Interestingly, regardless to the presence of transgene, females were detected in puberty at 5.66 ± 1.37 months whereas males at 4.67 ± 0.40.

No significant differences ($P > 0.05$) between T and NT males were observed in all semen parameters evaluated (Table 2). Regardless to the presence of transgene, the overall mean of the following variables were: ejaculate volume (0.8 ± 0.12 mL), concentration (3.81 ± 0.99 × 10^9 cells/mL), total sperm per ejaculate (3.17 ± 1.23 × 10^9 cells/mL), massal motility (4.0 ± 0.4), progressive individual motility (83.2 ± 1.6%), major defect (13.71 ± 2.49%), minor defect (1.68 ± 0.40), total defect (15.39 ± 2.88%).

Table 1

Body weight (BW) parameters of F1-generation hG-CSF transgenic and non-transgenic male and female goats before (up to 3 months old) and after weaning (3 to 10 months old)

Parameters	**Transgenic (n = 5)**	**Non-transgenic (n = 4)**
BW at birth (kg)	2.54 ± 0.29	2.34 ± 0.19
Mean BW gain before weaning (kg/week)	0.86 ± 0.09	0.83 ± 0.21
BW at weaning (kg)	13.72 ± 1.59	13.11 ± 2.26
Equation of BW gain before weaning (kg/week)	0.1236x + 2.43	0.1249x + 2.46
Mean BW gain after weaning (kg/week)	0.24 ± 0.32	0.20 ± 0.25
Equation of BW gain after weaning (kg/week)	0.0287x + 12.55	0.0287x + 10.28
BW at puberty (kg)	17.78 ± 3.03	14.90 ± 2.43

Within a row, values with different superscripts represent significant difference ($P < 0.05$).

3.3. Hematological and serum biochemistry evaluations

Total WBC count for T and NT goats throughout the experimental period is shown in Fig. 1A. Significant differences ($P < 0.05$) in mean WBC were detected between T and NT in their first day of life, during and after breastfeeding (Table 3). Although other cell counts were occasionally higher in T goats, differential counts showed that this difference was mainly due to an increased number of neutrophils, which represents 84.6, 67.2 and 56.8% of total WBC, respectively, in the three moments (Fig. 1B). The serum biochemical parameters of T and NT goats throughout the experimental period are presented (Table 4). In comparison with NT goats, no alterations occurred in serum concentrations of glucose, urea, creatinine, AST and ALT in T goats.

Table 2

Physical and morphological semen parameters from F1-generation hG-CSF transgenic and non-transgenic bucks (8 to 10 months old).

Parameter	Transgenic (n = 2)	Non-transgenic (n = 3)
Ejaculate volume (mL)	0.77 ± 0.12	0.84 ± 0.22
Concentration ($x10^9$ cells/mL)	4.14 ± 1.44	3.60 ± 0.81
Total sperm per ejaculate ($x10^9$ cells)	3.24 ± 1.45	3.12 ± 1.29
Massal motility (0 – 5)	4.15 ± 0.63	3.94 ± 0.99
Progressive individual motility (%)	81.00 ± 6.58	84.67 ± 4.42
Major defect (%)	15.55 ± 3.36	12.49 ± 3.15
Minor defect (%)	1.00 ± 0.53	2.13 ± 1.17
Total defect (%)	16.55 ± 3.46	14.61 ± 3.33

Semen was collected from all bucks once per week (five replicates).

Within a row, values with different superscripts represent significant difference ($P < 0.05$).

Table 3

Leukocyte count in peripheral blood of F1-generation hG-CSF transgenic (T) and non-transgenic (NT) goats.

Life period	Group	Cells $\times 10^3$ /µL (mean ± SD)					
		WBC	Neutrophils	Lymphocytes	Eosinophils	Basophils	Monocytes
First day of life	T (5)	174.6 ± 14.7 [a]	149.7 ± 19.3 [a]	16.9 ± 5.8 [a]	0.7 ± 0.8 [a]	0.0 ± 0.0 [a]	2.8 ± 1.0 [a]
	NT (4)	15.0 ± 4.0 [b]	12.6 ± 5.0 [b]	3.2 ± 3.3 [b]	0.1 ± 0.2 [a]	0.0 ± 0.0 [a]	0.2 ± 0.3 [b]
During breastfeeding*	T(5)	66.8 ± 21.1 [a]	44.9 ± 20.0 [a]	20.0 ± 6.8 [a]	0.8 ± 0.7 [a]	0.0 ± 0.0 [a]	0.7 ± 0.5 [a]
	NT (4)	17.0 ± 4.6 [b]	7.2 ± 3.2 [b]	12.2 ± 6.9 [a]	0.3 ± 0.3 [a]	0.0 ± 0.0 [a]	0.1 ± 0.1 [a]
After breastfeeding**	T(5)	36.6 ± 4.0 [a]	20.8 ± 3.4 [a]	13.5 ± 2.3 [a]	1.9 ± 0.9 [a]	0.0 ± 0.0 [a]	0.4 ± 0.2 [a]
	NT (4)	15.5 ± 2.2 [b]	6.2 ± 1.0 [b]	8.1 ± 1.6 [a]	0.5 ± 0.2 [a]	0.0 ± 0.0 [a]	0.1 ± 0.1 [a]
Normal range***		4.0 to 13.0	1.2 to 7.2	2.0 to 9.0	0.05 to 0.65	0 to 0.12	0 to 0.55

( ) number of animals;

*During breastfeeding: average obtained from the second day of life up to 3 months-old; ** After breastfeeding: average obtained from 3 to 10 months-old;

*** Reference value reported by Pugh, 2002. WBC: Total white blood cell count.

Within a column, values with different superscripts represent significant difference ($P < 0.05$).

Table 4

Serum biochemistry of F1-generation hG-CSF-transgenic (T) and non-transgenic (NT) goats.

Life period	Group	Biochemical dosages (mean ± SD)				
		Glucose [1] (mg/dL)	Urea [2] (mg/dL)	Creatinine [1] (mg/dL)	ALT [1] (IU/L)	AST [1] (IU/L)
First day of life	T (5)	91.02 ± 7.51	39.40 ± 6.55	0.95 ± 0.11	12.37 ± 5.42	61.14 ± 3.70
	NT (4)	100.37 ± 17.29	33.55 ± 3.52	0.91 ± 0.10	15.00 ± 6.75	63.04 ± 12.62
During breastfeeding*	T (5)	56.57 ± 5.32	36.80 ± 2.91	1.02 ± 0.04	19.80 ± 3.65	62.68 ± 4.00
	NT (4)	57.84 ± 3.53	36.61 ± 3.74	1.09 ± 0.17	18.63 ± 2.33	60.05 ± 4.30
After Breastfeeding**	T (5)	50.96 ± 4.24	37.89 ± 0.74	1.06 ± 0.22	15.60 ± 4.36	57.80 ± 8.66
	NT (4)	52.84 ± 4.32	30.92 ± 1.51	1.10 ± 0.07	18.50 ± 7.23	66.25 ± 12.17
Normal range		48.2 to 76.0	21.4 to 42.8	0.7 to 1.5	15.3 to 52.3	66.0 to 230.0

( ) number of animals;

[1] Reference value reported by Boyd, 1984. [2] Reference value reported by Kaneko, 1997. AST: Aspartate aminotransferase. ALT: Alanine aminotransferase. *During breastfeeding: average obtained from the second day of life up to 3 months-old; ** After breastfeeding: average obtained from 3 to 10 months-old; Within a column, values with different superscripts represent significant difference ($P < 0.05$).

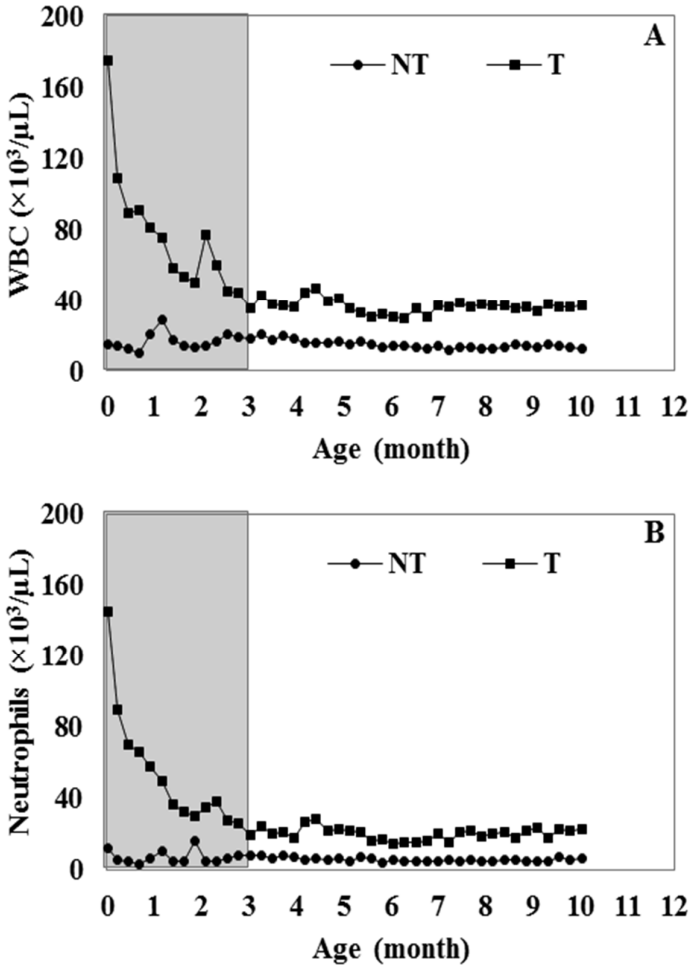

Fig. 1. Leukocyte profile o f F1-generation hG-CSF-transgenic (T; n=5) and non-transgenic (NT; n=4) goats during (shaded area) and after (white area) breastfeeding. (A) White blood cell (WBC) and (B) neutrophils count.

3.4 Human G-CSF analysis in serum

In all nine goats (T and NT), hG-CSF protein was not detected in any serum blood samples: in their first day of life, during or after breastfeeding.

4. Discussion

In this study, several parameters associated with growth and reproduction of F1-generation transgenic goats for hG-CSF were evaluated to assess the performance of these lines (10M and 12F) when compared with their NT siblings. As the hG-CSF transgenic line was generated by pronuclear microinjection (Freitas et al., 2012), randomly integrated foreign DNA would be of concern, since it may increase the risk of disruption of endogenous genes (Jackson et al., 2010) and disturb normal physiological processes. In this context, it has been suggested that three aspects of the welfare of a transgenic line should be evaluated including the effects of insertional mutagenesis, transgene expression and, if applicable, effects of *in vitro* reproduction technologies if somatic cell nuclear transfer techniques were used to generate the transgenic line (Van Reenen 2009).

None of the growth and reproductive traits was affected by presence of the transgene. No significant differences between T and NT were observed in the BW at birth, mean BW gain before weaning, mean BW gain after weaning, BW at weaning, and BW at puberty, suggesting that the site of integration of the transgene did not appear to interrupt an endogenous gene for growth in utero and absorption of nutrients after birth. During all experimental period, the mean BW gain observed in this study was 0.06 and 0.05 kg per day for T and NT, respectively, all within the range of 0.02–0.3 kg per day reported in the literature (National Research Council, 2007).

The age at puberty observed for males and females, for both T and NT were within the range related in literature for caprine species (3-7 months of age; Jackson et al., 2010) and the onset

of puberty initiated when expected considering their BW. Additionally, semen physical (ejaculate volume, concentration, total sperm per ejaculate, massal motility, progressive individual motility) and morphological (major, minor, and total defect) characteristics were similar between both groups. Values observed for these parameters are within the normal range for goats: volume of 0.1–1.5 mL, concentration of 2–6 billion sperm per mL of ejaculate, progressive individual motility of 82–93%, and normal morphology of 80–95% (Hafez and Hafez, 2000; Nur et al., 2005). Overall, the presence of the hG-CSF transgene in the genome of these animals does not appear to interfere on their normal reproductive parameters.

Human G-CSF regulates the proliferation, differentiation and maturation of hematopoietic cells (Crea et al., 2009). This molecule exerts biological functions through specific receptors expressed on the surface of target cells (Richt et al., 2006). We observed in this study, an increase of 11.6, 4.5 and 2.4-fold in WBC count of T compared to NT goats in their first day of life, during and after breastfeeding, respectively. This result suggests that G-CSF, although a human protein, is able to affect goat hematopoietic stem cells, stimulating their proliferation and differentiation, mainly for neutrophils. It is important to highlight that we did not detect the presence of this cytokine in the serum in any T goat, at different moments of evaluation. It is noteworthy that neither chronic nor acute kidney disease was detected, as demonstrated by normal concentrations of 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. Finally, this information can provide support contributing to the knowledge required to make science-based regulatory decisions regarding the use of transgenic animals.

5. Conclusion

Despite the increased white blood cell count due to neutrophilia, first-generation hG-CSF-transgenic male and female goats remained clinically healthy throughout the experimental period until now. Their body weight development was considered normal both prenatal and during their first months of age. Moreover, all the reproductive characteristics evaluated such as age at puberty and semen physiological and morphological analysis were not adversely affected by the insertion of the transgene. Therefore, it is possible to assume that all animals are normal and had no deleterious effects on either growth or reproductive parameters by the presence of the transgene or as a consequence of leukocyte profile alteration.

Conflict of interest statement

The authors wish to confirm that there are no known conflicts of interest associated with this publication.

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6 CAPITULO 3

Características fenotípicas da primeira geração de caprinos transgênicos para produção do Fator Estimulante de Colônias de Granulócitos humano no leite

Resumo

O fator estimulante de colônias de granulócito humano (hG-CSF) é um fator de crescimento hematopoiético usado em pacientes neutropênicos. Esta proteína é produzida em sistemas bacterianos ou células de mamíferos cultivadas. Como alternativa, foi demonstrada agora a expressão de hG-CSF na glândula mamaria da primeira geração (F1) de cabras transgênicas durante a lactação induzida. Apesar de menor produção de leite, as fêmeas transgênicas apresentaram composição do leite semelhante (gordura, proteína e lactose), quando comparados as não-transgênicas ($p > 0,05$). A concentração média (±DP) de hG-CSF recombinante no leite durante a lactação foi 360,0±178,2 mg mL^{-1}. Todos os parâmetros clínicos, bem como função renal e hepática indicaram que cabras transgênicas F1 eram saudáveis. Além disso, não foi detectada expressão ectópica de hG-CSF nos tecidos de machos F1 transgênicos. Em conclusão, cabras F1 transgênicas para hG-CSF foram capazes de expressar a proteína recombinante no leite em quantidades compatíveis com a sua utilização como bioreactores em um programa de produção de proteína em escala comercial.

Palavras-chave: biorreator , cabras , lactação induzida , Neutrofilia , Transgênese

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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. This protein is produced in bacterial systems or cultured mammalian cells. As an alternative, it was now demonstrated the hG-CSF expression in the mammary gland of first-generation (F1) transgenic goats during induced lactation. Despite lower milk production, transgenic females presented 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 360.0 ± 178.2 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. In conclusion, F1 hG-CSF-transgenic goats were able to express

recombinant protein in milk at quantities compatible with their use as bioreactors in a commercial-scale protein-production program.

Keywords Bioreactor, Goats, Induced lactation, Neutrophilia, Transgenesis

Introduction

Recombinant proteins of high economic value have been successfully 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 pounds 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) cell system 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 a couple of transgenic goats (10M and 12F lineages) containing hG-CSF fused to goat α-S1 casein (CSN1S1) promoter (Freitas et al. 2012). Later on, 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 ten-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 × *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 × *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 optical density was measured at 450 nm in a plate reader (Multiskan FC; Thermo Scientific).

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 eight days before and during the induced lactation, every two 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 two 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 for 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 26.0 ± 23.0 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 vs. 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 ± 0.8 vs. 4.9 ± 1.0%), dry extract (11.8 ± 2.0 vs. 12.3 ± 1.0%) 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}).

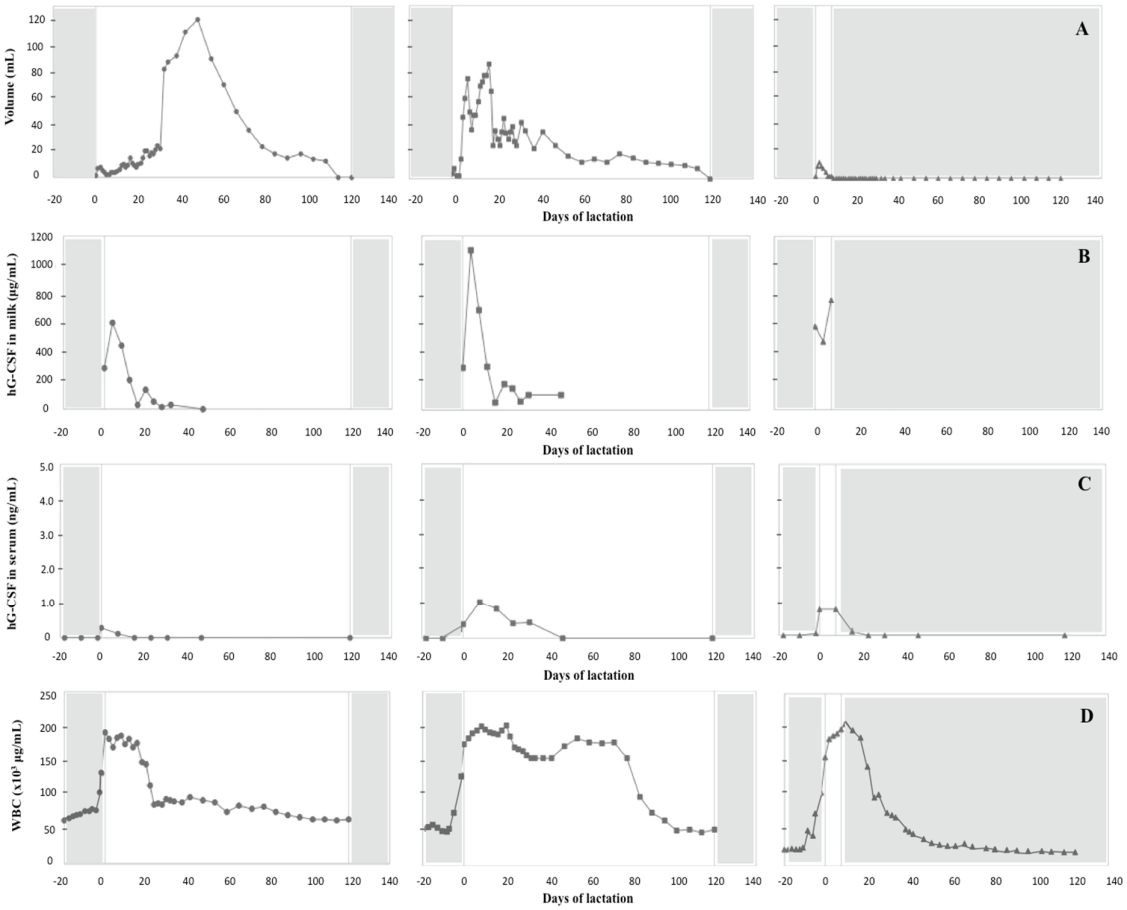


Fig. 1 Hormonally induced lactation of F1-generation hG-CSF-transgenic and non-transgenic (control) goats. Mean of milk production (A), hG-CSF concentration in both milk (B) and blood serum (C) and total leukocyte counts (D) of three transgenic (●) and three non-transgenic females (■) were plotted versus lactation period. Periods before (negative days) and after milk secretion were presented in shaded area.

hG-CSF quantification

Human G-CSF was successfully 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 360.0 ± 178.2 µg mL^{-1}. Human G-CSF concentration in the milk of T goats ranged from 25.1 to 1,098.8 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, two days before the beginning of milk production at levels of 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 count 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.

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

	Group	Cells $\times 10^3$ μL^{-1} (mean ± SD)					
		WBC	Neutrophils	Lymphocytes	Eosinophils	Basophils	Monocytes
Before	T	$48.5 \pm 18.6^{A,a}$	$39.7 \pm 23.9^{A,a}$	$15.6 \pm 5.1^{A,a}$	$3.3 \pm 2.0^{A,a}$	$0.0 \pm 0.0^{A,a}$	$0.9 \pm 1.8^{A,a}$
	NT	$13.8 \pm 3.9^{AB,b}$	$6.4 \pm 2.7^{A,b}$	$7.1 \pm 2.4^{AB,b}$	$0.3 \pm 0.4^{A,b}$	$0.0 \pm 0.0^{A,a}$	$0.1 \pm 0.2^{A,b}$
During	T	$139.6 \pm 49.4^{B,a}$	$118.3 \pm 46.5^{B,a}$	$20.4 \pm 9.8^{B,a}$	$2.2 \pm 2.2^{B,a}$	$0.0 \pm 0.0^{A,a}$	$1.1 \pm 1.6^{A,a}$
	NT	$16.6 \pm 2.8^{B,b}$	$8.8 \pm 2.6^{B,b}$	$7.2 \pm 2.0^{A,b}$	$1.8 \pm 5.2^{B,b}$	$0.0 \pm 0.0^{A,a}$	$0.2 \pm 0.2^{A,a}$
After	T	$63.2 \pm 50.4^{A,a}$	$46.4 \pm 20.0^{A,a}$	$15.8 \pm 7.5^{AB,a}$	$6.0 \pm 7.2^{A,a}$	$0.0 \pm 0.0^{A,a}$	$2.7 \pm 5.2^{A,a}$
	NT	$9.3 \pm 2.9^{A,b}$	$4.0 \pm 1.5^{C,b}$	$4.9 \pm 1.8^{B,b}$	$0.4 \pm 0.3^{AB,b}$	$0.0 \pm 0.0^{A,a}$	$0.1 \pm 0.1^{A,b}$
Normal range[1]		4.0 to 13.0	1.2 to 7.2	2.0 to 9.0	0.05 to 0.65	0 to 0.12	0 to 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 vs. 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 [1] (mg dL^{-1})	Urea [2] (mg dL^{-1})	Creatinine[1] (mg dL^{-1})	ALT [1] (IU L^{-1})	AST [1] (IU L^{-1})
Before	T	54.5 ± 7.1	35.1 ± 9.5	1.1 ± 0.2	15.3 ± 2.0	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 ± 0.2	15.2 ± 2.5	142.0 ± 64.7
	NT	53.7 ± 9.2	34.5 ± 4.7	1.0 ± 0.1	18.6 ± 4.7	88.6 ± 14.0
After	T	58.2 ± 7.5	31.7 ± 8.6	1.0 ± 0.1	16.4 ± 2.2	119.0 ± 52.6
	NT	54.7 ± 4.8	38.8 ± 7.0	1.0 ± 0.1	18.4 ± 1.5	94.2 ± 21.1
Normal range		48.2 to 76.0	21.4 to 42.8	0.7 to 1.5	15.3 to 52.3	66.0 to 230.0

Reference values reported by Boyd (1984)[1] and Kaneko et al. (1997)[2]. Aspartate aminotransferase (AST), Alanine aminotransferase (ALT). Within a column values with different superscripts differ significantly ($p < 0.05$).

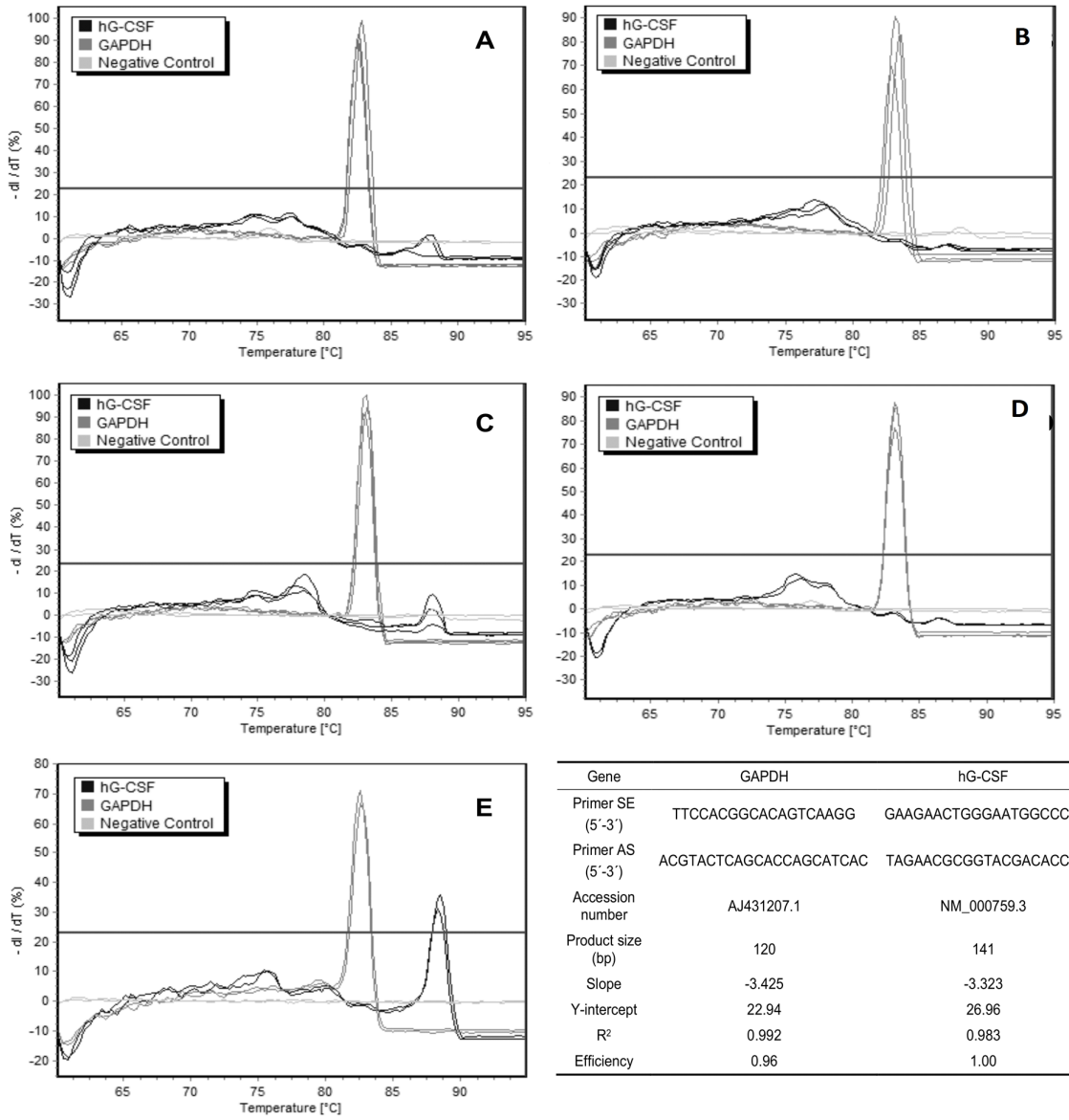
Table 3 Clinical monitoring of F1-generation hG-CSF-transgenic (T) and nontransgenic (NT) goats before, during and after during hormonally induced lactation

Period	Group	Clinical parameters (mean ± SD)		
		Rectal temperature (^{o}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 ± 3.0	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 ± 3.0	73.2 ± 7.6
Normal range		37.5 to 39.7	12.0 to 25.0	70.0 to 110.0

Reference value reported by Pugh, 2002. Within a column values with different superscripts differ significantly ($p < 0.05$).

Ectopic transgene expression investigation

No hG-CSF transcripts were detected in lymphocyte samples of T and NT females before (Fig. 2A and 2B) or during (Fig. 2C and 2D) 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.



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 and B) or during (C and D) lactation period from transgenic (A and C) or non-transgenic (B and 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 to 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.

Discussion

Overall, concerning hG-CSF expression, the milk of F1 transgenic females had similar order 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-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 (10M and 12F) is compatible with investments for recombinant production in milk on a commercial scale.

Some studies have shown that the productions of large quantities of recombinant protein in the mammary gland of transgenic goats compromise 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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9 CONCLUSÕES

- As fêmeas F1 transgênicas para hG-CSF, expressam a proteína recombinante no leite em quantidade compatível para serem usadas como biorreatores.
- A presença do hG-CSF em caprinos transgênicos da linhagem 10M e 12F não comprometeu os parâmetros produtivos e reprodutivos dos mesmos.
- A inserção do gene exógeno (hG-CSF) e a expressão do mesmo na glândula mamaria aumentaram a proliferação e a diferenciação das células da linhagem granulocítica, no entanto sem comprometer a saúde dos animais.
- A expressão do hG-CSF no leite das fêmeas F1 comprometeu a lactação, sem alterar a composição do leite.
- A expressão do hG-CSF induz um aumento de células somáticas do leite, mas sem ocorrência de mastite.
- Não foi verificada expressão ectópica em leucócitos, linfonodos, baço ou fígado dos caprinos transgênicos para o hG-CSF.

10 PERSPECTIVAS

Presente estudo demonstrou que a geração F1 das duas linhagem de caprinos transgênicos fundadores para o hG-CSF são saudáveis e expressam corretamente a molécula de interesse e numa concentração compatível para serem usadas como biorreatores. Com isso, a principal perspectiva é a purificação dessa proteína que se encontra diluída dentre centenas de outras proteínas componentes do leite.

Outra vertente que o presente trabalho proporciona é a necessidade do estudo sobre os mecanismos que comprometem a lactação pela expressão do transgene na glândula mamaria. Apesar de crescente uso de animais transgênicos para expressão de proteínas recombinantes na glândula mamária, este aspecto ainda é negligenciado na maioria dos estudos sobre a características fenotípicas de animais transgênicos.

Finalmente, torna-se também imprescindível a formação de um rebanho transgênico para o hG-CSF tornando próximo da realidade a produção da proteína recombinante em escala industrial.

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12 APÊNDICES

12.1 APÊNDICE A

ARTIGO PUBLICADO: *Acta Veterinaria Brasilica, v.8, Supl. 2, p. 402-406, 2014*

Transgênese em caprinos

(*Transgenesis in goats)*

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Resumo: Entre todos os mamíferos transgênicos produzidos até hoje, a espécie caprina tem representado um excelente modelo em transgênese quando são considerados os fatores como a demanda do mercado para a proteína, o volume de leite produzido por lactação e as taxas reprodutivas. Várias proteínas recombinantes foram obtidas a partir de cabras transgênicas, e entre estas, a antitrombina humana, foi a primeira proteína recombinante de origem animal a ser aprovada como um medicamento para a utilização clínica em seres humanos. Esta revisão tem por objetivo apresentar o estado-da-arte em caprinos transgênicos dando especial ênfase aos resultados obtidos por nosso grupo.

Palavras-chave: *Capra hircus*, engenharia genética, leite, proteína recombinante.

Abstract: Among all the transgenic mammalians produced so far, goats have represented an excellent model of transgenesis when considering the factors such as the market demand for protein, volume of milk produced per lactation and reproductive rate. Various recombinant proteins have been obtained from the transgenic goats, and among these, human antithrombin was the first recombinant protein of animal origin to be released as a drug for the clinical use in humans. This review aims to present the state-of-the-art in transgenic goats with special emphasis on results obtained by our group.

Keywords: *Capra hircus*, genetic engineering, milk, recombinant protein.

1. Introdução

Proteínas humanas têm sido utilizados na medicina mundialmente e, durante algum tempo, o seu fornecimento foi limitado devido às poucas fontes para a sua extração (CLARK, 1998). Com o advento da genética engenharia, essa realidade mudou, pois genes de interesse podem ser isolados, inseridos em vetores de expressão e transferidos para células ou organismos que podem, dessa forma, serem transformados em produtores de proteínas em escala industrial (HOUDEBINE, 2003). As primeiras tentativas de produção de proteínas de valor terapêutico a partir de genes clonados foram em microrganismos. No entanto, dessa maneira, algumas proteínas humanas são sintetizados apenas em pequenas quantidades, outras tornam-se insolúveis fazendo sua purificação de difícil realização. Além disso, os produtos podem apresentar-se como imunogênicos ou com pouca atividade biológica devido ao processamento pós-traducional incorreto (KUES & NIEMANN, 2004).

Desde 1982, com o nascimento do primeiro mamífero (camundongo) expressando níveis elevados do hormônio do crescimento humano (PALMITER et al., 1982), verificou-se que os animais podiam ser utilizados como biorreatores. Posteriormente, vários animais transgênicos

foram obtidos em diferentes espécies: coelhos, suínos e ovinos (HAMMER et al., 1985), caprinos (EBERT et al., 1991) e bovinos (KRIMPENFORT et al., 1991).

De acordo com CLARK (1998), para a produção de proteínas recombinantes, alguns aspectos devem ser considerados na escolha da espécie como reator biológico, como a demanda do mercado pela proteína, o volume de leite produzido por lactação e as taxas de reprodução da espécie. De todos os biorreatores mamíferos já produzidos, os caprinos (*Capra hircus*) representam um excelente modelo para transgênese, já que a produção de animais fundadores e custos operacionais são significativamente mais fáceis de gerenciar em comparação com bovinos, por exemplo.

Neste manuscrito serão apresentados os aspectos inerentes à produção de caprinos transgênicos. Além disso, uma ênfase especial será dada aos resultados do nosso grupo na obtenção de caprinos transgênicos para o Fator Estimulante de Colônia de Granulócitos humano (hG-CSF).

2. Métodos para a produção de caprinos transgênicos

Caprinos transgênicos já foram obtidos por microinjeção pronuclear e transferência nuclear de células somáticas (TNCS). As principais etapas dessas duas técnicas são apresentadas na Figura 1.

A microinjeção pronuclear é o método tradicional para a produção de caprinos transgênicos e consiste na microinjeção de uma construção gênica (gene de interesse + gene de expressão em glândula mamária) em um dos pró-núcleos do embrião recém-fecundado. A microinjeção pronuclear é realizada em microscópio invertido munido de micromanipuladores. Uma micropipeta contendo a construção de DNA (cerca de 500-5000 cópias em 1-2 pL) penetra na zona pelúcida e a injeção é realizada em um dos pró-núcleos (HOUDEBINE, 2003). A microinjeção deve ser realizada entre 15 e 20 h após a provável fecundação. Além disso,

devido à grande quantidade de gotas lipídicas no citoplasma, os embriões caprinos devem ser primeiramente submetidos à uma centrifugação. No entanto, a microinjeção pronuclear é um método que apresenta uma baixa eficiência e, geralmente, menos de 10% da prole é transgênica (BALDASSARRE & KARATZAS, 2004).

A TNCS, combinada com técnicas de biologia molecular e celular, mostra uma variedade de aplicações. Entre as diferentes áreas, a transgênese é, possivelmente, a que mais se beneficiou com os avanços nesta biotécnica, no sentido de aumentar a eficácia e reduzir os custos. Desde o nascimento da ovelha Dolly (WILMUT et al., 1997), a tecnologia de TNCS continua praticamente a mesma e consiste na transferência de núcleo de células doadoras (carioplastos) para oócitos enucleados (citoplastos) com reconstrução posterior do embrião através da fusão celular. A TNCS pode produzir animais transgênicos através da transfecção de núcleos com vetores de DNA ou através da clonagem de animais transgênicos fundadores (BALDASSARRE & KARATZAS, 2004).

No método de TNCS, utilizando a transfecção de núcleos, o DNA exógeno é incorporado aleatoriamente no genoma utilizando pressão seletiva. Além disso, as células transgênicas podem ser completamente caracterizadas com relação ao local de integração, o número de cópias integradas e integridade do transgene antes da etapa de transferência nuclear propriamente dita. Embora a capacidade para desenvolvimento dos embriões reconstruídos é baixa, a maioria da descendência é transgênica, o que torna esta técnica muito mais eficiente do que a microinjeção pronuclear (BEHBOODI et al., 2005).

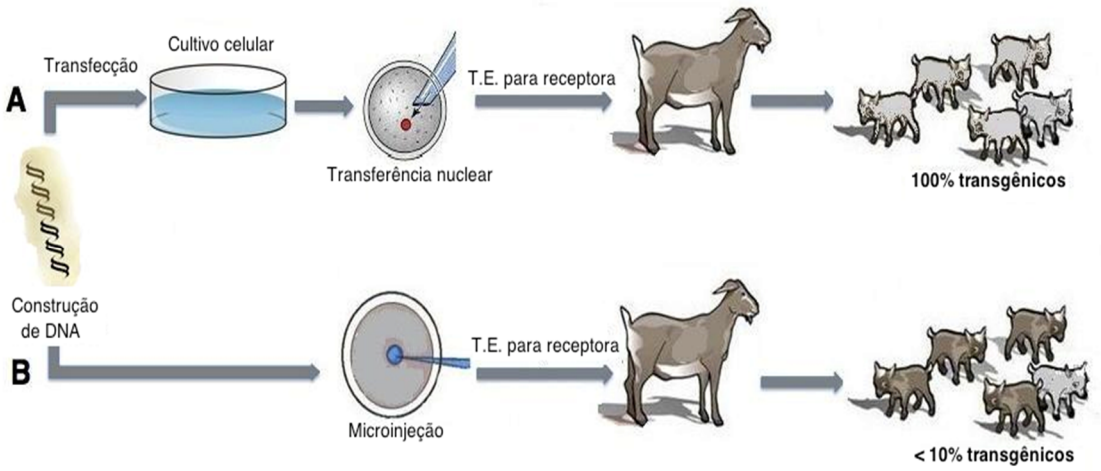

Figura 1: Métodos para a produção de caprinos transgênicos: A) transferência nuclear de células somáticas (TNCS) e B) microinjeção pronuclear.

3. A glândula mamária como biorreator

A glândula mamária é uma glândula cutânea de característica apócrina/exócrina, com composição túbulo-alveolar e suas unidades secretoras consistem de alvéolos que levam a pequenos ductos excretores. Os grupos de unidades secretoras formam lóbulos que são posteriormente agrupados em estruturas maiores chamadas lobos, formando o parênquima mamário.

Os alvéolos são as unidades secretoras fundamentais de leite e são compostos por células epiteliais capazes de sintetizar as gorduras, carboidratos e proteínas, expulsando o produto para o interior do lúmen dos alvéolos. As proteínas secretadas pela glândula mamária são agrupados em duas classes, caseínas e proteínas do soro. De acordo com PARK et al. (2007), os ruminantes secretam principalmente quatro caseínas (αS1, αS2, β e κ) e duas proteínas séricas (β-lactoglobulina e α-lactoalbumina). Os genes que codificam estas proteínas estão em cópias simples e são transcritas a níveis elevados, em especial durante a prenhez e lactação (CLARK, 1998). Assim, trabalhos pioneiros nos anos 1980 exploraram a capacidade da produção de proteínas recombinantes no leite de animais transgênicos (GORDON et al., 1987;

SIMMONS et al., 1987). Assim, promotores e regiões regulatórias de genes específicos para proteínas do leite foram utilizados para direcionar a expressão do gene na glândula mamária (MAGA & MURRAY, 1995). Um exemplo dessas construções, com objetivo de secretar proteínas recombinantes no leite, pode ser visualizado na Figura 2, e foi utilizado na obtenção de camundongos e caprinos transgênicos para o hG-CSF.

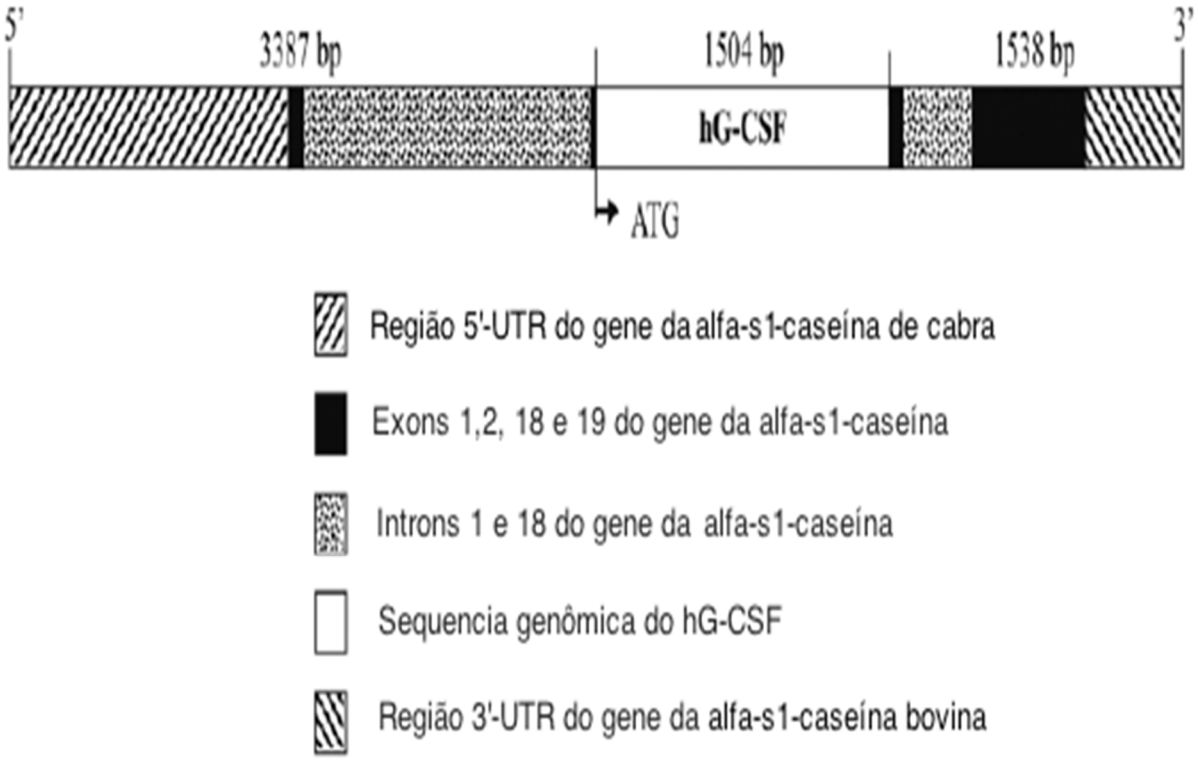

Figura 2: Construção gênica para direcionar a expressão do hG-CSF no leite de camundongos e caprinos (DVORIANCHIKOV et al., 2005)

Como explicitado anteriormente, vários caprinos transgênicos já foram obtidos em diferentes laboratórios ao redor do mundo, sendo que a maioria foi planejada para a secreção de proteínas de valor terapêutico no leite desses animais (Tabela 1).

Tabela 1: Nível de expressão, do mais alto para o mais baixo, de proteínas humanas recombinantes secretadas no leite de caprinos transgênicos.

Proteína humana	Promotor	Nível de expressão (mg/mL)	Referência
Antitrombina	β-caseína caprina	0,09 – 12,5	ZHOU et al., 2005
Butirilcolinesterase	β-caseína caprina	0,1 – 5,0	HUANG et al., 2007
Lactoferrina	β-caseína caprina	0,77	ZHANG et al., 2008
Lisozima	α-s1-caseína caprina	0,27	MAGA et al., 2006
G-CSF	α-s1-caseína caprina/bovina	0,62	FREITAS et al., 2012
G-CSF	β-caseína caprina	0,05	KO et al., 2000
Fator IX	β-caseína bovina	$9,5 \times 10^{-5}$	HUANG et al., 1998

4. Resultados obtidos no Brasil

O Brasil vem se apresentando como uma referencia internacional na obtenção de caprinos transgênicos através de dois grupos localizados em Fortaleza (CE). O primeiro, pioneiro com trabalhos nesta espécie, é o grupo do Laboratório de Fisiologia e Controle da Reprodução-LFCR da Universidade Estadual do Ceará (UECE). Este grupo obteve caprinos transgênicos para o hG-CSF em duas ocasiões: 2006 e 2008 (FREITAS et al., 2007; FREITAS et al., 2012). Já o segundo grupo, do Laboratório de Biologia Molecular e do Desenvolvimento da Universidade de Fortaleza (UNIFOR). Este grupo obteve caprinos transgênicos para a lisozima (BERTOLINI, comunicação pessoal, 2012).

Nas pesquisas realizadas no LFCR foram utilizadas primeiramente cabras da raça Saanen. Em um segundo momento, objetivando agregar valor a uma raça naturalizada do Nordeste, utilizou-se cabras Canindé. Na comparação entre estas duas raças, foi verificada uma leve superioridade (não significativa) dos caprinos naturalizados sobre os exóticos (FREITAS et al., 2014), como pode ser observado na Tabela 2. Vale ressaltar que animais transgênicos foram obtidos somente com receptoras que receberam embriões oriundos de doadoras Canindé.

Tabela 2: Diagnóstico de prenhez, a sobrevivência embrionária e fertilidade ao parto de receptoras sem raça definida que receberam embriões previamente colhidos de doadoras Canindé e Saanen e microinjetados com uma construção de DNA.

Raça	Diagnóstico de prenhez 30 dias		Ao parto	
	Prenhes	Sobrevivência	Recep. parindo	Sobrevivência
Canindé	14/20 (70,0%)	20/61 (31,8%)	12/20 (60,0%)	18/61 (29,5%)
Saanen	5/12 (41,7%)	7/30 (23,3%)	3/12 (25,0%)	5/30 (16,7%)

Com os dois animais fundadores, obtidos por microinjeção pronuclear em 2008, o grupo realizou estudos para a caracterização fenotípica e genotípica desses animais. Um desses estudos foi o uso da indução hormonal da lactação na fêmea fundadora e a verificação do nível de expressão do hG-CSF no leite. Assim, foi observado, por dosagem ELISA que a fêmea fundadora produz em média (± DP) 620,92 ± 179,93 μg/mL de leite. Esta produção é cerca de 10 vezes superior ao outro caprino transgênico para o hG-CSF obtido por pesquisadores sul-coreanos (KO et al., 2000).

Adicionalmente, os dois animais obtidos neste experimento demonstraram ser verdadeiros fundadores, já que através de sua reprodução com caprinos não transgênicos foram obtidas

crias transgênicas por herança mendeliana normal: 54,4% das crias do macho fundador foram transgênicas e 37,5% das crias da fêmea fundadora possuíam o gene exógeno (FREITAS et al., 2012).

5. Considerações finais

Uma vez obtidos os primeiros caprinos transgênicos no mundo, esta espécie tem sido utilizada como um modelo para o a produção de proteínas recombinantes. Durante este tempo, mesmo após ter sido adquirido um conhecimento amplo dos vários aspectos que influenciam a produção de animais transgênicos, as taxas de sucesso continuam baixas. No entanto, várias proteínas para uso terapêutico em humanos foram produzidas, purificadas e caracterizadas.

No Brasil, com a produção dos primeiros caprinos transgênicos, espera-se que o mesmo possa juntar-se ao seleto grupo de países que dominam essa tecnologia e que seja possível reduzir os custos de produção de proteínas recombinantes de interesse em saúde humana.

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12.2 APÊNDICE B

III Animal Lab, 2011, Fortaleza. Animal Sciences. Fortaleza: Editora UECE, 2011. v. 1. 3.

AVALIAÇÃO DA PRESERVAÇÃO DE BULBOS CAPILARES CAPRINOS ATRAVÉS DE QPCR COM SONDA TAQMAN® PARA AMPLIFICAÇÃO DO GENE DA BETA-LACTOGLOBULINA

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Bulbos capilares são amostras teciduais de coleta pouco invasiva, frequentemente utilizadas para análises de DNA genômico (DNAg) de animais de produção. Neste sentido, a preservação adequada do tecido durante o transporte do local de coleta até o laboratório é importante para a manutenção da integridade do DNAg por longos períodos. Assim, o objetivo deste estudo (aprovado pelo CEUA/UECE, n.09172814-2) foi avaliar a melhor condição de preservação de bulbos capilares de caprinos através da quantificação do gene da β-Lactoglobulina (BLG) por PCR em tempo real (qPCR). Seis amostras contendo cerca de 50 bulbos cada foram coletadas de fêmeas caprinas da raça Canindé e estocadas a temperatura ambiente sem preservante (TA), com etanol (TA-EtOH) ou a -20° C sem preservante (T-20), durante 24 h ou 7 dias. O DNAg foi purificado utilizando o Wizard SV Genomic DNA Purification kit (Promega). As reações de qPCR ocorreram em triplicata para cada pool de seis amostras e consistiram de 10 µL contendo 5 µL TaqMan Gene Expression Master Mix (Applied Biosystems), 0,5 µL PrimeTime 20X (IDT, 0,25 µM de cada primer senso e antisenso para BLG caprina e 0,25 µM de sonda ligada a FAM) e 0,5 µL de amostra. Uma

curva padrão foi gerada utilizando uma amostra de DNA extraído de tecido auricular quantificada por espectrofotometria (Biophotometer, Eppendorf). Os dados foram expressos em média ± erro padrão e comparados pelo teste T, com significância de 5%. O número de cópias de BLG nas amostras foi calculado a partir do tamanho do genoma diplóide caprino (**C-value** = 6,48). A amplificação das reações para BLG apresentaram altos valores de linearidade (R^2=0,99) e eficiência (1,09). O limite de amplificação foi de 7,74 pg de DNAg, o referente a uma célula diplóide. O número de cópias de BLG por µL foi significativamente menor em amostras preservadas por 24 h em TA-EtOH (2.156,25 ± 254,92) quando comparadas à TA (3.360,12 ± 220,34), a qual não diferiu da preservação à T-20 (4.545,72 ± 642,62). Após 7 dias de armazenamento à TA sem preservante, a quantidade total de BLG foi significativamente inferior àquela detectada em 24 h (1.416,60 ± 182,25 **versus** 3.360,12 ± 220,34, respectivamente). Contudo, quando comparadas entre si, após 7 dias de armazenamento, não houve diferença quantitativa entre as três condições testadas (1.416,60 ± 182,25; 1.901,56 ± 513,93 e 2.548,56 ± 279,98, para TA, TA-EtOH e T-20, respectivamente). Em conjunto, nosso resultados indicam que amostras de bulbos capilares caprinos que irão ser submetidas à obtenção de DNAg dentro do período de 24 h a partir da coleta podem ser igualmente bem preservadas à -20°C ou à temperatura ambiente. As quantidades de DNAg obtidas de amostras após 7 dias de preservação à temperatura ambiente, com ou sem etanol, ou à -20°C parecem ser similares. No entanto, a degradação à temperatura ambiente sem etanol parece ser mais intensa dentro desse período, quando comparada às demais condições. Assim, só havendo condições de preservação à temperatura ambiente, indica-se que as amostras sejam processadas dentro de 24 h a partir da coleta. Adicionalmente, tempos intermediários entre 24 h e 7 dias devem ser avaliados para o acesso à velocidade de degradação do DNAg nas diferentes condições de preservação. Finalmente, a coleta desse tipo

de amostra não demanda qualquer procedimento anestésico, podendo ser realizada a qualquer momento, sem praticamente induzir dor ou quaisquer ferimentos nos animais.

12.3 APÊNDICE C

RESUMO PUBLICADO: *Acta Scientiae Veterinariae*, **39 (Supl. 1):** 411, 2011.

IN VITRO EMBRYO PRODUCTION (IVEP) AFTER LAPAROSCOPIC OOCYTE RECOVERY (LOR) IN CANINDÉ GOATS

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Currently, a major concern of livestock is the biodiversity preservation. In Northeast Brazil, there are several naturalized goat breeds at risk of extinction, including the Canindé. Reproductive biotechnologies could participate in this process. From these, IVEP after LOR may accelerate the genetic material recovery. Nevertheless, few studies demonstrate the real efficiency of this system in goats. Therefore, the aim of this study was to evaluate the IVEP coupled with LOR as biotechnique to create an embryo bank for the preservation of Canindé goats. Thus, 20 adult and cyclic goats (five females per session) received intravaginal sponges with 60 mg medroxyprogesterone acetate (Progespon, Buenos Aires, Argentina) for 11 days associated with 70μg cloprostenol (Prolise, Buenos Aires, Brazil) in the eighth day. Thirty-six hours before sponge removal, animals received 70 mg pFSH (Folltropin, Ontario, Canadá) and 200 IU eCG (Novormon, Buenos Aires, Argentina). The follicles, visualized by laparoscopy, were classified as small (< 3mm), medium (3 to 4 mm) and large (> 4mm) and aspirated just after the sponge removal using an aspiration system for small ruminants

(Watanabe, Cravinhos, Brazil). *Cumulus* -oocyte complexes (COCs) were recovered and classified (grade I to IV) based in the presence of *cumulus* cells and cytoplasm homogeneity. Grade I and II structures were matured in modified TCM199, for 24 h at 38.5°C and 5% CO_2. After this period, COCs were fertilized with fresh spermatozoa ($2x10^6$ sperm/mL) in SOF-FIV medium supplemented with heparin for 16 h in the same maturation conditions. The presumptive zygotes were cultured in SOF-CIV medium, in the same fertilization conditions, for seven days. A total of 245 follicles were punctured and distributed in small (31.5%), medium (35.9%) and large (32.6%). The oocyte recovery rate was 74.3% (182/245) with an average of 9.1 oocytes per goat. Regarding to oocyte quality, 13.2% (24/182), 68.1% (124/182), 5.5% (10/182) and 13.2% (24/182) were classified as grade I, II, III and IV, respectively. The average of COCs submitted to maturation (grade I and II) was 7.5 per goat. From the presumptive zygotes in vitro incubated, 58.3% (84/144) cleaved after 48 h of culture. The blastocyst rate was 52.1% (75/144) regarding the total number of structures in culture. The total percentage of blastocyst in relation to cleaved embryos was 89.3% (75/84). In conclusion, the IVEP-LOR system was efficient to produce Canindé goat blastocysts and may be used in the creation of an embryo bank in order to preserve the breed.

Keywords: goat, Canindé, embryo

12.4 APÊNDICE D

RESUMO PUBLICADO: *Transgenic Research,* **21 (4)**: 21, 2012.

USE OF TWO METHODS FOR EMBRYO RECOVERY IN AN EMBRYO TRANSFER PROGRAM FROM A FOUNDER TRANSGENIC GOAT FOR HUMAN GRANULOCYTE COLONY-STIMULATING FACTOR (hG-CSF)

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In a transgenesis program, after obtaining the founder, its breeding is fundamental to create a transgenic herd secreting the recombinant protein. This study aimed to compare the embryo recovery method (surgical vs. transcervical) to expedite the production of transgenic progeny from a founder transgenic doe for hG-CSF. It was used a transgenic Canindé doe, two non-transgenic Canindé bucks and nine undefined breed recipients. Four *in vivo* embryo production sessions were performed: two by surgical (laparotomy) and two by transcervical method. The embryo donor superovulated using ahormonal treatment consisting of vaginal sponges impregnated with progestagen coupled to pFSH and cloprostenol injections. From the second session, it was used flunixin-meglumine in the prevention of premature regression of corpora lutea (PRCL). The recipients received progestagen and cloprostenol associated with an eCG injection. Donor mating was performed at estrus onset and 24 h later. The embryo recovery was performed 7 days post-estrus and, just before, a laparoscopy was done to assess

the number and quality of CL. Thetranscervical recovery was performed with a circuit and catheter for small ruminants and the donor received a cloprostenol injection at 12 h before collection. The transgenic kids were identified by PCR. During one surgical session and one transcervical, the collection was not performed due to the occurrence of PRCL and follicular cysts, respectively. In total, it was verified 24 CL and the recovery rate was 69.2% (9/13) and 72.7% (8/11), using surgical and transcervical method, respectively. Sixteen embryos were transferred to the recipients and eight kids were born. Two males (surgical method) and 1 female (transcervical method) were identifying as transgenics. In summary, embryo recovery (surgical or transcervical), coupled with embryo transfer, expedited the production of progeny from a transgenic founder doe.

12.5 APÊNDICE E

RESUMO PUBLICADO: *Acta Scientiae Veterinariae*, **39 (Supl. 1):** 448, 2011.

USE OF EMBRYO TRANSCERVICAL COLLECTION IN A FOUNDER TRANSGENIC GOAT FOR HUMAN GRANULOCYTE COLONY-STIMULATING FACTOR (hG-CSF)

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Goats are used as model of transgenic animals secreting recombinant proteins in their milk. After the founder production, its multiplication is indispensable in order to obtain a transgenic herd, which can be achieved by the transfer of *in vivo* produced embryos. However, embryo collection in goats is usually done by surgery, which can promote adhesions, limiting the number of times that it can be applied in the same animal. Thus, the study aimed to use the embryo transcervical collection in a founder goat obtained by our group (Freitas et al., 2010, Transgenic Res, 19, 146) for embryo transfer into synchronized recipients. This study was approved by UECE biosafety and ethic committee. One transgenic founder Canindé doe as a donor, two non-transgenic bucks of the same breed and four u breed recipients were used. The embryo donor goat was submitted to estrus synchronization and superovulation using progestagen (Progespon®, Buenos Aires, Argentina), pFSH injections (Folltropin®, Ontario, Canada) and cloprostenol (Prolise®, Buenos Aires, Argentina). In the prevention of premature regression of corpora lutea (CL), flunixin-meglumine (Flumedin®, Varginha,

Brazil) was used. Donor fertilization was performed at estrus onset and 24 h later. The number of CL was assessed by laparoscopy before embryo collection. Embryo transcervical collection was performed seven days post-estrus. Twelve hours before collection, the donor received 37.5 μg cloprostenol for cervix dilatation. The embryo collection was performed with a circuit and catheter for small ruminants (Circuit/catheter to collect embryos for sheep and goats®, Embrapa, Brasília, Brazil). It was possible to recover almost all the collection medium at the end of process. The transgenic donor goat had 11 CLs and eight structures were recovered, with a collection rate of 72.7%. Five blastocysts grade I, one grade II and one compacted morula were transferred. The recovered embryos were transferred by semi-laparoscopy into recipients that received progestagen and cloprostenol associated with an eCG injection (Novormon®, Buenos Aires, Argentina). Pregnancy diagnosis was assessed by ultrasonography 30 and 45 days post-estrus. Identification of transgenic offspring was performed by PCR. On day 30 post-estrus, pregnancy rate was 75.0% (3/4) and dropped to 50.0% (2/4) at 45 days. From the two pregnant recipients, one showed twin pregnancy, achieving a total of three kids. One transgenic female was identified by PCR. In this study, 33% of kids born were transgenic. In conclusion, embryo transcervical collection showed to be an efficient method for propagation of founder transgenic goat for hG-CSF.

Keywords: transgenesis, goat, hg-csf.

12.6 APÊNDICE F

RESUMO PUBLICADO: *Acta Scientiae Veterinariae*, **39 (Supl. 1):** 440, 2011.

ESTABLISHMENT OF AN hG-CSF TRANSGENIC GOAT LINE ORIGINATED FROM A MALE FOUNDER AND DETECTION OF AGE AT PUBERTY IN F1

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The growing worldwide demand for human Granulocyte Colony Stimulating Factor (hG-CSF) stimulated our laboratory to produce transgenic goats harboring this gene (Freitas *et al*., 2010; Transgenic Res., 19, 146). From the birth of founders, obtained by pronuclear microinjection, the imminent aim was the establishment of a transgenic herd for a commercial scale production of the protein. The objectives of this study were: a) to obtain transgenic goats from a male founder and b) to determine the age at puberty of the progeny (F1). For this, seven non-transgenic Canindé females were fertilized by the transgenic founder male. The characterization of F1, for the presence of foreign gene was performed by conventional PCR. All animals received breastfeeding and supplementation with commercial concentrate and Tifton hay until three months of age (weaning). Thereafter, the detection of puberty was performed on both males and females. In males, sexual behavior was evaluated weekly, using a female in estrus until the onset of the first ejaculate containing spermatozoa. In females, blood samples were taken weekly to determine serum progesterone (P4). A total of 12 kids

were born, with a foreign gene transmission rate of 50.0% (6/12) and the same ratio sex (three females and three males). Concerning the non-transgenic animals born, 66.7% (4/6) were males. In total, five kids from two pregnancies (one triple and one double) were stillborn, one of which was transgenic. The offspring born live remain healthy until now. Both transgenic males had motile spermatozoa in the ejaculate for the first time at 144 days of age, with 17.2 and 15.8 kg, corresponding to 42.2 and 38.7% of the adult weight of an adult Canindé male, respectively. Concerning the three non-transgenic males, the same found occurred at 119, 119 and 165 days, with 15.8, 12.6 and 13.6 kg, respectively. The transgenic females showed serum levels of P4 > 1 ng/mL (suggestive of ovulation) at 119 and 150 days of age, being 48% (14.9 kg) and 58% (17.9 kg) of the adult weight of an adult Canindé female, respectively. These results are consistent with the average age at puberty of naturalized breeds in Northeast Brazil. Moreover, indicate that the presence of foreign gene in F1, transmitted from the transgenic male thorough Mendelian inheritance, does not compromise the age at puberty in both males and females. Additional studies with a greater number of animals born in this line, as well as verification of fertility, are still needed.

12.7 APÊNDICE G

RESUMO PUBLICADO: *Animal Reproduction*, **9(3)**: 587, 2012.

EFFECT OF THE NUMBER OF LAPAROSCOPIC OOCYTE RECOVERY SESSIONS IN CANINDÉ GOATS ON THE EFFICIENCY OF OOCYTE PRODUCTION IN AN IVEP SYSTEM

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In vitro embryo production (IVEP) can become an excellent tool for genetic improvement and preservation of goat breeds. However, for this, it is necessary the use of gametes (spermatozoa and oocytes) derived from genetically superior and pure specimen. Concerning the female, laparoscopic oocyte recovery (LOR) could be the appropriate method. However, in Canindé goats there is no study on the effect of repeated LOR in the same oocyte donor. The objective of this study was to observe the response of donors after successive sessions for the oocyte production in an IVEP system. For this purpose, it was used 16 adult and cyclic Canindé goats, which were submitted to two or three treatments for hormonal ovarian stimulation followed by LOR. All oocyte donors received intravaginal sponges containing 60 mg MAP (Progespon®, Syntex, Buenos Aires, Argentina) for 11 days, combined with an intramuscular (im) injection of 50 μg d-cloprostenol (Ciosin®, Coopers, São Paulo, Brazil) on day 8 of

progestagen treatment. For ovarian stimulation, goats received a single im injection of 70 mg NIH-FSH-P1 (Folltropin-V®, Vetrepharm, Belleville, Canada) plus 200 IU eCG (Novormon®, Syntex, Buenos Aires, Argentina) 36 h before sponge removal. The interval between each hormonal treatment/LOR was 14 days. LOR was performed under volatile anesthesia and according the procedure cited by Avelar *et al* (2012 Anim Reprod, *in press*). The vacuum pressure was set at 30 mmHg and all follicles larger than 2 mm were aspirated. The collection medium used was TCM199 supplemented with HEPES, heparin and gentamicin. Once the LOR was completed, each ovary was gently flushed with a heparinized saline. The effect of repeated LOR was analyzed using repeated-measures ANOVA and Tukey's test. There were no statistical differences among the three LOR sessions in the number of visualized/punctured follicles (15.3 ± 5.1/12.7 ± 4.5, 15.5 ± 4.2/12.8 ± 3.9 and 14.7 ± 6.4/11.9 ± 4.9, $P > 0.05$). Concerning the recovery rate, there was also no statistical difference between the different sessions, with an average of 71.2, 74.8 and 74.4% ($P > 0.05$) for the first, second and third session, respectively. In conclusion, three LOR sessions were not enough to decrease the oocyte production in Canindé goats submitted to hormonal ovarian stimulation aiming subsequent IVEP.