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Ultrasonographic evaluation of hG-CSF transgenic goat conceptus*

Avaliação ultrassonográfica de conceptos transgênicos para o hG-CSF

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Abstract

The objective of this study was to evaluate the development of transgenic (T) goat embryos and fetuses for human Granulocyte Colony Stimulating Factor (hG-CSF) by ultrasonography. Four pregnancies in non-transgenic (NT) goats were obtained after fertilization (either fixed-time artificial insemination or natural mating) using the T male for hG-CSF. Ultrasound examinations were carried out at 30, 40 (transrectal via), 50, 60, 90 and 120 days of pregnancy (transabdominal via). Some parameters were observed such as morphology, organogenesis and formation of skeletal fetuses, viability with cardiac activity and fetuses' movements. Measurements were taken of the crown-rump length, diameter of embryonic vesicle, thorax, abdomen, umbilical cord and placentomes. After parturition, DNA testing was conducted in all offspring and 4 T and 2 NT kids were identified. The conceptus started their differentiation at 40 days. The heart was detected in all examinations and the heart chambers were assessed at 50 days. Gastric compartments, liver and kidneys were observed at 60 days, the same period that all bony structures were visualized. Average values of all evaluated parameters had a gradual increase with the progression of pregnancy. T and NT goat embryos and fetuses had a similar growth and all remained viable throughout the experimental period.

Keywords: caprine, fetal morphology, hG-CSF, transgenesis, ultrasound.

Resumo

O objetivo deste estudo foi avaliar o desenvolvimento de embriões e fetos transgênicos (T) para o Fator Estimulante de Colônias de Granulócitos humano (hG-CSF) por ultrassonografia. Quatro gestações em cabras não transgênicas (NT) foram obtidas pos fecundação (inseminação artificial em tempo fixo ou monta controlada) utilizando o bode T para o hG-CSF. Exames ultrassonográficos foram realizados aos 30, 40 (via transretal), 50, 60, 90 e 120 dias de gestação (via transabdominal). Alguns parâmetros foram observados como morfologia, organogênese e formação do esqueleto fetal, viabilidade por meio de atividade cardíaca e movimento fetal. As seguintes mensurações foram realizadas: comprimento crânio caudal, diâmetro da vesícula embrionária, do tórax, do abdomen, do cordão umbilical e dos placentomas. Após o parto, o exame por PCR foi conduzido em todas as crias e 4 T e 2 NT foram identificadas. O concepto iniciou sua diferenciação aos 40 dias. O coração foi detectado em todos os exames e as câmeras cardíacas foram identificadas aos 50 dias. Compartimentos gástricos, fígado e rins foram observados aos 60 dias, o mesmo período que todas as estruturas ósseas foram visualizadas. Valores médios de todos os parâmetros avaliados tiveram um aumento gradual com o avanço da gestação. Embriões e fetos T e NT tiveram um crescimento similar e todos permaneceram viáveis durante o período experimental.

*Palavras-chave:*caprino, morfologia fetal, hG-CSF, transgênese, ultrassonografia.

Introduction

The market for the production of therapeutic proteins is booming (Pavlou and Reichert, 2010; Zhang et al., 2010), and the transgenic (T) animal has been an outstanding advance in biotechnology (Houdebine, 2005). Dairy goats are preferable to cattle for the production of transgenic proteins in milk (biopharming), due to their shorter gestation, earlier sexual maturation, and smaller size. In 2006, the first T goat (for human Granulocyte Colony Stimulating Factor, hG-CSF) in Latin America was born (Freitas et al., 2007), a Saanen male. Soon after, a couple (one male and one female) of T founders of the Canindé breed were obtained for the same protein (Freitas et al., 2012). The hG-CSF is important for immune defense based on neutrophils, due to its regulatory role in growth, differentiation, survival and activation of these cells and their precursors (Barreda et al., 2004).

Besides the benefits brought by T animals, it is noteworthy that the use of animals as bioreactors has been questioned concerning human health. Therefore, studies have evaluated the normality of physiological parameters related to health and reproduction of T animals (Jackson et al., 2010). Thus, it is essential to use minimally invasive methods and ultrasound imaging is a safe method for the assessment of structures and tissue consistency of abdominal organs, without affecting pregnancy (Noia et al., 2002). The optimal moment for visualization of fetal structures is important to evaluate their integrity and to allow early diagnosis of diseases or congenital defects that may occur (Léga et al., 2003). In addition, it is possible to estimate gestational age (Lee

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et al., 2005), evaluate conceptus movements and fetal heart rate (FHR) to assess fetal viability (Léga et al., 2007).

Interestingly, prenatal ultrasonography can provide sequential measurements of the same conceptus throughout different developmental stages. The evaluation of T animals by ultrasound may represent an important tool to check their development. However, there are no reports in the literature that evaluates the embryonic/fetal development of T animals by the use of real-time ultrasound. Our hypothesis was that it is possible to evaluate normal embryo/fetus development of T goats. Therefore, the objective of this study was to evaluate the morphometric and morphological development of T goat embryos and fetuses for hG-CSF by the use of real-time ultrasound at different gestational ages.

Materials and Methods

Bioethics

The experiment was approved by the Committee of Ethics in the Use of Animals of the State University of Ceará (CEUA/UECE, no. 10244447-1/24) and by Brazil's National Technical Committee of Biosafety (CTNBio, Biosafety Quality Certificate - CQB, no. 0228/06).

Location and experimental animals

The experiment was conducted from May to October (winter/spring) in Fortaleza-CE, Brazil, at 3° 47'38" S and 38° 33'29" W latitude and longitude, respectively. All animals were maintained in a semi-intensive system, having access once a day to pasture consisting of Tifton (*Cynodon dactylon*), receiving supplementation with commercial concentrate (minimum of 18% crude protein) and Tifton hay once a day. Mineralized salt and water were available *ad libitum*.

Eight non-transgenic (NT) Canindé does were subjected to estrus synchronization treatment (Souza-Fabjan et al., 2013) and were transcervical artificially inseminated (AI) with fresh semen. Goats that returned to estrus were naturally mated (NM), using in both cases the Canindé T male for hG-CSF. The day of conception (day of AI or NM) was considered as day zero of pregnancy. Two pregnancies were obtained after AI and two more after NM, checked 20 days and confirmed at 30 days after conception.

Ultrasonographic examinations

All does were transrectally and transabdominally examined. The transrectal via was used at 30 and 40 days of pregnancy whereas the transabdominal via at 50, 60, 90 and 120 days of pregnancy. Transrectal exams were conducted with does in a standing position, using the Falco 100 apparatus (Pie-Medical, Netherlands) a real-time, B mode ultrasound scanner, equipped with a 6.0/8.0 MHz linear array transducer. After the manual removal of feces, a solution of carboxymethylcellulose gel was applied in the rectum, using a 10 mL syringe. The transducer was carefully inserted in the rectum and the entire reproductive tract was inspected by moving the probe gently backwards and forwards and rotating it 90° clockwise and counter-clockwise. The transabdominal exam was performed using a dual frequency convex transducer of 3.5/5.0 MHz. The animals were restrained in standing position and the transducer was placed on the hairless area of the ventral abdominal wall just above the udder. Using both approaches (transrectal and transabdominal transducers) ultrasonographic images of embryonic vesicles (anechoic structures of circular or elliptical shape containing the embryo/fetus; Martinez et al., 1998) were recorded and transferred to a computer for later analysis.

Embryo and fetal morphology

During each ultrasound examination, conceptus development was evaluated by its morphological characterization by the organogenic differentiation (head, body and limbs) and formation of bone structures (Léga et al., 2003). The presence of an anatomical head and eyes (with orbits) was detected. In the body, the thoracic cavity was observed to evaluate the heart and its chambers whereas in the abdominal cavity, the gestational period was determined by the evaluation of organ's morphology, as gastric compartments (Gonzalez-Bulnes et al., 1998; Léga et al., 2003), kidneys (Ali and Hayder, 2007) liver, and bladder (Léga et al., 2003). The formation of bone structures was recorded by an increased echogenicity in those areas which they could be formed; thus, as the pregnancy advanced, mineralization occurred and then bones were visualized. The formations of skull, orbits, ribs, vertebral column and long bones were evaluated by ultrasound (Medan et al., 2004; Suguna et al., 2008).

Ultrasonographic fetometry

The evaluation of ultrasonographic fetometry was performed with the measurement of parameters such as the diameter of embryonic vesicle (DEV), crown-rump length (CRL), diameter of thorax (DT), diameter of abdomen (DA), diameter of umbilical cord (DUC) and diameter of placentomes (DPL). For DEV, the embryonic vesicle was measured in its maximal diameter (Martinez et al., 1998). The CRL was measured as a straight line from the most upper part of the skull to the tail (end of the sacrum or first coccygeal vertebra) (Abdelghafar et al., 2007). The DT was determined by the side view (ventral-dorsal) as the distance between the sternum and thoracic vertebrae through the heart, being the largest measured transverse from the abdomen to the junction of the umbilical vein (Lee et al., 2005). The DA was defined by the distance between the ends of the trunk, in the lateral view, measured from the last rib to the level of the umbilical cord (Léga et al., 2007). The DUC was determined by measuring the insertion site of the umbilical cord in the body (Lee et al., 2005). As pregnancy progressed, the DPL was measured in C- or O-shaped gray images, depending on the section imaging in uterine fluid. The average diameter of four placentomes located in different areas in the uterus was used (Lee et al., 2005).

All variables were evaluated and measured using recorded images (Image J software; National Institutes of Health, United States of America), with prior calibration for each frequency. For images captured at a frequency of 6.0 and 8.0 MHz, it was established that 5 mm would correspond to 10.00 and 21.00 pixels, respectively. For the frequencies of 3.5 and 5.0 MHz, it was established that 10 mm consisted of 13.00 and 16.00 pixels, respectively.

Embryo and fetal viability

The conceptus viability was evaluated to characterize their normal development during pregnancy. There are two parameters indicative of viability: cardiac activity and fetal movements (Amer et al., 2010). For the cardiac activity, the embryonic or FHR was measured by the use of M-mode (Curran and Ginther, 1995). The embryonic/fetal movements were evaluated during all examinations with their intensity varying according to the gestational age.

Detection of the hG-CSF transgene

The hG-CSF transgene was detected in skin biopsies from the ears of two-week-old founders using PCR amplification according to the method reported by Freitas et al. (2007). Only after obtaining these results, the images previously obtained by ultrasound were allocated into sonograms of T and NT goats.

Statistical analysis

T and NT goats were compared by ultrasound appearance and through measurements by non-parametric Kruskal-Wallis test. Values were expressed as mean ± SEM and differences were considered to be statistically significant at a level of $P<0.05$. Statistical analysis was performed using Prism 5.0a software.

Results

Four pregnancies were obtained after AI and NM (50%) and all goats remained healthy throughout the entire experiment, i.e., no pregnancy loss occurred in the period evaluated in the study. During all ultrasonographic evaluations T and NT conceptus showed a similar and progressive development. Conceptus images had a normal echogenicity for the age evaluated. As the fetuses developed, it was possible to evaluate the formation and differentiation of some organs, which will be described below.

Detection of the hG-CSF transgene

After PCR, 4 T (two males and two females) and 2 NT males were identified, demonstrating that the T male was able to transmit to offspring the exotic gene (Freitas et al., 2012).

Embryo and fetal morphology

At 30 days, embryos had a rounded shape, without differentiation of cranial and caudal regions. The onset of differentiation of these regions occurred after 40 days. At this time, the head was observed, and with the onset of mineralization, the body was present with a rib cage, spine and limbs. Conformation of T and NT fetuses remained similar and within normal limits throughout the study period (Figure 1; Figure 2).

At the abdominal cavity, the gastric compartments were identified at 60 days as an anechoic chamber at the cranial cavity of the abdomen. At the same period of time, the liver showed an area between the chest and abdomen with homogeneous echogenicity. The kidneys were located using the rumen as a reference. The bladder was visualized in the caudal part of abdomen. All the organs both in T and NT fetuses had normal echogenicity and development during the period of the study. In the chest, the heart development and its cardiac chambers were observed at 30 and 40 days of pregnancy, respectively, as a small anechoic area showing intense movements. After the initial 50 days, the heart chambers could be assessed with more details and four well-defined anechoic areas were identified.

The limbs were seen as small hyperechogenic points at 40 days and continued its development. Bone structures were identified as hyperechogenic areas and the calcification began at 40 days with skull and spine mineralization. At the same period, the face and eyes were also observed. During fetal bone calcification, at 50 days of pregnancy, it was possible to clearly observe the orbits, ribs and long bones. The two orbits were hypoechogenic circular areas in the upper region of the skull. At 60 days, all bone structures were fully visualized.

Ultrasonographic fetometry

In T and NT conceptus fetometry, all parameters measured were within the normal range within different gestational ages. The initial parameters measured were DEV and CRL at days 30, 40, 50 and 60. The embryonic vesicle was characterized by a circular area filled with anechoic fluid. Both DEV and CRL showed a progressive growth (Figure 3). However, due to fetus growth, it was not possible to identify and measure the transverse plane of these parameters after this period.

Other parameters to monitor fetal growth during the second trimester of gestation were evaluated. After the initial 50 days it was possible to assess the DT, DA, DUC and DPL. As expected, throughout pregnancy fetuses grew. The DT, DA and DUC of T and NT fetuses are shown in Figure 4 A-C. An increase with the advance of pregnancy was observed. The placentomes were

Figure 1: Ultrasonographic images screening the early pregnancy detection in goats showing a fluid filled vesicle in the uterine lumen: (A) embryo at 30 and (B) 40 days of pregnancy evaluated by transrectal probe, 8 MHz

Figure 2: Typical images observed by transabdominal real time ultrasonography with a 3.5 MHz linear array transducer: beginning of the ossification in the head and ribs. Embryo at (A) 60, (B) 90 and (C) 120 days of pregnancy.

not seen on days 30 and 40 (transrectal) but were present at 50, 60, 90 and 120 days of pregnancy (transabdominal probe) (Figure 4D). In the last observation (120 days) there were greater numbers of placentomes, making the observation of fetuses more difficult (Figure 2).

Embryo and fetal viability

During the evaluation of embryo and fetal viability it was possible to assess FHR (Figure 5) and thus to record it. At Day 30, the fetal heartbeat was detected, but its heart rate was measured only after 40 days after fertilization. With the advance of pregnancy, there was a decrease in the heartbeat up to the last ultrasonographic evaluation made. Fetal heart rate was important for evaluation of fetal viability. All fetuses presented movements in all ultrasound examinations. In the beginning of pregnancy (30 days) these movements were very discrete. At 40 days they were more frequent and even more intense at 50 days of pregnancy. At 120 days the fetal movements compromised the fetal heart rate measurement.

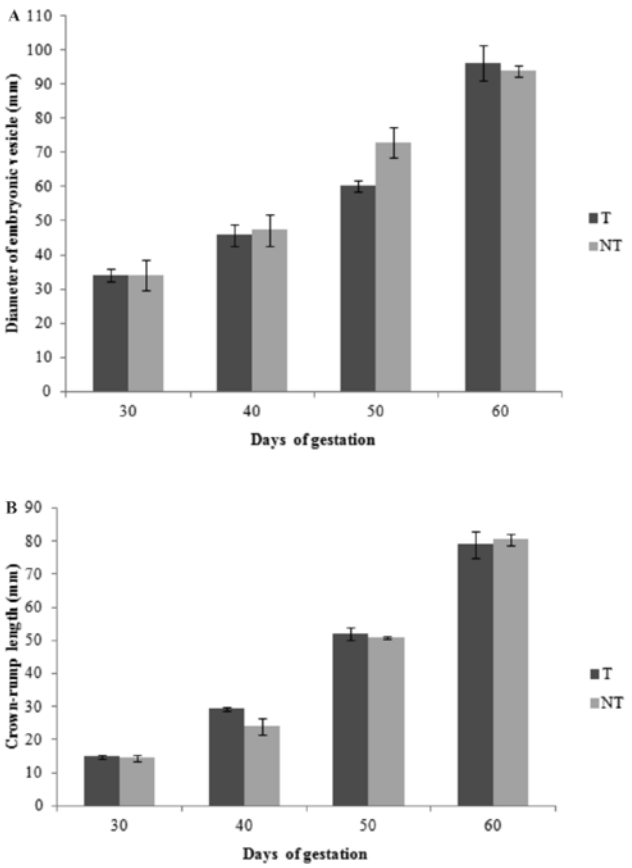

Figure 3: Fetometry results. (A) Diameter of embryonic vesicle, and (B) Crown-rump length of embryos and fetuses of transgenic (T) and non-transgenic (NT) goats at days 30, 40, 50 and 60 of pregnancy ($P > 0.05$; Kruskal-Wallis test)

Discussion

This is the first report which evaluates the embryonic and fetal development of T goats by the use of real-time ultrasound evaluation. It is possible to assume that the hG-CSF protein did not adversely affect the embryos and fetuses morphology, confirming our hypothesis that they would have normal development throughout pregnancy. In the current study, the T kids were generated by fertilization (AI or NM) and no embryo manipulation occurred, supporting our hypothesis. However, it was essential to prove the normality of their pre-natal life evaluated by ultrasound. At 20 days after fertilization, pregnancy diagnosis was carried out and confirmed at 30 days, with no pregnancy loss detected. In the consulted literature, there are no reports on ultrasound evaluation of T embryo/fetuses (in any species), being difficult to generalize about T embryo/fetuses development, considering also the different possible methods of their obtention. It is reasonable to assume that T embryo/fetuses for hG-CSF do not have any critical period of development, as reported in cloning (Chavatte-Palmer et al., 2006).

The gestational sacs were detected at 20 days and at 30 days after conception the embryos were seen surrounded by the embryonic vesicle with the presence of heartbeats, as earlier demonstrated by Martinez et al. (1998) in Anglo-Nubian goats in the same period. We observed the heart as an anechoic area between the ribs, containing hyperechogenic lines dividing it into the four heart chambers, corroborating with Medan et al. (2004).

The differentiation of the conceptus conformation in our study was observed at 40 days, similar to that reported by Ali and Hayder (2007) in Ossimi ewes, who observed the head, body and tail at 38 days of pregnancy. The authors highlighted that the accessibility of different fetal organs and parts by ultrasonic exams depended on the day of pregnancy. In the current study, the limbs were initially visualized at 40 days. This period is close to that was previously reported, from 34 to 36 days of pregnancy (Léga et al., 2003). The beginning of calcification of the bone structure occurred at 40 days, with its increase at 60 days, first identifying the skull, ribs and then the spine and long bones as previously described (Léga et al., 2003). Suguna et al. (2008) determined in cross-bred goats that the skull, rib cage and spine were seen for the first time with 56 days and were detected up to 130 days. However, images were distorted and thus were not measured. According to Medan et al. (2004) after two months these structures and the long bones could be clearly noted. It was possible to visualize the orbits at 50 days, similar to 52 days previously reported (Léga et al., 2003). Lee et al. (2005) working with Korean goats measured fetal orbits after 60 days of pregnancy.

The anechoic cavity of the stomach was visualized at 60 days of pregnancy. The results are similar to those reported by Léga et al. (2003) that identified the gastric compartments from 55 to 57 days as an anechoic structure with invaginations in the lumen for the differentiation of the four compartments of the ruminant stomach. It was earlier indicated that the stomach could be monitored after 50 days of pregnancy, with a linear increase in diameter up to 90 days (Gonzalez-Bulnes et al., 1998). Other organs such as liver, kidneys and bladder were observed in the same period. Ali and Hayder (2007) noticed the kidneys for the first time at 73 days. Léga et al. (2003) observed the bladder for the first time from 49 to 51 days, showing an anechoic appearance and discretely full. These slight differences could be related to the breed used in each study.

After 60 days, both the CRL and the DVE could not be completely visualized in the monitor and thus measured, due to the fetus' large size. The same time period was previously observed in Ossimi sheep (Ali and Hayder, 2007) or in Egyptian native

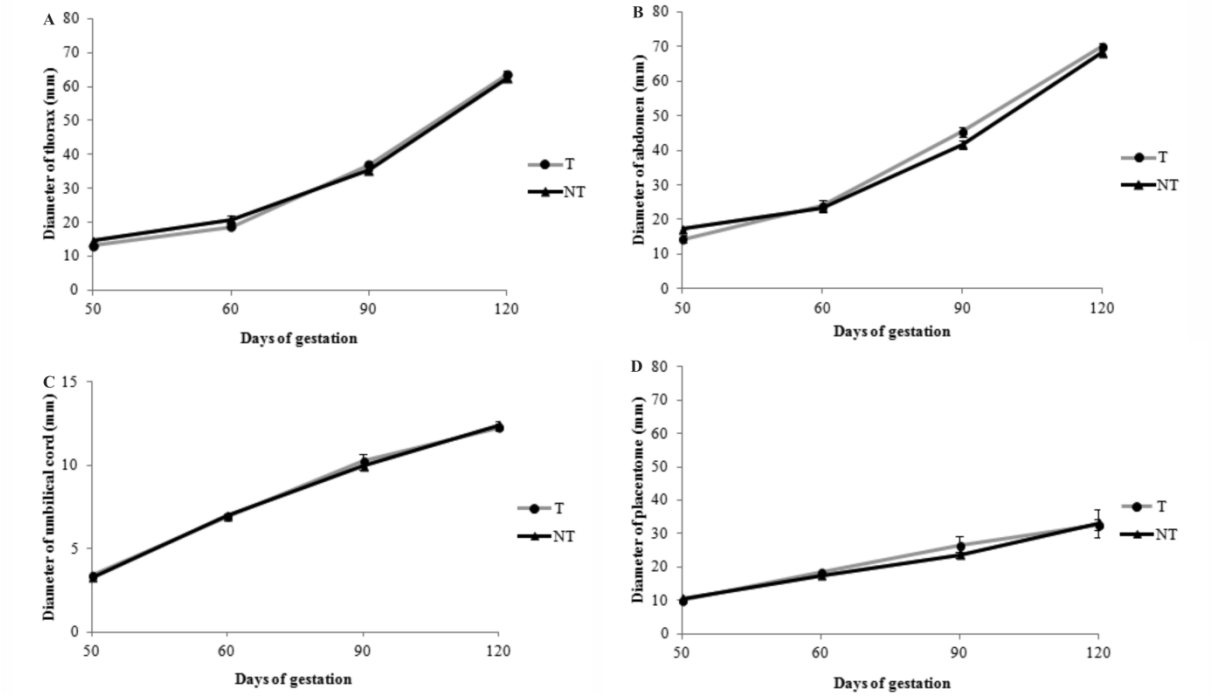

Figure 4: Fetometry results. (A) Diameter of the thorax, (B) Diameter of the abdomen, (C) Diameter of umbilical cord, and (D) Diameter of placentome of embryos and fetuses of transgenic (T) and non-transgenic (NT) goats at days 50, 60, 90 and 120 of pregnancy ($P > 0.05$; Kruskal-Wallis test).

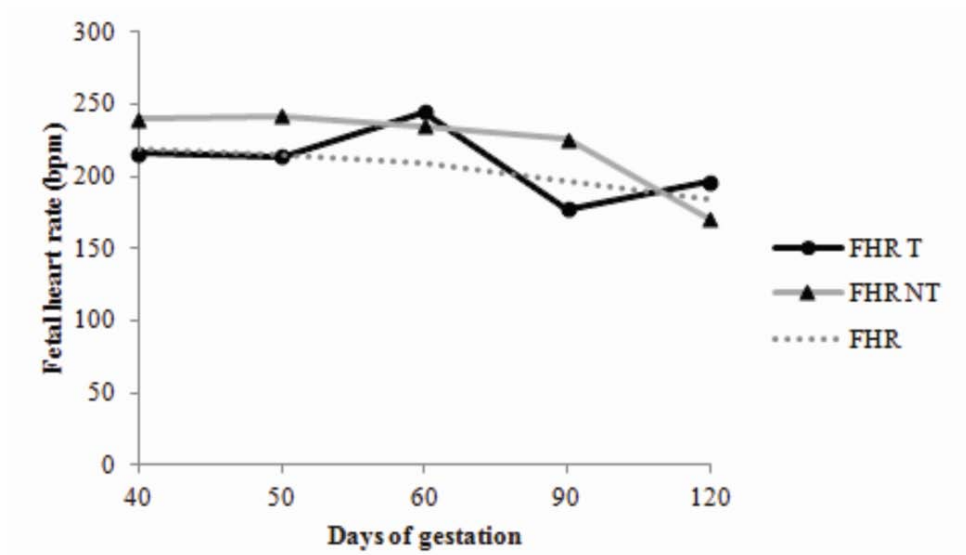

Figure 5: Fetal heart rate (FHR) of transgenic (T) or non-transgenic (NT) goat embryos and fetuses at days 40, 50, 60, 90 and 120 of pregnancy.

goats (Karen et al., 2009). In Santa Inês sheep, the embryonic vesicle diameter was 25.6 mm at 35 days and 44.4 mm at 45 days (Ramos et al., 2007). Chalhoub et al. (2001) evaluated the average CRL of sheep fetuses and obtained results similar to ours at 30 (13.3 mm) and 40 days (33.1 mm) of pregnancy. Amer (2010) reported a diameter of 36 mm (40 days) in dairy goats. These values were also similar to those found by Abdelghafar et al.(2007) working with Saanen goats, at 53 (71.6mm) and 60 days (94.7 mm).Mean DT of T goat fetuses was close to that earlier described in Saanen goats (Léga et al.,2007). The mean values of DA were similar to those observed by Abreu et al. (2007) in Serrana goats on days 54 and 61 of pregnancy and Léga et al. (2007) in 90 and 120 days of pregnancy. Mean DCU of T and NT goat fetuses were similar to those reported by Lee et al. (2005).

The placentomes were only detected and measured (9.96 mm) after 50 days of pregnancy and with advancing pregnancy they became more echogenic and brighter. Conversely, Ali and Hayder (2007) first identified them at 37 days as small areas

slightly elevated on the surface of the endometrium. Suguna et al. (2008) first detected them at 42 days, in a similar diameter (10 mm) as ours. The authors suggested that the second month of pregnancy is the best time to identify these structures. The diameter of the placentomes in this study increased to similar size as observed in earlier reports at 54 and 61 days of pregnancy (Abreu et al., 2007) and at 90 and 120 days of pregnancy (Léga et al., 2007).

The cardiac activity was within the normal range for all fetuses. In this study the fetal heartbeat was detected at day 30 and fetal heart rate was measured after 40 days. It is noteworthy that the FHR decreased as pregnancy progressed. The first observation at 40 days was 215.50 bpm (T) and 239.50 bpm (NT) whereas the last at 120 days, 196.25 bpm (T) and 170 bpm (NT). Léga et al. (2003) reported that it was only possible to measure it after 37 days of pregnancy, using a mechanical sector transducer and/or linear with frequency of 5.0 MHz and the values ranged from 208 to 226 bpm after this period. However, some authors have detected about 22 to 24 days of pregnancy (Medan et al., 2004; Padilla-Rivas et al., 2005). The decrease in FHR was also observed by Karen et al. (2009) and Suguna et al. (2008). The ultrasound exams allowed the identification of the heart rate and fetal movements, which are reliable parameters to check fetus viability (Amer et al., 2010). Thus, all T and NT goat fetuses were viable throughout the gestational period.

Conclusions

The transgenic goat embryos and fetuses for hG-CSF remained viable and showed growth similar to the non-transgenic ones during pregnancy. Therefore, the integration of exogenous gene in the genome of these animals does not compromise embryo or fetal development during the period evaluated.

Acknowledgments

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