

Morphology and α -SMA, F-actin and JC1 Protein Expression in the Mammary Gland of Goats in Early Hormonal Lactation*

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ABSTRACT

Background: Mammary remodeling is determined by a combination of cell differentiation, proliferation and programmed death controlled not only by systemic hormones, but also by proteins produced either in the stromal or in the epithelial compartments. However, few works were undertaken to use phenotypic markers for cellular components of the lactating caprine mammary gland and to determine structural–functional relationships. Thus, the aim of this research was to evaluate the morphology and α -SMA, F-actin and JC1 protein expression in the mammary gland of Canindé goats in early hormonal lactation.

Materials, Methods & Results: Fourteen 2 years old female Canindé goats were used and distributed into two groups: non-lactating (n = 4) and lactating animals (n = 10). Lactation was induced by using estrogen, progesterone and prednisolone according to previous protocol. All subjects were housed indoors and had four hours of daily access to solarium. Mammary gland biopsies were obtained at days 5 (D5) and 26 (D26) of the early stage of lactation and were assessed by histological and immunohistochemical analysis. The microstructure of mammary gland in lactating goats was analyzed by conventional histologic techniques. Immunohistochemistry was used to identify α -SMA in myoepithelial cells and JC1 in epithelial cells. α -SMA and F-actin expression were assessed by confocal microscopy. Concerning microscopic features, the alveoli structure was evaluated in respect to number, size and cell population. The structural units of the lactating mammary gland consisted of variably shaped lobules. When comparing to lactating tissue, sections of the non-lactating mammary gland showed a lower number and size of alveoli, separated by wide connective tissue. Lactating tissues demonstrated numerous and well-developed alveoli, separated by thin trabeculae of connective tissue. In relation to lactating goats, no difference ($P > 0.05$) was observed in the alveoli size between D5 and D26. Immunostaining in lactating mammary glands using α -SMA and JC1 expression demonstrated that from D5 to D26, the number of epithelial, myoepithelial and proliferating epithelial cells per alveolus increased by 64.0%, 82.6% and 79.2%, respectively. Cytoplasmic F-actin was detected by Alexa Fluor 488-phalloidin in myoepithelial cells as individual as associated to α -SMA expression.

Discussion: At the early stage of the hormonal lactation in goats, a high increase was observed regarding to population of epithelial, myoepithelial and proliferating epithelial cells, which were characterized by immunological markers. These results revealed the intense mitotic process which occurs at early lactation. Mitosis is an essential process which ensures the mammary gland to have enough secretory and myoepithelial cells for production and ejection of milk, respectively. Based on our results it is presumable that the presence of F-actin in association with α -SMA highlights the power of contractile force of MECs of caprine mammary gland. In the present study, the goat mammary gland during early hormonal lactation demonstrated morphological features similar to those reported for natural lactation. To the best of our current knowledge, this is the first paper to demonstrate the F-actin expression in myoepithelial cells of goat mammary gland. The analysis of histological findings, and α -SMA, F-actin and JC1 expression suggests that epithelial and myoepithelial cells play essential role in the early hormonal lactation in goats.

Keywords: Goats, mammary gland, epithelial cells, α -SMA, mitogen-regulated protein 1.

INTRODUCTION

The mammary gland undergoes histological modifications which can be associated with the different stages of the lactation cycle. Mutual increments of lobule-alveolar system throughout gestation, as well as a reduced amount of stroma tissue are reported. In addition, during early and mid-lactation there is an increase of alveolus number, size and differentiation and the presence of epithelial cells with a columnar shape and secretory vesicles. At late lactation, a reduction of epithelial cells number is evident [11]. Mammary remodeling is determined by a combination of cell differentiation, proliferation and programmed cell death controlled not only by systemic hormones, but also by proteins produced either in the stromal or in the epithelial compartments [3]. However, few works were undertaken to use phenotypic markers for cellular components of the lactating caprine mammary gland and to determine structural-functional relationships [12].

Hormonal lactation has been successfully demonstrated in goats [2,13]. Procedures to induce animals into lactation may be a tool to study mammary biology during short-time periods of growth and during lactogenesis [7]. Then, some proteins of strong functional relevance, such as contractility (α -SMA and F-actin) and proliferation (JC1, also designated mitogen-regulated protein 1) [1,5,10] were studied. Thus, the aim of this research was to evaluate the morphology and α -SMA, F-actin and JC1 protein expression in the mammary gland of Canindé goats in early hormonal lactation.

MATERIALS AND METHODS

Animals and hormonal lactation

Fourteen 2 years old female Canindé goats weighing 31.2 ± 2.0 kg (Mean \pm SEM) were used and distributed into two groups: non-lactating (n = 4) and lactating animals (n = 10). Animals received Tifton (*Cynodon dactylon*) hay in pens and were supplemented with commercial concentrate (20% crude protein). In addition, goats were housed indoors, had four hours of daily access to outside park and free access to water and mineralized salt.

Ten animals were hormonally induced by using estrogen, progesterone and prednisolone, according to protocol earlier described [2], hand-milked daily until D30 (start of milk production is day 0 = D0) and there-

after at crescent intervals until the lactation stopped. No animal had been previously utilized for lactation hormonally induced. The volume of milk produced was measured for each teat of individual goats.

Mammary gland biopsies

Biopsies were performed in does, after milking (lactating goats), at both D5 (Biopsy 1) and D26 (Biopsy 2) of milk production, on the right and left teats, respectively. Does were denied access to feed 24 h and water 12 h prior to biopsy. Animals were anaesthetized using 0.1 mg/kg EV of xylazine (Kensol)¹ at 2% combined with 5 mg/kg ketamine (Quetamina)² at 10%. An incision was made in the skin of the udder and a small piece of the parenchyma (0.5 cm³) was taken. The animals were injected intramuscularly with antibiotic terramycin (Terramicina/LA)³ at 20 mg/kg.

Histological evaluation

Mammary gland samples collected by biopsy procedures from lactating and non-lactating goats were fixed in 10% neutral formol saline during 24 h, and subsequently dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin, sectioned at 3 μ m thickness and stained with hematoxylin and eosin [14].

Immunohistochemistry

Primary antibodies used in the present study were obtained commercially by Santa Cruz Biotechnology⁴. A monoclonal anti-mouse α -SMA was used as phenotypic marker for myoepithelial cells. A monoclonal anti-human proliferin (JC1, a nuclear antigen, also designated mitogen-regulated protein 1) was employed to detect proliferating epithelial cells. For light microscopy, secondary biotinylated antibodies and 3,3'-Diaminobenzidine (DAB) were supplied in avidin-biotin complex (ABC) kit⁴ and used as described below.

Specimens mounted on positively charged and coated slides were dewaxed, rehydrated and subjected to inactivation of endogenous peroxidases by incubation in 3% hydrogen peroxide (H₂O₂) for 30 min. Then, the sections were placed in citrate buffer (pH 6) and heated in a microwave (98°C) for 5 min for antigen retrieval. The slides were blocked with 5% bovine serum albumin (BSA) and 3% Triton X100 in PBS for 30 min and were incubated with the primary antibody (anti- α -SMA or JC1) at 1:100 (v:v) dilution in BSA

in an humidified chamber for one hour, at room temperature. After three washes with PBS, the tissues were incubated with biotinylated secondary antibody, for 30 min at room temperature. A kit based on ABC method was used to detect the secondary antibody, following the manufacturer's instructions. After incubation with a biotinylated horseradish peroxidase (HRP), the color was developed using DAB substrate. Specimens were counterstained with Mayer's hematoxylin for 3 min, washed in water, dehydrated through graded ethanol, cleared in xylene, mounted with medium for light microscopy and photographed with a digital camera⁵ [1 adapted].

For immunohistochemical negative controls, primary antibody was omitted. For positive control in staining with JC1, tissue of mammary malignant tumor from canine was used. For all microscopic analyses, fifteen fields were detected randomly at 40 \times and then the fields were examined at 200 \times . Concerning microscopic features, the alveoli structure was evaluated in respect to number, size and cell population.

Confocal microscopy

Slides were prepared for α -SMA detection as described above for light microscopy, but a rabbit secondary IgG conjugated with Alexa Fluor 647⁶ was used to detect α -SMA. Additionally, counterstaining was conducted for the nucleus with 4'-6-diamino-2-phenylindole dilactate (DAPI)⁷ and F-actin with Alexa Fluor 488-phalloidin⁶. Slides were examined and photographed under a confocal laser scanning microscope (LSM 710)⁸ with objective lens Plan-Apochromat 20 \times , 40 \times and 100 \times . The excitation lines used were 405, 488 and 633 nm, for nuclei (blue), F-actin (green) and α -SMA (red), respectively. Laser power and photomultiplier tube gain were always constant to allow image analysis. The immunoreaction specificity was accessed by omission of the primary antibody.

Statistical analysis

It was used the unpaired Student's *t* test to compare non-lactating and lactating goats, and to compare D5 and D26 of early lactation in respect to alveoli size. Differences were considered to be statistically significant at a level of $P < 0.05$. Values of milk production and alveoli size were expressed as mean \pm SEM. Data of alveoli cell population was presented in percentage. Statistical analysis was performed using GraphPad InStat 3.0.

RESULTS

Hormonal lactation

All females responded to hormonal treatment for induction of lactation in the first attempt. During 30 days of lactation, the daily average milk production (\pm SEM) was 177.4 \pm 44.6 mL per goat. At the peak lactation, the daily average milk production (\pm SEM) was 234.4 \pm 57.2 mL. The milk yield increased by 44.5% from D5 to D26 of early lactation. Drying process was initiated after second biopsy session and even so milk production only stopped after 5 months.

Histological findings

Concerning histological features, the structural units of the lactating mammary gland consisted of variably shaped lobules, composed of tubulo-alveolar secretory units that drain into small intralobular ducts (simple cuboidal epithelium), which leave the lobule and open into a large interlobular duct (bistratified cuboidal epithelium).

The histological analysis also showed several differences between lactating (Figure 1. A) and non-lactating sections (Figure 1. D). Sections of the non-lactating mammary gland showed a lower number of alveoli, separated by wide connective tissue. In addition, the alveoli size was significantly lower (14.6 \pm 0.7 and 8.6 \pm 0.3 μ m for long and short diameter, respectively) than lactating ($P < 0.05$). Lactating mammary gland demonstrated numerous and well-developed alveoli, separated by thin trabeculae of connective tissue. However, when comparing D5 and D26 of lactating goats, no difference ($P > 0.05$) was observed in the alveoli size (57.9 \pm 2.5 versus 59.9 \pm 1.9 μ m for long diameter and 36.0 \pm 1.3 versus 36.6 \pm 1.1 μ m for short diameter). The number of epithelial secretory cells per alveolus increased by 64.0% from the D5 to D26 (25.0 \pm 3.4 and 41.0 \pm 7.6 cells per alveolus, respectively) of early lactation.

α -SMA and JC1 expression

In general, MECs and the vascular smooth muscle cells expressed a strong immunostaining for α -SMA, but not the others parenchymal and stromal components of the mammary gland, at lactation or not. The spatial distribution of MECs varied in the different segments of the alveolar-ductal system. Ductal MECs were spindle-shaped and oriented parallel to the long axis of ducts as a continuous layer, whereas alveolar MECs were stellate-shaped and did not form a continuous layer between the secretory epithelium and the surrounding basement

membrane. Additionally, the MECs in lactating and non-lactating tissues showed a similar morphology (Figure 1. B, E, C, F). An increase of 82.6% was observed from D5 to D26 regarding number of MECs per alveolus (D5: 5.8 ± 0.3 and D26: 10.6 ± 1.6 cells per alveolus).

Epithelial cells of caprine lactating mammary gland and of positive control (Figure 2) expressed strong immunoreactivity for JC1. According to labeled-cell counts performed in D5 and D26, the number of proliferating epithelial cells per alveolus was increase by 79.2% from the D5 to D26 (6.1 ± 0.5 and 10.9 ± 1.2 cells per alveolus, respectively) of early lactation.

Evaluation of α -SMA and F-actin expression by confocal microscopy

To characterize the MECs in lactating goat mammary tissue the same antibody against α -SMA was used (Figure 3). MECs expressing α -SMA binding sites were located on the basal surface of alveolus and recognized by their stellate-shaped morphology. The cytoskeleton fluorescent stain is showed through the binding of phalloidin to F-actin (Figure 3. D). Thus, MECs expressing α -SMA were co-localized with phalloidin staining (Figure 3. F).

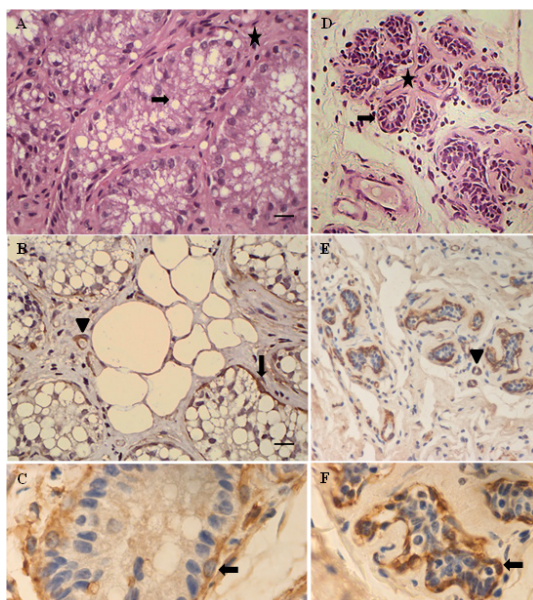


Figure 1. Immunohistochemical detection of α -SMA on goat mammary glands. Light microscopy analysis of lactating (A, B and C) and non-lactating (D, E and F) tissues were performed after either hematoxylin-eosin (A and D) or α -SMA (B, C, E and F) staining. Alveoli (arrows) separated by trabeculae of connective tissue (stars) were indicated in lactating (A) and non-lactating (D) photomicrographs. Staining of α -SMA was presented in both myoepithelial (arrows) and vascular smooth muscle (arrowheads) cells in both lactating (B and C) and non-lactating (E and F) tissues. Magnifications of 400 \times (A, B, D and E) and of 1000 \times (C and F) were used. [Scale bars = 10 μ m].

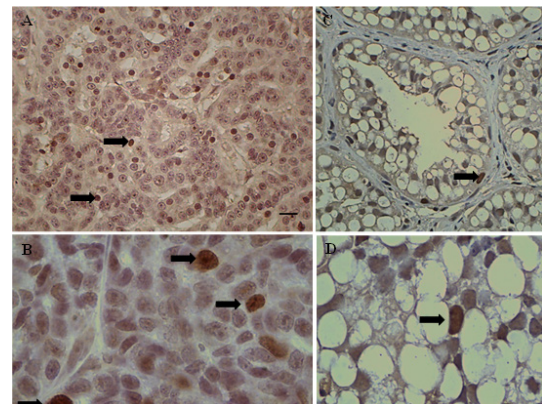


Figure 2. Photomicrographs of JC1 immunostaining in goat and canine mammary glands. Both canine malignant tumour (A and B), a positive control, and healthy lactating goat mammary gland (C and D) presented strong JC1 staining. Proliferating epithelial cells (arrows) were detected in canine tumour as well as in caprine lactating tissues. Magnifications of 400 \times (A and C) and 1000 \times (B and D) were used. [Scale bars = 10 μ m].

DISCUSSION

Mammary gland is a complex organ in both structure and function. The glandular parenchyma is responsible for milk production and is constituted by tubule-alveolar glands. In goats, histological modifications of the mammary gland during the course of lactation has been more investigated [3,4], however, few works employed immunological markers for cell types [12]. Therefore, the purpose of this study was to evaluate the morphological findings, and α -SMA, F-actin and JC1 expression in mammary glands of Canindé goats in early hormonal lactation.

The light microscopic structure of the lactating goat mammary gland is in agreement with previous studies in this specie [3,4]. As expected, the non-lactating mammary gland had a higher stroma-parenchyma proportion in comparison to lactating gland. Likewise, the alveoli size was smaller in non-lactating mammary gland and early lactation was characterized by marked increase of alveoli number, size and differentiation. These results are consistent with the common microscopic pattern reported for mammary gland during natural lactation [11].

The number of the secretory cells per alveolus, described as the best indicator of mammary gland lactogenic activity increased from D5 to D26 of early lactation at the present study. Indeed, it was observed an increment on milk yield at D26 of lactation. These data indicate that the increase in milk production is followed by increased cellular number in the mammary parenchyma. Similarly, at 28 days of natural lactation in

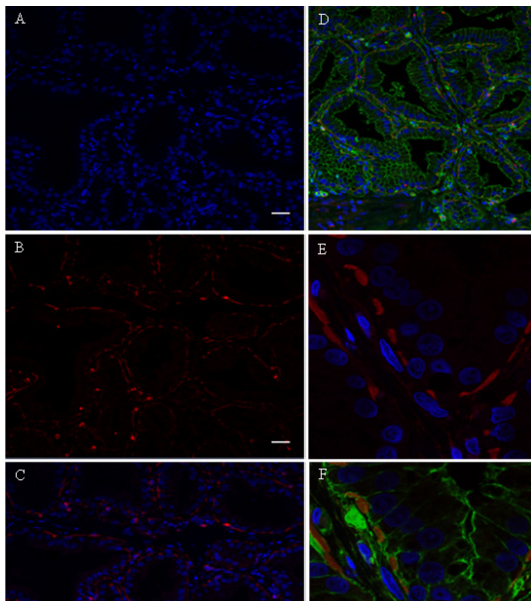


Figure 3. Confocal microscopy of myoepithelial cells on goat lactating mammary gland. (A) The nuclei were counterstained with DAPI (blue). (B) The myoepithelial cells were stained with α -SMA (red). (C) Merged images of panels A and B. (D) DAPI, α -SMA, and F-actin stained with phalloidin (green). (E) Filaments of α -SMA were found in myoepithelial cells. (F) Cytoplasmic filaments of F-actin were detected in myoepithelial cells and cytoskeleton. The orange color represents the co-expression of α -SMA and F-actin. Magnifications of 400 \times (A to D) and 1000 \times (E and F) were used. [Scale bars = 10 μ m].

Damascus goats, an increase on milk yield and epithelial cells was reported [3]. Thus, these results suggest that, during the early stage of hormonal lactation, the caprine mammary gland shows histological findings similar to those reported for natural lactation.

In addition to histological investigation, cell characterization can be obtained more suitably using monoclonal antibodies which act as markers of cell types and cell cycle related antigens. For examples, Ki67, anti-PCNA and JC1 have been described as markers of proliferation [5,6]. We report here the successful use of JC1 for stain proliferating epithelial cells on lactating mammary gland of goats. The current trial showed a high increase in the number of mammary proliferating epithelial cells at D26 of the early lactation. These results revealed the intense mitotic process which occurs at early stage. Mitosis is an essential process which ensures to have enough secretory cells producing milk. Moreover, a previous study showed that metabolic activity of epithelial cells also increased in this period [3].

α -SMA is the most abundant actin isoform in mammary MECs. The present study showed that in

addition to the expression of α -SMA, MECs of goat mammary gland also express F-actin. Previous surveys also reported α -SMA staining in MECs of the caprine mammary glands [12], however, the F-actin expression not yet demonstrated in goats. Actin exists mainly as a fibrous polymer, F-actin, and can assemble into contractile structures in non-muscle cells, termed stress fibers, to generate contractile force [8,16]. F-actin is stained by phalloidin, a naturally peptide found in the death cap toadstool, *Amanita phalloides* [10].

In an earlier study in mice [9], the stress fibers were observed in phalloidin-stained MECs expressing α -SMA or not, however, the level of contractile force generated was significantly less in MECs lacking α -SMA. In goats, we believe that the presence of F-actin in association with α -SMA highlights the power of contractile force of mammary MECs. Nevertheless, future studies will be necessary to determine the role of F-actin in MECs of goat mammary gland.

The increasing MECs number indicates that it was necessary an important increase on the number of these cells to provide the milk ejection in goats. Based on our data, it is possible to infer that at the early stage of the hormonal lactation in goats, high levels of hyperplasia are evident and that the mitotic process is markedly present at a molecular/cellular level in epithelial and myoepithelial cells.

CONCLUSION

The analysis of histological findings, and α -SMA, F-actin and JC1 expression suggests that epithelial and myoepithelial cells play essential role in the early hormonal lactation in goats.

To the best of our current knowledge, this is the first paper to demonstrate the F-actin expression in myoepithelial cells of goat mammary gland. The α -SMA and F-actin expression in myoepithelial cells of mammary gland suggest the power of contractile force required for milk ejection in goats.

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