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Acute and chronic effects of cortisol on milk yield, the expression of key receptors, and apoptosis of mammary epithelial cells in Saanen goats

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ABSTRACT

Cortisol (CORT) induces mammary development in late gestation and is fundamental to the differentiation of mammary epithelial cells and lactogenesis. The objective of this study was to investigate the relationship between CORT, insulin, prolactin, growth hormone, and insulin-like growth factor-1 in milk as well as the effect of CORT on the expression of receptors of insulin (*INSR*), prolactin (*PRLR*), growth hormone (*GHR*); we also studied the insulin-like growth factor-1 (*IGF1R*), glucocorticoid (*NR3C1*), mineralocorticoid (*NR3C2*), B-cell lymphoma 2 (*BCL2*), BCL-2-like protein X (*BAX*) genes, and the apoptosis rate of mammary epithelial cells of lactating Saanen goats in vivo and in vitro. The following experiments were conducted: (1) comparing hormone release in milk and blood after ACTH or a placebo administration; (2) evaluating the effect of acute CORT increases in mammary gland expression and milk yield in vivo; and (3) evaluating the effect of a chronic increase in CORT concentration in epithelial mammary cell apoptosis in vitro. In vivo, ACTH administration significantly increased CORT release but did not affect insulin, prolactin, growth hormone, and insulin-like growth factor-1 release in plasma and milk versus placebo. The results show also that a low CORT release after ACTH administration increased the expression of *GHR* and *PRLR* genes in the mammary tissue. Indeed, CORT release significantly increased the milk yield from goats subjected to ACTH versus goats subjected to the placebo. However, a higher amount of CORT added in vitro upregulated the *NR3C1*, *GHR*, *PRLR*, and *BAX* genes and downregulated the *IGF1R* and *INSR* genes, which could negatively modulate the apoptosis of mammary epithelial cells. Finally, the effect of CORT in vivo after ACTH administration demonstrated the increased expression of the *PRLR* and *GHR* genes, which may improve epithelial cell responsiveness and be associated with the positive effect of CORT observed on milk yield at mid-end lactation.
Key words: cortisol, mammary gland, receptors, apoptosis, milk yield

INTRODUCTION

Cortisol (**CORT**) induces mammary development during late gestation and initiates the start of the synthesis of milk components in mammary epithelial cells (Neville et al., 2002; Casey and Plaut, 2007; Kobayashi et al., 2017). Some authors have argued that CORT action in different tissues is regulated by the intracellular crosstalk between the glucocorticoid (*NR3C1* gene) and mineralocorticoid (*NR3C2* gene) receptors in target cells (Wintermantel et al., 2005; Bertucci et al., 2010; Chida et al., 2011). In Saanen goats, we demonstrated in vivo that low cortisol concentration did not have an effect on proliferation or apoptosis in the mammary tissue at early lactation; however, high cortisol concentration in vitro significantly increased the expression of the *BAX* gene and decreased the number of mammary epithelial cells in culture (Bomfim et al., 2018). Taking into account that the BAX:BCL2 ratio is often used as an indicator of cell susceptibility to apoptosis (Perlman et al., 1999; Nørgaard et al., 2008), the relationship between CORT, its receptors, *BAX*, *BCL2*, and cell apoptosis in the mammary gland remains unclear.

Cortisol is classically considered fundamental to lactogenesis (Casey and Plaut, 2007; Kobayashi et al., 2016; Kobayashi et al., 2017). Furthermore, CORT, prolactin (**PRL**), and growth hormone (**GH**) released upon suckling and milking by adequate mammary gland stimulation (Negrão, 2008) are associated with the maintenance of lactation (Ollier et al., 2016; Ponchon et al., 2017). However, CORT is also involved in the physiological response to several stressors throughout lactation (Trevisi and Bertoni, 2009; Caroprese et al., 2010; Chen et al., 2015). An increase in CORT release during acute stress promotes positive physiological adjustments that allow for dairy animal adaptation; whereas, chronic CORT release caused by different

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stressors harms the immune system and compromises animal health (Mormède et al., 2007; Trevisi and Bertoni, 2009; Chen et al., 2015; Putman et al., 2018). Some contradictions related to the CORT effect have been reported after glucocorticoid administration, and several studies have demonstrated that glucocorticoids reduce milk yield (Maciel et al., 2001; Ollier et al., 2016; Ponchon et al., 2017). Other authors found no negative effects of glucocorticoid administration on milk yield (Shamay et al., 2000; Bomfim et al., 2018) or reported that CORT and ACTH administration in goats can increase milk yield (Stewart and Thompson, 1984). These inconsistent findings show that CORT's effect on milk yield is influenced by the specific glucocorticoid tested, dosage, frequency, and the lactation phase.

In addition to the direct actions of CORT, large doses of glucocorticoids decreased insulin (**INS**), PRL, GH, and IGF-1 release in blood and reduced milk yield (Maciel et al., 2001; Ollier et al., 2016; Ponchon et al., 2017). Some studies have also suggested that CORT's effect on milk synthesis is related to its intracellular interaction with receptors of INS (*INSR*), PRL (*PRLR*), GH (*GHR*), and IGF-1 (*IGF1R*) in the mammary gland (Sakamoto et al., 2005; Flint et al., 2008; Menzies et al., 2010; Rudolph et al., 2011; Shao et al., 2013). However, it is not clear how CORT interacts with receptors of these hormones in the mammary gland. Consequently, we hypothesized that acute, intermittent, and limited exposure to CORT will positively change the mammary gland's responsiveness, proliferation, and apoptosis rate and milk yield; however, chronic and prolonged exposure to CORT negatively affects the expression of the *BAX* and *BCL2* genes and increases the rate of apoptosis in mammary epithelial cells in vitro. In this context, the objective of this study was to investigate the relationship between CORT, INS, PRL, GH, and IGF1 in milk; the effect of CORT on milk yield; as well as the expression of the *NR3C1*, *NR3C2*, *INSR*, *PRLR*, *GHR*, *IGF1R*, *BAX*, and *BCL2* genes and apoptosis rates in the mammary epithelial cells of lactating Saanen goats in vivo and vitro.

MATERIALS AND METHODS

All animal procedures complied with the ethical codes of the Faculty of Animal Science and Food Engineering (FZEA) of the University of São Paulo (USP), which adheres to Brazilian Federal Law. The experiments were carried out at the Laboratory of Animal Physiology in the Department of Basic Sciences of FZEA, USP, in Pirassununga, Brazil (21°57′02″ S, 47°27′50″ W). In this area, the climate is subtropical with an average annual temperature of 23°C, humidity of 73%, and a rainy season from November to March (with annual rainfall ranging from 1,300 to 2,000 mm). The experimental interventions and sample collections were carried out between July and December. The natural light-dark cycle was from 10:14 in July to 13:11 in December. Experimental pens were illuminated from 0700 to 1800 h by fluorescent lights with approximately 100 lx of intensity at the goats' eye level.

Animals, Housing, Diet, and Milking Routine

All goats were kept in group pens at a density of 3.6 m^2/animal. The pens had a feeding trough and mineral salt; water was provided ad libitum. The total diet contained 40% concentrate (corn grain, soybean meal, soybean oil, mineral, and vitamin mix) and 60% roughage (corn silage), which together satisfy 100% of the animal's daily nutritional requirements (NRC, 2007). Milking was performed twice daily (at 0600 and 1800 h) by the same team throughout the lactation. The milking machine was regulated with a vacuum level of 48 kPa and a pulse rate of 120 cycles/min. Before milking, the goats were attached to the milking parlor, and their teats were predipped and dried. The teat cups were attached at time 0 and detached at the end of milking. Afterward, the teats were postdipped, and the individual milk yield was recorded; the goats then exited the milking parlor. During sampling procedures, the goats were immobilized in individual tiestalls in the milking parlor with access to concentrate and water. The goats had visual, vocal, but limited physical contact with neighboring goats throughout all procedures.

Organization of Experiments

Three experiments were conducted: (1) comparing the levels of hormones released in the milk and blood after ACTH or placebo administration; (2) evaluating, in vivo, the effect of an acute increase of CORT (intermittent and time-limited exposure) on mammary gland gene expression and milk yield; and (3) evaluating, in vitro, the effect of a chronic increase of CORT (prolonged exposure to different CORT concentration) on mammary epithelial cell apoptosis.

Experiment 1: Hormones in Milk After ACTH Administration

Healthy, nonpregnant, purebred Saanen goats (n = 20; mean ± SD, BW of 50.0 ± 1.2 kg, BCS of 3.0 ± 0.5, parity of 2.5 ± 0.5) were used in this study to evaluate the effect of ACTH (Sigma) administration on hormone concentrations in plasma and milk after ACTH administration. Experimental goats were randomly distributed according to previous parity number,

milk yield, BW, BCS, and the number of fetuses in parturition to 2 treatments: placebo (saline solution) or ACTH administration (experimental challenge). The ACTH dose used in the present study (0.6 IU of ACTH by BW per animal) was diluted in 6 mL of saline solution (0.9% NaCl) and administered by i.v. injection. The ACTH dose used causes a rapid peak and return of CORT to basal concentrations similar to those observed after exposure to acute stressors related to farm management (Fulkerson and Jamieson, 1982; Larzul et al., 2015; Bomfim et al., 2018).

On d 70 of lactation, 10 goats were subjected to ACTH administration, whereas 10 other goats were subjected to placebo administration. Experimental milkings were performed at 6, 7, 8, 12, and 18 h. Blood samples (collected by a puncture from the jugular vein), and milk samples were taken before at 0 h (before milking performed at 6 h), and 1, 2, 6, and 12 h after ACTH administration (at milking performed respectively at 6, 7, 8, 12, and 18 h).

Hormones were measured in duplicate in plasma and milk by immunoassay analysis (EIA). Cortisol was measured using a commercial kit according to the manufacturer's instructions (GenWay). Milk INS and IGF1 were measured using a commercial kit according to the manufacturer's instructions (Monobind and Enzo, respectively). Ovine PRL and ovine GH, and their antibody were generously provided by Guy Kahn (INRA, Nouzilly, France). The PRL and GH were measured using an HRP conjugation kit according to the manufacturer's protocol and instructions for ELISA measurement (Thermo Fisher Scientific). All kits were validated by demonstrating parallel curves between standard concentrations and serially diluted plasma and milk samples (Supplemental Figure S1; https://doi.org/10.17632/n77wc67k4t.1; Negrao, 2021). In plasma, the intra- and interassay coefficients of variation (CV) were less than 3.5 and 6.0%, respectively, for all hormones. Hormone concentrations in milk were substantially lower than those measured in plasma. Therefore, we centrifuged the milk sample to separate the fatty and aqueous fractions. Afterward, each fraction was lyophilized, and the milk samples were 10-fold less diluted than the plasma samples. Some hormone concentrations in the milk samples remained lower than the minimal concentrations indicated by the kit being used; consequently, 15% of the milk samples in experiment 1 could not be reliably measured. Regarding the milk, the interassay CV was less than 10% for all hormones, whereas the intra-assay CV were 6.8, 7.3, 8.0, 7.2, and 6.4% for CORT, INS, PRL, GH, and IGF-1, respectively.

Experiment 2: Effect of Acute CORT Increases in Vivo

Another group of 30 healthy, nonpregnant Saanen goats (means ± SD, BW of 58.0 ± 0.8 kg, BCS of 3.5 ± 1.0, and parity order of 3.0 ± 0.5) was used in this experiment. These 30 goats were randomly distributed into 2 groups blocked by parity, previous milk yield, BW, BCS, and the number of kids in parturition to the following treatments: ACTH (0.6 IU of ACTH by BW per animal) or placebo administration (saline). These treatments were performed by i.v. injection on d 90, 120, 150, and 180 of lactation at morning milking to evaluate the effect of acute and intermittent CORT increases on mammary gland expression of *NR3C1*, *NR3C2*, *INSR*, *PRLR*, *GHR*, *IGF1R*, *BAX*, and *BCL2* genes; milk yield; and milk quality.

On lactation d 180, mammary gland biopsies were performed 1 h after challenge with ACTH or placebo. Details of the biopsy procedures and animal care are described by Hooper et al. (2020). Three mammary tissue samples (approximately 2.0 × 0.5 cm of mammary tissue) were collected and washed in sterile PBS and immediately processed. The first sample was fixed in formaldehyde (10%), embedded in paraffin, and used for histological analysis. The second sample was frozen in liquid nitrogen and stored at −80°C until gene expression analysis. The third sample was cut into small pieces and epithelial cells were isolated for in vitro study using 0.25% trypsin and 0.02% EDTA solution (details concerning the isolation of mammary epithelial cells are presented in the description of subitem experiment 3, effect of chronic CORT increases in vitro).

The apoptosis rate of the epithelial cells was determined in histologic samples using a terminal deoxynucleotidyl transferase (**TdT**)-mediated dUTP-biotin nick end labeling (**TUNEL**) kit according to the manufacturer′s instructions (Roche). The rate of proliferation of epithelial cells was determined using a proliferating cell nuclear antigen (**PCNA**) kit according to the manufacturer′s instructions (Invitrogen). After TUNEL or PCNA labeling, the histological slides were analyzed by an Axioscope fluorescence microscope (Zeiss). For each slide, 10 photomicrographs were captured by digital camera Axiocam (Zeiss), and analyzed by Zen commercial software (2011, Zeiss). To confirm the representativity of parenchyma tissue obtained by biopsy of the mammary gland, a tissue sample was stained with hematoxylin and eosin to identify the alveoli and epithelial cells (Supplemental Figure S2; https://doi.org/10.17632/n77wc67k4t.1; Negrao, 2021). The epithelial cells were counted by fluorescent microscopy

to obtain the percentage of cell proliferation and cell apoptosis in representative digital slides of each goat. For quantification of apoptotic cells by TUNEL, 1,000 epithelial cells were counted in at least 20 fields; the epithelial cells with nucleus stained by intense green were considered apoptotic cells (positive), and those cells with a pale green stained nucleus were considered nonapoptotic (negative). For quantification of proliferating cells by PCNA, 1,000 epithelial cells were counted in 20 fields: the epithelial cells with nucleus stained by intense brown were considered proliferating cells (positive), and cells with a pale-brown-stained nucleus were considered nonproliferating cells (negative; Supplemental Figure S2).

The total RNA of mammary tissue (80 to 100 mg) was extracted and purified using a PureLink RNA Mini Kit (Invitrogen). The material was treated with RNase-free DNase (Promega) to prevent genomic DNA contamination. The RNA concentrations were determined using Qubit 2.0 Fluorometric Quantification (Thermo Fisher Scientific). The quality of each RNA sample was evaluated by the optical density at the 260 and 280 nm absorbance waves and the 260:280 absorption ratio was approximately 2. The integrity of the RNA was analyzed on a denaturing agarose gel. Total RNA (1.0 μg) was reverse-transcribed in a final volume of 20 μL using the Improm II Kit (Promega) according to the manufacturer's protocol. The expression levels of target genes were measured using real-time quantitative PCR (**RT-qPCR**) conducted using the StepOne Real-Time system (Invitrogen). The primer sequences for the genes of interest (*NR3C1*, *NR3C2*, *INSR*, *PRLR*, *GHR*, *IGF1R*, *BAX*, and *BCL2*) can be found in Table 1.

Each gene was amplified in a separate reaction and in duplicate; the 20-μL reaction mixture was composed of 1 μL of cDNA (mean concentration of 10 ng/μL), 10 μL of SYBR Green (Invitrogen), 0.4 μ*M* primer pair, and 8.2 μL of ultrapure water. For all genes, the quantitative PCR conditions were as follows: incubation stage (95°C for 10 min); 40 cycles of increasing temperature and separation of double-stranded nucleic acids (95°C for15 s); annealing and primer binding (60°C for 1 min); and dissociation. Each primer pair tested had one single peak in the melt curve, and the housekeeping gene had a similar expression between treatments and across time. Three housekeeping genes were tested (*GAPDH*, *ACTB*, *UBC*); *GAPDH* and *UBC* were selected as endogenous controls based on the efficiency of amplification and expression stability between treatments.

The PCR cycles were as follows: 42°C for 60 min followed by 15 min at 70°C and cooling to 4°C. For all primers, the PCR efficiency can be found in Table 2, and the specificity was confirmed by melting curve analysis and detection of an appropriate size band using 1.5% agarose gel electrophoresis. Gene expression was quantified using the $2^{-\Delta\Delta CT}$ method, where CT is the cycle threshold (Schmittgen and Livak, 2008), comparing the expression of the target genes with the geometric mean of reference genes (*GAPDH* and *UBC*). To evaluate the effects of treatments (ACTH or placebo) in experiment 2 on gene expression, the fold

Table 1. Sequence of primers used in the reverse transcription quantitative PCR

Gene[1]	Primer sequence	Accession number[1]	PCR efficiency (%)
NR3C1	5′-CCATTTCTGTTCACGGTGTG-3′ 5′-CTGAACCGACAGGAATTGGT-3′	XM_005683087	101.4
NR3C2	5′-GGGTCTATCTGTAGCCCTGGAA-3′ 5′-TGTCTGGACTGGGAACCACAT-3′	XM_005691196	88.3
INSR	5′-CCTGGTGTCACTTTCCTTCT-3′ 5′-TTAGGTTCTGGTTGTCCAAGGCGT-3′	XM_005682398	104.0
PRLR	5′-GCCCCAACTCCTGCTACTTT-3′ 5′-GAGGCTCTGGTTCAACTATGT-3′	NM_001285669	99.9
GHR	5′-CGTCTCTGCTGGTGAAAACA-3′ 5′-AACGGGTGGATCTGGTTGTA-3′	NM_001285648	109.5
IGF1R	5′-TTAAAATGGCCAGAACCTGAG-3′ 5′-ATTATAACCAAGCCTCCCAC-3′	XM_005694951	97.1
BCL2	5′-GATGACCGAGTATCTGAACCG-3′ 5′-GACAGCCAGGAGAAATCAAACA-3′	NM_001166486	98.4
BAX	5′-TCGGTCTCAACGGCTACA-3′ 5′-CCACTCCAGCCACAAAGA-3′	NM_173894	97.8
ACTB	5′-GATCTGGCACCACACCTTCT-3′ 5′-CCAGAGGCATACAGGGACAG-3′	NM_173979	99.7
UBC	5′-ATGCAGATCTTTGTGAAGAC-3′ 5′-CTTCTGGATGTTGTAGTC-3′	NM_001206307.1	100.6
GAPDH	5′-GGTGATGCTGGTGCTGAG-3′ 5′-TGACAATCTTGAGGGTGTTG-3′	AJ431207	101.5

[1]https://www.ncbi.nlm.nih.gov/genbank/.

change relative to the placebo goats was calculated as $\Delta\Delta Ct = \Delta Ct$ ACTH $- \Delta Ct$ placebo.

Milk yield (kg/d) was recorded for 210 d of lactation and presented as the average produced in the morning and afternoon milking. Weekly, milk was aseptically taken from both teats in a sterile tube for microbiological content analysis, and another sample was taken from the milk collector to determine the milk composition and SCC at the morning and evening milking, and these parameters were presented as the average of 2 teats measured from the morning and evening milking. The percentage of fat, protein, and lactose in the milk samples was determined by infrared spectroscopy (MilkScope Expert). The SCC in the milk was determined via a direct method (using Carnoy solution and methyl green pyronin) and counted under an optical microscope (Raynal-Ljutovac et al., 2007). The SCC was determined with 10 μL of milk by counting the leukocytes on the microscope slide, multiplying their number by the dilution factor, and converting the result into a log scale. Microbiological analyses of the milk were performed on the bacterial count in standard plate count agar, Baird-Parker agar, and MacConkey agar to express the count (cfu/mL) for total counts of bacteria, *Enterobacteriaceae* and *Staphylococcus* spp., respectively.

Experiment 3: Effect of Chronic CORT Increases In Vitro

This in vitro experiment was performed with the mammary samples from experiment 2 to evaluate the effect of chronic CORT increase (prolonged exposure to different CORT concentrations) on mammary epithelial cell apoptosis without affecting the mobilization of body reserves and metabolic adjustments related to cortisol response to stress that could also change milk yield. As 70% of all experimental goats presented similar BW, body score, and parity, 14 goats (7 goats from placebo and 7 goats from ACTH treatment groups) were evenly distributed to milk production to have 14 representative mammary samples. There was no effect of previous treatment (ACTH or placebo) in cell culture. Therefore, our in vitro results study reported the effect of cortisol addition without taking into account the effect of previous treatment.

The mammary epithelial cells from goats (n = 14) were subjected to 4 treatments in vitro: 0, 10, 100, and 1,000 μg/mL cortisol (hydrocortisone, Sigma; diluted in the basal medium Dulbecco's modified Eagle's medium Nutrient Mixture F-12 Ham (DMEM/F12, Gibco Life Technologies). The concentrations of cortisol tested were based on previous studies that used from 0 to 1,000 μg/mL of cortisol to study hormone actions on mammary epithelial cells in vitro (Keys et al., 1997; Pantschenko et al., 2000; Yang et al., 2005; Bomfim et al., 2018). As reported by van der Kolk (1990), 200 IU of ACTH are equivalent to 31 ng/mL of plasma cortisol. For these reasons, we chose to add 100 μg/mL to mimic a low cortisol concentration in plasma, and 1,000 μg/mL of cortisol to mimic a high cortisol concentration in plasma. Therefore, we considered that the effect of 1,000 μg/mL of cortisol in primary epithelial cells cultured for 5 d mimics a

Table 2. Milk yield, protein, fat, and lactose percentage, SCC, and total counts of bacteria, *Enterobacteriaceae*, and *Staphylococcus* spp. of goats subjected to ACTH or placebo treatments[1]

	In vivo treatment[2]			
Item	ACTH (n = 15)	Placebo (n = 15)	SEM	*P*-value
Milk yield (kg/d)	2.65[a]	2.21[b]	0.12	0.01
Fat (%)	3.41	3.37	0.07	0.67
Protein (%)	3.04	3.07	0.12	0.70
Lactose (%)	4.12	4.09	0.02	0.27
SCC[3] (cells/mL)	1,339	1,130	88.65	0.28
Total count of bacteria[4] (cfu/mL)	17.60	19.92	14.97	0.19
Enterobacteriaceae[5] (cfu/mL)	—	—	—	—
Staphylococcus spp.[4] (cfu/mL)	24.23	27.45	6.45	0.18

[a,b]Means within a row with different superscripts differ ($P \leq 0.05$).

[1]Data concerning milk yield and milk composition correspond to the mean (kg/d) measured from 90 to 180 d of lactation for ACTH and placebo goats. Data are presented as means ± SE of mean.

[2]ACTH or placebo were administered on d 90, 120, 150 and 180; milk yield was presented as the mean produced at morning and evening milking; milk composition and SCC were presented as the mean between the 2 teats, and between morning and evening milking.

[3]Values $\times 10^3$/mL.

[4]Values $\times 10^3$/mL.

[5]The count was not high enough to perform statistical analysis.

high cortisol concentration similar to those of chronic stressors.

For the isolation of mammary epithelial cells, we used the protocol described by Pantschenko et al. (2000), with minor modifications. Briefly, between 75 and 100 mg of mammary tissue obtained by biopsies (n = 14) were washed, cut, and placed individually on culture in 35-mm plates with a basal medium of DMEM/F12 (Gibco Life Technologies) with 10% fetal bovine serum (Gibco Life Technologies), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B. The cells were cultured in an incubator at 37°C under 5% CO_2, and the basal medium was changed every 48 h and cultured for 5 d. Culture dishes were monitored closely, and epithelial cells were detached with 0.25% trpysin-0.02% EDTA and transferred to new culture dishes. Subsequently, the fibroblasts were removed with 0.25% trypsin solution. The growth and morphology of the cultured cells were observed daily using inverted phase-contrast microscopy (Supplemental Figure S3; https://doi.org/10.17632/n77wc67k4t.1; Negrao, 2021). The synthesis of lactose by epithelial cells was also measured to test the cell viability 2, 4, and 6 d after incubation in culture medium, and lactose concentration in culture medium. The lactose concentration in the culture medium indicated that epithelial cells obtained from experimental goats were viable before the cortisol addition (Figure 1). The lactose was measured by a colorimetric assay kit according to the manufacturer's instructions (Abnova).

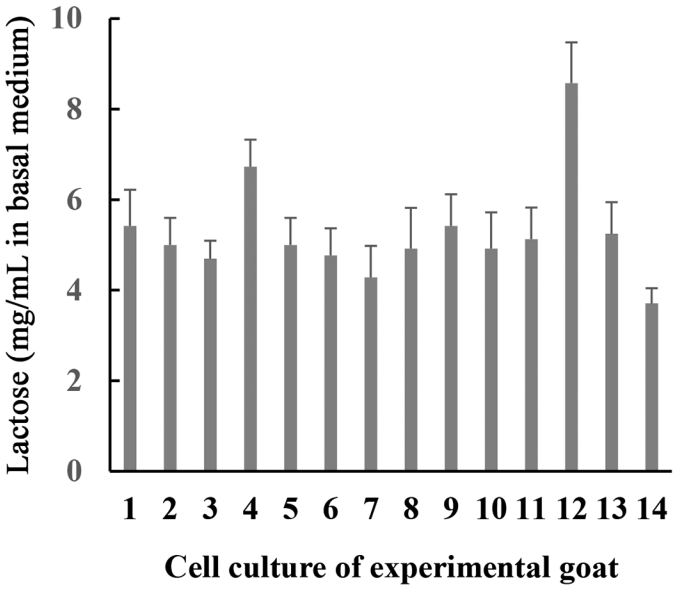

Figure 1. Synthesis of lactose (mg/mL) in the basal medium by mammary epithelial cells isolated from 14 experimental goats. The lactose concentration in culture medium indicated that epithelial cells obtained from experimental goats were viable before the cortisol addition. Data are presented as means ± SEM.

For the treatments in vitro, 1.0×10^5 live mammary epithelial cells from each experimental goat were placed on an individual plate onto a basement membrane matrix (25 μL/cm^2 BD Biosciences) with basal medium (as described above) supplemented with 5 μg/mL of bovine INS (Sigma), 1 μg/mL of ovine PRL (Sigma), and 4 CORT (hydrocortisone, Sigma) treatments in vitro (0, 10, 100, and 1,000 μg/mL of CORT), and incubated at 37°C with 5% CO_2. The incubation time was 5 d. After incubation, the total number of cells (cell/mL) in culture was determined using cell counting equipment (Thermo Fisher Scientific), and the apoptosis rate (%) was determined using a TUNEL kit according to the manufacturer's instructions (Roche) for cell culture. The gene expression of *GAPDH* (housekeeping gene), *NR3C1*, *NR3C2*, *INSR*, *PRLR*, *GHR*, *IGF1R*, *BAX*, and *BCL2* was analyzed via RT-qPCR, as described for the in vitro experiment. To evaluate the treatments effects (0, 10, 100, or 1,000 μg/mL cortisol) in experiment 3, the fold change relative to the control goats was calculated as shown: ΔΔCt = (ΔCt 10, 100, or 1,000 μg/mL −ΔCt 0 μg/mL).

Statistical Analysis

The data were analyzed using SAS software (version 9.1; SAS Institute Inc.). The normality of the data was confirmed using Shapiro-Wilk test. Afterward, the hormone concentration was subjected to an ANOVA by the MIXED procedure of SAS, which separated the treatment (ACTH or placebo), time of sampling, and animal as potential causes of variation. In the model, the treatment effect was considered fixed, time of sampling, and animals were considered random effects. Milk yield, milk composition, and quality were subjected to an ANOVA by the MIXED procedure of SAS, which separated the treatment (ACTH or placebo), day of lactation, and animal as potential causes of variation. In the model, the treatment effect was considered fixed, and the effects of the day of lactation, time of sampling, and animals were considered random effects. Gene expression in vivo (ΔΔCt in experiment 2 = ΔCt ACTH − ΔCt placebo) and in vitro (ΔΔCt in experiment 3 = ΔCt 10, 100, or 1,000 μg/mL − ΔCt 0 μg/mL) was subjected to an ANOVA by the GLM procedure of SAS, which separated the treatments and animals as causes of variation. In this model, the treatment effect was considered fixed, and the effects of the animal were considered random. There was no effect of previous treatment (ACTH or placebo) on gene expression in cell culture. Therefore, our in vitro study reported the effect of cortisol addition without taking into ac-

count the effect of previous treatment. Several errors of covariance structures were tested, and the one that best fit the data according to the Bayesian information criterion was selected. The means were compared using Fisher's exact test when there was a significant effect. Statistical significance was defined as $P \leq 0.05$.

RESULTS

Experiment 1: Hormones in Milk After ACTH Administration

Placebo-treated control goats maintained their basal CORT concentration in plasma and milk. However, ACTH administration had a significant effect on the CORT levels in milk (Figure 2A) and plasma (Figure 2B) relative to placebo. The highest CORT concentrations were detected at 1 and 2 h in plasma and milk, respectively. However, there was no effect of treatment on CORT in plasma and milk 6 and 12 h after ACTH administration. Indeed, there was no significant effect of ACTH administration on INS, PRL, GH, and IGF1 concentrations in milk (Figure 2C) and plasma (Figure 2D).

Experiment 2: Effect of Acute CORT Increases In Vivo

There was a significant CORT-related effect on milk yield; goats subjected to ACTH produced more milk than goats subjected to placebo (Table 2). From d 90 to 180 of lactation, ACTH goats produced more milk than the placebo goats (Figure 3). However, there was no treatment effect on fat, protein, or lactose percentages, SCC, or and bacterial counts in the milk samples (Table 2). In mammary tissue, CORT release had a significant effect on the *GHR* and *PRLR* genes with higher gene expression in goats subjected to ACTH compared with the control goats (Table 3). On the other hand, there was no effect of repeated CORT increase in vivo on the expression of the *NR3C1*, *NR3C2*, *INSR*, *IGF1R*, *BAX*, and *BCL2* genes nor the percentage of cell proliferation and apoptosis measured in the mammary tissue (Table 3).

Experiment 3: Effect of Chronic CORT Increases In Vitro

The CORT addition had a significant effect on the expression of *NR3C1*, *GHR*, *PRLR*, *INSR*, *IGF1R*, *BCL2*, and *BAX* genes in mammary epithelial cells in vitro (Table 4), but it did not affect the expression of the *NR3C2* gene in mammary cells. Considering the CORT concentration added in culture, the 1,000 μg/mL treatment significantly increased the expression of *NR3C1*, *GHR*, and *PRLR* genes. However, the other treatments (0, 10, and 100 μg/mL) did not affect the expression of these genes in mammary cells. In contrast, CORT addition significantly decreased the expression of the *INSR* and *IGF1R* in epithelial cells: The lowest expression was measured in the 1,000 μg/mL treatment (Table 4). The lowest expression of the *BCL2* gene was observed in the 10 and 100 μg/mL compared with 0 and 1,000 μg/mL treatments. However, there was no difference in expression of the *BCL2* gene at the 0 and 1,000 μg/mL treatments. Moreover, the addition of 1,000 μg/mL CORT significantly increased the expression of the *BAX* gene versus all other treatments. Furthermore, *NR3C1*, *PRLR*, *GHR*, and *BAX* genes were significantly upregulated in mammary epithelial cells when cultured with 1,000 versus 0 μg/mL. Indeed, the *IGF1R* gene was downregulated in mammary epithelial cells when cultured with 1,000 versus 0 μg/mL (Table 4). Finally, CORT addition significantly increased the apoptosis rate and decreased the number of epithelial cells in vitro (Table 4).

DISCUSSION

Goats subjected to ACTH administration showed greater CORT release in plasma and milk than goats given a placebo. The fast increase and return of CORT to baseline as described here is classically used to define acute stress (Mormède et al., 2007; Negrao, 2008; Chen et al., 2015). Although the CORT concentration was higher in plasma than in milk, the profile observed in both fluids was similar to those measured during different farm management practices considered stressful to dairy goats (Negrao and Marnet, 2003; Romero et al., 2015; Bomfim et al., 2018). Other studies have shown that a large dose of glucocorticoids or ACTH decreased PRL, INS, GH, and IGF1 release and milk yield (Maciel et al., 2001; Ollier et al., 2016; Ponchon et al., 2017). In experiment 1, the ACTH dose administrated on d 70 of lactation significantly increased CORT release without affecting INS, PRL, GH, and IGF1 release in plasma and milk. On the other hand, the same ACTH dose administrated on d 90, 120, 150, and 180 of lactation significantly increased the expression of *GHR* and *PRLR* genes in the mammary gland and milk yield on experiment 2, suggesting that ACTH administration positively changed the responsiveness of the mammary gland to these hormones. Previously, at early lactation, the same ACTH dose administrated on 30 and 60 d of lactation did not show a significant effect on the expression of *GHR*, *IGF1R*, proliferation and

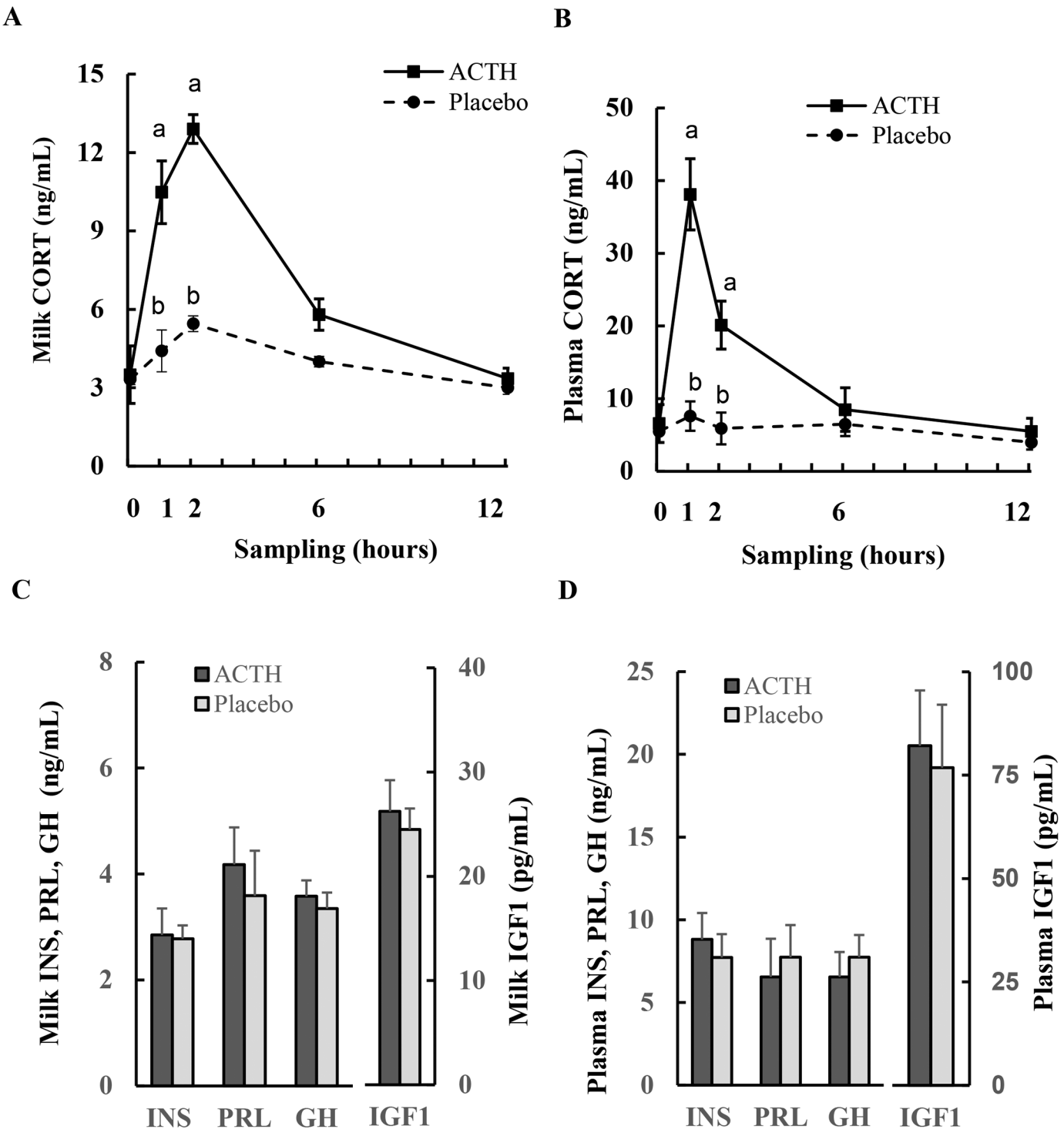

Figure 2. Cortisol (CORT) release in milk (A) and plasma (B) of dairy goats after ACTH (n = 10) or placebo (n = 10) administration on d 70 of lactation. Insulin (INS), prolactin (PRL), growth hormone (GH), and insulin-like growth factor 1 (IGF1) in milk (C) and plasma (D) after ACTH or placebo administration. Data are presented as means ± SEM. Means with different letters (a, b) differ ($P \leq 0.05$).

apoptosis rate, and milk yield (Bomfim et al., 2018). These inconsistencies suggest that CORT's effect on milk yield could be influenced by the lactation stage.

In fact, CORT release significantly increased the milk yield of goats subjected to ACTH versus goats subjected to the placebo from d 112 to 140 and from d

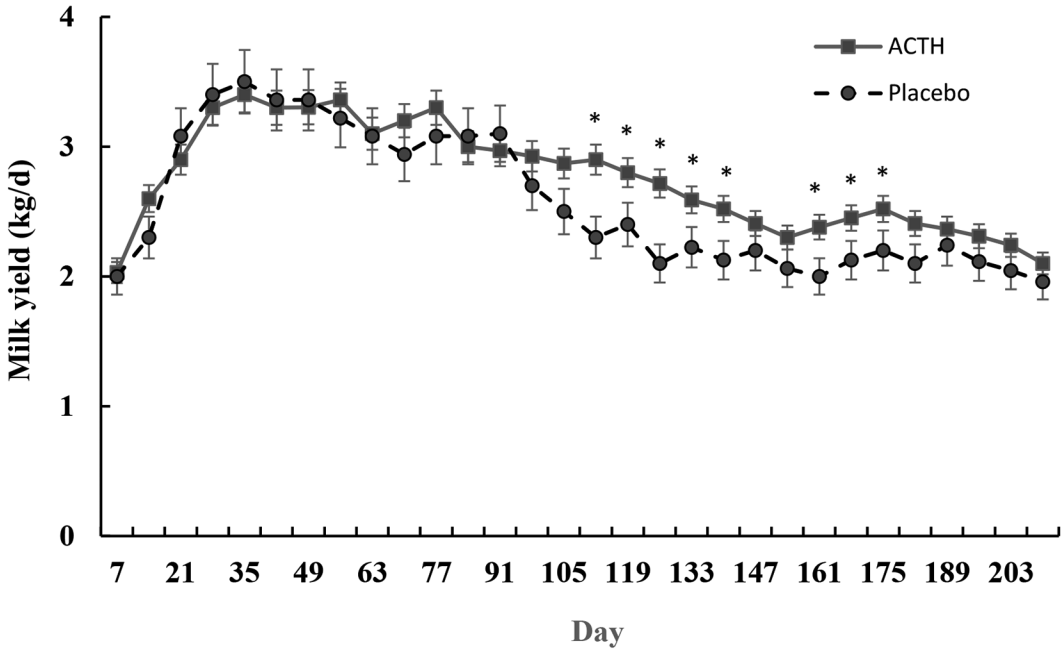

Figure 3. Milk yield (kg/d) of goats treated with ACTH (■, n = 15) and placebo (●, n = 15) was recorded during 210 d of lactation. Data are presented as means ± SEM. *Differs between treatments at $P \leq 0.05$.

161 to 175 of lactation. Previous studies support that CORT can act directly or indirectly on carbohydrates, fat, and protein metabolism to provide the energy necessary to maintain animal homeostasis (Trevisi and Bertoni 2009; Chen et al., 2015; Collier et al., 2017). Other studies have demonstrated that CORT increases glucose and fatty acid concentrations in the plasma of healthy dairy ruminants (Stewart and Thompson, 1984; Shamay et al., 2000); both of which are key precursors for milk synthesis in epithelial cells. Rapid CORT release promotes positive physiological adjustments related to energy metabolization and the redistribution of nutrients to active tissues to allow animals to adapt to acute stress (Trevisi and Bertoni 2009; Chen et al., 2015). Therefore, we can argue that the physiological actions of CORT also increase the amount of nutrients that reach the mammary gland to facilitate milk synthesis in Saanen goats.

Other studies have reported that a high CORT concentration or pharmacological doses of glucocorticoid

Table 3. Gene expression (mRNA, $2^{-\Delta\Delta CT}$ method) of target genes, percentage of cell proliferation, and cell apoptosis in the mammary tissue of goats subjected to ACTH or placebo treatments[1]

	In vivo treatments			
Item	ACTH (n = 15)	Placebo (n = 15)	SEM	*P*-value
Gene (mRNA)				
NR3C1	2.01	1.00	0.51	0.21
NR3C2	2.12	1.05	2.87	0.74
INSR	4.59	1.00	0.92	0.18
PRLR	4.67	1.01	0.57	0.02
GHR	1.35[a]	1.00[b]	0.22	0.02
IGF1R	1.44	1.00	0.09	0.30
BAX	1.28	0.99	0.17	0.32
BCL2	0.71	1.01	0.26	0.88
Cell proliferation (%)	5.22	4.01	0.71	0.57
Cell apoptosis (%)	2.19	1.81	0.37	0.72

[a,b]Means within a row with different superscripts differ ($P \leq 0.05$).

[1]To evaluate the effects of treatments (ACTH or placebo) in experiment on gene expression, the fold change relative to the placebo goat was calculated as shown: ΔΔCt = ΔCt ACTH − ΔCt Placebo. Mammary biopsies were obtained at 180 d of lactation. Data are presented as means ± SEM.

can decrease the immune response (Caroprese et al., 2010: Larzul et al., 2015; Mehdid et al., 2019) and increase the SCC in milk with a risk of clinical mastitis in dairy animals (Barrón-Bravo et al., 2013; Sorgolon et al., 2015; Mehdid et al., 2019). Here, CORT release had no negative effect on milk composition, SCC, or bacterial count in milk. These contrasting results underscore the importance of studying the different relationships between acute and chronic stressors and physiologic and pharmacologic CORT effects in dairy animals.

Furthermore, low CORT release did not affect the proliferation and apoptosis rate of mammary tissue in vivo from d 90 to 180 of lactation, and we cannot confirm a positive effect of CORT on cell proliferation at lactation as observed by other authors (Casey and Plaut, 2007; Chida et al., 2011; Kobayashi et al., 2016). We furthermore cannot confirm a negative effect of CORT on cell apoptosis caused by several stressors (Bertucci et al., 2010; Tao et al., 2015; Mehdid et al., 2019). Although Stewart and Thompson (1984) demonstrated that a single ACTH and CORT administration increased milk yield in goats, our results suggest that CORT improves milk yield without changing the proliferation and apoptosis rates of mammary epithelial cells. These results were unexpected because we postulated that ACTH administration may change the balance between proliferation and apoptosis in epithelial cells via cortisol. In part, the differences between our study and those in the literature can be explained by the different ACTH doses used, lactation phase, and time after ACTH administration at which our biopsy was performed. For the other side, an acute CORT release is associated with a metabolic increase that promotes positive physiological adjustments in different tissues (Trevisi and Bertoni 2009; Chen et al., 2015); thus, an increase in the secretory activity of mammary epithelial cells could improve milk synthesis (Capuco et al., 2001, 2003; Nørgaard et al., 2008). Our in vivo results suggest that cortisol has a positive effect on milk yield; however, further studies are necessary to understand how cortisol modulates the molecular pathways of milk component synthesis.

In our study, CORT did not change the expression of *NR3C1* and *NR3C2* genes in mammary tissue. However, CORT significantly increased milk yield and the expression of *GHR* and *PRLR* in the mammary gland, and it is possible argue that CORT improved the responsiveness of these receptors. On the other hand, some authors have associated the higher GH and PRL concentration in plasma with the higher expression of the BCL2 gene and cell survival (Yang et al., 2005). Other authors have reported that CORT and PRL or PRL and CORT improve α-lactalbumin and casein synthesis (Sakamoto et al., 2005; Shao et al., 2013; Kobayashi et al., 2017). Meanwhile, other authors have demonstrated that INS and IGF1 are part of the functional and molecular mechanism that controls milk synthesis (Flint et al., 2008; Menzies et al., 2010; He et al., 2016). However, CORT release did not affect INS and IGF1 in plasma and milk nor did it affect the expression of the *INSR* and *IGF1R* genes in the mammary tissue. Consequently, we cannot speculate on the interaction

Table 4. Gene expression (mRNA, $2^{-\Delta\Delta CT}$ method) of target genes, cell number, and percentage of apoptosis in the mammary epithelial cells subjected to 4 treatments in vitro: 0, 10, 100, and 1,000 μg/mL of cortisol

	In vitro cortisol treatment[2]					
Gene (mRNA)[3]	0 μg/mL (n = 14)	10 μg/mL (n = 14)	100 μg/mL (n = 14)	1,000 μg/mL (n = 14)	SEM	*P*-value
NR3C1	1.00[a]	1.30[a]	1.36[a]	3.17[b]	0.43	0.01
NR3C2	0.98	1.33	1,25	0.28	0.65	0.15
INSR	1.00[b]	1.73[a]	0.82[b]	0.45[b]	0.21	0.01
PRLR	1.00[a]	1.20[a]	1.24[a]	6.47[b]	0.83	0.01
GHR	1.01[b]	1.03[b]	1.51[ab]	2.68[a]	0.41	0.03
IGF1R	1.00[a]	0.70[a]	0.37[ab]	0.22[b]	0.20	0.04
BAX	0.99[b]	0.59[b]	0.68[b]	2.42[a]	0.19	0.01
BCL2	1.00[a]	0.36[b]	0.42[b]	1.18[a]	0.13	0.01
Cell number[3] (cell/mL)	1.03[a]	0.88[ab]	0.74[b]	0.46[c]	0.47	0.04
Cell apoptosis (%)	1.14[b]	1.29[b]	1.75[b]	7.37[a]	0.27	0.01

[a,b]Means within a row with different superscripts differ ($P \leq 0.05$).

[1]Mammary biopsies were obtained at 180 d of lactation. The epithelial cells were cultured for 5 d. To evaluate the effects of treatments (0, 10, 100 or 1,000 μg/mL of cortisol) the fold change relative to the placebo goats was calculated as shown: ΔΔCt = ΔCt 10, 100 or 1,000 μg/mL − ΔCt 0 μg/mL. Data are presented as means ± SEM.

[2]As goats presented similar BW, body score, and parity, 14 goats were evenly distributed to milk production to have 14 representative mammary samples in culture.

[3]Values × 10^5/mL.

of CORT with the INSR and IGF1R receptors or about their interactions in higher milk production observed in goats given ACTH.

The low amounts of CORT did not show any significant effects on *NR3C1* gene expression in vitro. However, the addition of 1,000 μg/mL CORT increased the expression of the *NR3C1* gene and was associated with an increase in the apoptotic rate of epithelial cells. Thus, other authors have argued that glucocorticoid receptor is the main CORT receptor under stress conditions (Meijer et al., 2018; Monczor et al., 2019). Furthermore, CORT addition caused the upregulation of *GHR* and *PRLR* genes and the downregulation of *IGF1R* genes in vitro. In a previous study performed by our team, CORT addition was related to the higher expression of *IGF1R* gene in mammary epithelial cells (Bomfim et al., 2018); however, this difference can be explained in part by the lactation phase in which the biopsy was performed: on lactation d 60 in the previous study (Bomfim et al., 2018) and on lactation d 180 in present study. In other cell models, a large dose of glucocorticoids decreases the receptor number or expression of the *GHR*, *PRLR*, and *IGF1R* genes (Lembessis et al., 2004; Yokoyama et al., 2008; Feng et al., 2013). However, our results showed that CORT interacts simultaneously and differently with *PRLR*, *INSR*, *GHR, INSR,* and *IGF1R* genes in mammary epithelial cells. Indeed, mammary cells subjected to CORT addition may be coping with prolonged exposure to CORT via compensatory responses to maintain their homeostasis and survival at the expense of their specific function.

Although, we postulate that the addition of 1,000 μg/mL of cortisol in epithelial cells cultured for 5 d mimics the high cortisol concentration seen with the chronic stressor. A limitation of our in vitro model is that cortisol addition to the culture medium does not represent the natural dynamics of cortisol release through the hypothalamic-pituitary-adrenal axis, which involves other in vivo aspects, for example, the effect of the type and duration of the stressor on cortisol concentration. Therefore, other studies are necessary to understand how cortisol increased the milk yield in vivo.

The addition of 1,000 μg/mL CORT significantly increased the expression of the *NR3C1* and *BAX* (an apoptotic factor) genes; the *BAX*:*BCL2* ratio was measured at the 1,000 μg/mL treatment and showed that an excessive amount of CORT can induce apoptotic mechanisms in mammary epithelial cells. Some authors have reported that the *BAX*:*BCL2* ratio is a good indicator of cell susceptibility to apoptosis (Nørgaard et al., 2008; Bomfim et al., 2018). Furthermore, a higher expression of the *BAX* gene was previously related to the apoptosis of mammary epithelial cells (Bertucci et al., 2010; Tao et al., 2015; Singh et al., 2016; Bomfim et al., 2018). Our results showed that although the number of epithelial cells in the culture decreased gradually with CORT addition, the percentage of apoptosis jumped to 7% when 1,000 μg/mL of CORT was added to the culture. These differences can be attributed to the physiological and pharmacological CORT added to the culture, whereas a low CORT concentration did not significantly affect the apoptosis rate, an excessive amount of CORT negatively affected the survival of epithelial cells in vitro.

Simultaneous to the increase in the apoptosis rate, the highest expression of *NR3C1* and *PRLR* genes and the lowest expression of the *IGF1R* gene were measured after treatment with 1,000 μg/mL of CORT. Our results suggest that the CORT-induced downregulation of the *IGF1R* gene in mammary epithelial cells may explain their higher apoptosis rate measured in vitro, because the overexpression of the *IGF1R* gene in mammary tissue protected these cells from apoptosis (Flint et al., 2005; Murney et al., 2015); however, our work did not show a positive relationship under large and chronic CORT increases. Finally, our in vivo study showed that acute CORT release increased the expression of *PRLR* and *GHR* genes and milk yield at mid-end lactation. However, a large and chronic CORT addition in vitro upregulated the *NR3C1*, *GHR*, *PRLR*, and *BAX* genes and downregulated the *IGF1R* genes to increase apoptosis among mammary cells. Further studies are necessary to understand how CORT modulates the molecular elements of the milk component synthesis and apoptosis pathways in epithelial cells.

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