Effect of acute stressors, adrenocorticotrophic hormone administration, and cortisol release on milk yield, the expression of key genes, proliferation, and apoptosis in goat mammary epithelial cells

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ABSTRACT

Cortisol is essential to milk synthesis; however, different acute stressors and the exogenous administration of adrenocorticotrophic hormone (ACTH) decrease milk yield. Therefore, the effect of cortisol on milk yield and its influence on the survival of mammary epithelial cells have not been fully elucidated. In this context, the objective of this study was to evaluate the effect of cortisol on the expression of growth hormone receptor (*GHR*), insulin-like growth factor type 1 (*IGF1*), insulin-like growth factor type 1 receptor (*IGF1R*), insulin-like growth factor-binding protein 3 and 5 (*IGFBP3* and *IGFBP5*), *BAX*, and *BCL2* genes on the proliferation and apoptotic rates of mammary epithelial cells, and on milk yield in Saanen goats. In the present study, 3 experiments were conducted: (1) comparing the in vivo effects of first milking, vaccination, vermifugation, preventive hoof trimming, and the administration of ACTH or a placebo on cortisol release in dairy goats; (2) studying the in vivo effects of immediate increases in cortisol on the mammary gland of lactating goats; and (3) studying the in vitro effects of a prolonged increase in cortisol on mammary epithelial cells obtained from lactating goats. Cortisol release by goats increased significantly after ACTH administration compared with that observed after a placebo, and the cortisol profiles after first milking, vaccination, vermifugation, hoof trimming, and ACTH administration were similar. However, there was no effect of the immediate increase in cortisol in vivo on IGF-1 release, milk yield, milk quality, or the apoptosis and proliferation rates, nor was there any effect on the expression of the target genes. Furthermore, no interaction was observed between IGF-1 and cortisol in either the in vivo or in vitro experiments. However, the addition of cortisol in vitro

significantly increased the expression of the *GHR* and *IGF1R* genes, which stimulate cell proliferation, and the *BAX* gene, which causes apoptosis. These contrasting results can explain why cortisol did not change the rates of proliferation or apoptosis in epithelial cells. Indeed, cortisol supplementation in vitro did not change the number or apoptotic rate of epithelial cells over the course of 5 d. Finally, further studies must be performed to understand the effect of cortisol on the expression of the *GHR*, *IGF1R*, and *BAX* genes by epithelial cells and the roles of these genes in milk synthesis during early lactation.

Key words: milk yield, mammary gland, cortisol, cell apoptosis

INTRODUCTION

Cortisol is essential for copious milk synthesis (Neville et al., 2002; Casey and Plaut, 2007). However, an increase in hypothalamic-pituitary-adrenocortical activity is a characteristic of the stress response (Mormede et al., 2007; Negrao, 2008; Brown and Vosloo, 2017). Furthermore, exogenous glucocorticoids and ACTH administration decrease the plasma concentration of IGF-1 and milk yield in cows (Maciel et al., 2001; Ollier et al., 2016; Ponchon et al., 2017). In fact, administration of a large amount of glucocorticoid over a long period disrupts the growth hormone (GH)/IGF-1 axis, resulting in inhibition of the action of GH and IGF-I (Lembessis et al., 2004; David et al., 2011; Feng et al., 2013). Indeed, GH and IGF-1, IGFBP-3, and IGFBP-5 also influence the expression of *BCL2* (anti-apoptotic) and *BAX* (pro-apoptotic) genes and have important roles in the regulation of epithelial cell survival (Plath-Gabler et al., 2001; Sakamoto et al., 2007; Flint et al., 2008). Therefore, the effect of cortisol on milk yield and its influence on the survival of mammary epithelial cells have not been fully elucidated.

At the same time, farm management practices such as vaccination, vermifugation, first milking, weaning, hoof trimming, and transport may cause acute stress,

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cortisol release, and decreased milk yield (Sevi et al., 2001a,b; Canaes et al., 2009; Caroprese et al., 2010). Usually, stress is negatively associated with milk yield, but it is important to understand the relationship between cortisol release and milk yield, because cortisol is necessary to maintain homeostasis and promote animal adaptation (Negrao and Marnet, 2003; Trevisi and Bertoni, 2009; Brown and Vosloo, 2017). In this context, stress and cortisol release are associated with the expression of proteins that regulate cell survival, including BCL2, which prevents apoptosis, and BAX, which initiates the cascade that promotes apoptosis in mammary epithelial cells (Green and Streuli, 2004; Portt et al., 2011; Tao et al., 2015). These contrasting findings concerning the physiological responses related to lactation and stress show that the effects of cortisol on milk synthesis and the survival of epithelial cells remain controversial.

Although exogenous ACTH and glucocorticoid administration has been shown to decrease milk yield in cows (van der Kolk, 1990; Ollier et al., 2016; Ponchon et al., 2017) and ewes (Caroprese et al., 2010), and increase the SCC in milk in dairy ewes (Sevi et al., 2001a,b; Caroprese et al., 2010), the effects of ACTH and glucocorticoid administration on milk yield in goats remains ambiguous. Several authors have reported the absence of a negative effect of administering ACTH and glucocorticoid on the milk yield of dairy goats (Stewart and Thompson, 1984; Anderson et al., 1991; Shamay et al., 2000). In this context, the objective of this study was to evaluate the effect of cortisol on the expression of the *IGF1*, *IGF1R*, *GHR*, *IGFBP3*, *IGFBP5*, *BAX*, and *BCL2* genes, the rates of proliferation and apoptosis of mammary epithelial cells, milk quality, and milk yield in Saanen goats.

MATERIALS AND METHODS

All the animal procedures complied with the ethical code of the Faculty of Animal Science and Food Engineering of the University of São Paulo, Faculty of Animal Science and Food Engineering (FZEA) of the University of São Paulo (USP). The experiment was carried out at the Laboratory of Animal Physiology of the Basic Sciences Department in the Faculty of Animal Science and Food Engineering (FZEA/USP) in Pirassununga, Brazil (at latitude 21° 57' 02" S, longitude 47° 27' 50" W). The climate in this area is subtropical, with an average annual temperature of 23°C and humidity of 73%, and a rainy season from November to March (with annual rainfall varying from 1,300 to 2,000 mm), and the experimental collections were carried out between June and September.

Housing, Diets, and Milking Routine

All experimental goats were distributed into covered, collective pens with feeding troughs, mineral salt, and water. The feed contained 60% concentrate and 40% roughage, providing 100% of the animals' requirements (NRC, 2007). Milking was conducted twice daily by the same team throughout the experimental period. The milking machine was regulated to maintain a vacuum level of 48 kPa and a pulse rate of 120 cycles/min. The milking routine was performed in accordance with the following protocol: before milking, the animals were attached to a milking machine, and their teats were predipped and dried. The teat cups were attached at time 0 and detached at the end of milking. Afterward, their teats were postdipped, the individual milk yield was recorded, and the animal exited the milking parlor.

Organization of Experiments

In the present study, 3 experiments were conducted: (1) comparing in vivo the effects of milking, vaccination, vermifugation, hoof trimming, and the administration of ACTH or a placebo on cortisol release in dairy goats; (2) studying in vivo the effects of immediate increases in cortisol on the mammary gland of dairy goats; and (3) studying in vitro the effects of a prolonged increase in cortisol levels on the epithelial cells of dairy goats.

Experiment 1 (Acute Stress and ACTH Administration)

Twenty-four healthy, homogeneous Saanen goats (mean $\pm$ SD; BW of 55.6 ± 1.3 kg, BCS of 3.5 ± 1.0 , lactating and not pregnant) were used to conduct this experiment. Experimental goats were randomly distributed according to the previous number of parturitions, milk yield, BW, and BCS. These 24 goats were subjected to 6 treatments: first milking, vaccination, vermifugation, preventive hoof trimming, and placebo (saline solution) or ACTH administration. The ACTH dose tested (0.6 UI of ACTH/kg of BW per animal) was based on previous studies that demonstrated that cortisol returned to basal levels just 6 h after ACTH administration (Fulkerson and Jamieson, 1982; Alam et al., 1986; Schwinn et al., 2016). This cortisol profile was considered similar to other acute stressors related to farm management (Fulkerson and Jamieson, 1982; Negrao et al., 2004; Negrao, 2008).

At 0800 h on the experimental day, 4 goats were subjected to first milking, 4 goats were subjected to vaccination, 4 goats were subjected to vermifugation, 4 goats were subjected to preventive hoof trimming,

4 goats were injected with ACTH, and 4 goats were injected with the placebo. The experimental goats were maintained and brought to the equipment in groups with 6 other goats that did not participate in the experiment. Each procedure was performed with the appropriate equipment, and during the procedures, the goats were immobilized in individual tiestalls. Goats had visual, vocal, and limited tactile contact with neighboring goats during all procedures. The experimental procedures were performed by a trained team, and the time spent to milk each goat was 3 ± 0.5 min, to vaccinate was 2 ± 0.2 min, to vermifugate was 1.5 ± 0.2 min, and to trim hooves was 10 ± 3.1 min. Afterward, the experimental goats were maintained with the other 6 goats that did not participate in the experiment in a collective pen directly after blood sampling.

Blood samples were collected by a puncture from the jugular vein at 30 min (30 min before), 0 min (just before) and 60, 120, 360, and 1,440 min after first milking, vaccination, vermifugation, hoof trimming, and either ACTH or placebo administration. Blood samples were centrifuged at $1,500 \times g$ and 4°C for 17 min, and the plasma was stored at -20°C until cortisol was analyzed. Cortisol was measured by immunoassay analysis using a kit (Monobind, Lake Forest, CA) according to the manufacturer's instructions. In the present study, the cortisol intra- and interassay coefficients of variation were 2.8 and 5.5%, respectively.

Experiment 2 (Effect of Immediate Increase in Cortisol In Vivo)

An additional herd of 30 homogeneous experimental Saanen goats (BW of 57.5 ± 1.5 kg, body score of 3.5 ± 1.0 , lactating and not pregnant) were used to conduct this experiment. The goats were randomly distributed according to the previous number of parturitions, milk yield, BW, and BCS and subjected to 2 treatments: ACTH and a placebo. On d 30 and 60 of lactation, 15 animals from each group were subjected to the following treatments: (1) ACTH challenge (administration of 0.6 UI/kg of BW per animal) and (2) placebo (saline solution). This in vivo experiment was performed to study the effect of the immediate increase in cortisol on the mammary gland.

Individual milk yield (kg) was recorded daily. Milk samples were taken weekly from each of the teats of the experimental goats to analyze the milk composition (fat, protein, and lactose) and milk quality (SCC and microbiological analyses). On d 30 and 60 of lactation, milk samples were taken at baseline (0 h, just before treatment), 12 (evening milking), 24, 36, and 48 h after

ACTH or placebo administration. The percentage of fat, protein, and lactose was determined by infrared equipment. The SCC was determined by direct microscopy (using Carnoy solution and pyronin Y-methyl green). Microbiological analyses were performed on the milk. After serial dilutions of the milk, each sample was plated and tested on 3 media, standard plate count agar, Baird Parker agar, and MacConkey agar, to count total colony formation. The results were expressed as colony-forming units per milliliter of *Enterobacteriaceae* and *Staphylococcus* sp.

On d 30 and 60 of lactation, blood samples were collected from the jugular vein at -30 min (30 min before), 0 min (just before) and 60, 120, and 360 min after ACTH or placebo administration. Blood samples were centrifuged at $1,500 \times g$ and 4°C for 17 min, and the plasma was stored at -20°C until the cortisol and IGF-1 analyses were performed. Cortisol was analyzed as described for experiment 1, and IGF-1 was measured by immunoassay analysis using a kit (Enzo, Farmingdale, NY) according to the manufacturer's instructions. In this study, the IGF-1 intra- and interassay coefficients of variation were 4.2 and 6.0%, respectively.

On d 60 of lactation, biopsies of the mammary glands of 8 goats were also performed after challenge with ACTH or the placebo. The 8 goats were randomly distributed, with 4 in each treatment. Each biopsy was performed 30 min after the administration of ACTH or the placebo. After trichotomy, antiseptics, and anesthetic procedures, the skin incision was performed at the medial part of the gland with a scalpel. The disposable biopsy needle was inserted into the incision to a depth of approximately 3.0 cm, and the equipment was triggered to cut and collect a sample of approximately 2.0×0.5 cm of mammary tissue. Three tissue samples were collected. The first was collected to use in determining the rates of apoptosis and proliferation, the second was used for the analysis of gene expression by reverse-transcription (RT) PCR, and the third was used for cell cultures and the subsequent administration of in vitro treatments.

The rate of apoptosis of the epithelial cells was determined using a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) kit according to the manufacturer's instructions (Roche, Indianapolis, IN). Apoptotic cells were counted on slides when their nuclei were intense green. The rate of proliferation of epithelial cells was determined using a proliferating cell nuclear antigen (PCNA) kit according to the manufacturer's instructions (Invitrogen, Burlington, ON, Canada), and proliferating cells were counted on slides when their nuclei

Table 1. Primer sequences for the genes studied

Gene ¹	Sequence (5' to 3')		GenBank accession	Amplicon (bp)
<i>GAPDH</i>	Forward	5'-GGTGATGCTGGTGCTGAG-3'	AJ431207	181
	Reverse	5'-TGACAATCTTGAGGGTGTG-3'		
<i>GHR</i>	Forward	5'-CGTCTCTGCTGGTGAACA-3'	NM_001285648	148
	Reverse	5'-AACGGGTGGATCTGGTTGTA-3'		
<i>IGF1R</i>	Forward	5'-TTAAAATGGCCAGAACCTGAG-3'	XM_005694951	314
	Reverse	5'-ATTATAACCAAGCCTCCAC-3'		
<i>IGF1</i>	Forward	5'-TGCTCTCCAGTTCGTGTG-3'	FJ716104	142
	Reverse	5'-CATCTCCAGCCTCCTCAG-3'		
<i>IGFBP3</i>	Forward	5'-AAAGGTCATGCCAAGGACAG-3'	JQ341161	281
	Reverse	5'-TGCCCGTACTTATCCACACA-3'		
<i>IGFBP5</i>	Forward	5'-TGCGAGCTGGTCAAGGAG-3'	JF720883	257
	Reverse	5'-TCCTCTGCCATCTCGGAG-3'		
<i>BCL2</i>	Forward	5'-GATGACCGAGTATCTGAACCG-3'	NM_001166486	120
	Reverse	5'-GACAGCCAGGAGAAATCAAACA-3'		
<i>BAX</i>	Forward	5'-TCGGTCTCAACGGCTACA-3'	NM_173894	189
	Reverse	5'-CCACTCCAGCCACAAAGA-3'		

¹Genes were glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), growth hormone receptor (*GHR*), insulin-like growth factor type 1 (*IGF1*), insulin-like growth factor type 1 receptor (*IGF1R*), insulin-like growth factor-binding proteins 3 and 5 (*IGFBP3* and *IGFBP5*), *BAX*, and *BCL2*.

were dark brown. The percentages of proliferating and apoptotic cells were determined by observing 1,000 cells in randomly selected fields.

Gene expression of mammary tissue was performed by extracting RNA with a PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA), and subsequent cDNA synthesis was conducted by RT, where the total RNA (1.0 µg) was reverse-transcribed in a final volume of 20 µL employing the Improm II kit (Promega, Madison, WI). In summary, the protocol began with the incubation of RNA with 0.5 µg oligo-dT for 5 min at 70°C. Then, 1× buffer, 3.0 mM MgCl₂, 0.5 mM dNTP, 40 units of RNase inhibitor, and 1.0 µL of Improm reverse transcriptase were added to the reaction tube. The reaction was conducted at 42°C for 60 min, followed by 15 min cooling at 70 and 4°C. Then, the samples were subjected to RT and subsequent analysis of the amplified gene fragments. The expression of the target genes was determined by RT-PCR using the primers described in Table 1.

Each gene studied was amplified in a separate reaction, and each reaction was performed in duplicate. Each 20 µL reaction contained 1.0 µL of cDNA sample, 0.4 µL of a mixture of a forward primer and reverse primer, 0.4 µL of the analyzed genes [*GAPDH* (housekeeper), *GHR*, *IGF1*, *IGF1R*, *IGFBP3*, *IGFBP5*, *BAX*, and *BCL2*], 8.2 µL of RNA-free water, and 10.0 µL of SYBR Green Mix (Invitrogen, Burlington, ON, Canada). The expression of the target genes was normalized to that of *GAPDH* as calculated by the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). The samples were then subjected to RT-PCR and subsequent analysis of the amplified gene fragments.

Experiment 3 (Effect of Prolonged Increase in Cortisol In Vitro)

This in vitro experiment was performed with the goats from experiment 2 to study the effect of a prolonged increase in cortisol on epithelial cells. Compared with experiment 2 in vivo, the experiment 3 studied the effect of large amount of cortisol (0, 10, 100, and 1,000 µg/mL) for 5 d in vitro to observe the epithelial cell survival in extreme situations, without affecting other mechanisms, tissues, or glands involved in response to cortisol. The concentrations of cortisol tested were based on previous studies that used between 1 and 10 µg/mL of cortisol to establish epithelial cells culture in vitro (Keys et al., 1997; Panticenko et al., 2000; Yang et al., 2005), and on our previous in vitro research (data not published) using 0, 0.1, 1, 10, and 100 µg/mL of cortisol. On d 60 of lactation, samples of the mammary glands of 8 goats (4 goats subjected to ACTH and another 4 goats subjected to the placebo) were used to establish primary cultures. Individually, the primary cultures of the mammary tissue of the 8 goats were established in a basal medium of Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 Han (DMEM/F12, Gibco Life Technologies, Grand Island, NY) with 10% fetal bovine serum (Gibco Life Technologies), 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 2.5 µg/mL of amphotericin B and were incubated at 37°C with 5% CO₂ until the cells filled the same plate background. The mammary cells from each experimental goat were counted using automated cell counting equipment (Invitrogen, Waltham, MA) to ensure that all experimental cultures had a minimum

of 10×10^4 live cells at the beginning of the 8 in vitro treatments. Afterward, mammary epithelial cells from each of the 8 goats were subjected individually to 8 hormone treatments in vitro (0, 10, 100, or 1,000 $\mu\text{g/mL}$ of cortisol together with either 0 or 100 ng/mL of IGF-1) in triplicate. Eight wells of each treatment studied were harvested for RNA after 5 d in culture.

For the in vitro treatments, mammary epithelial cells were placed on an individual plate containing a basement membrane matrix (25 $\mu\text{L/cm}^2$, BD Biosciences, San Jose, CA) with basal medium (as described above) supplemented with 5 $\mu\text{g/mL}$ of insulin (Sigma Chemical Co., St. Louis, MO), 1 $\mu\text{g/mL}$ of prolactin (Sigma Chemical Co.), and one of the 8 in vitro hormone treatments. This experimental medium was changed at 48 h, and the epithelial cells were incubated for 5 d to analyze the expression of the *GAPDH*, *GHR*, *IGF1*, *IGF1R*, *IGFBP3*, *IGFBP5*, *BAX*, and *BCL2* genes as described for the in vivo experiment.

Statistical Analysis

The data were analyzed using Statistical Analysis System software (ver. 9.2, SAS Institute Inc., Cary, NC) after checking their normality according to the residual of the Shapiro-WilkPROC single variable. Cortisol release was subjected to the ANOVA by the mixed procedure in the SAS software, which separated the treatment (first milking, vaccination, vermifugation, hoof trimming, and placebo or ACTH administration), time of sampling and animal as causes of variation. In the model, the treatment effect was considered fixed, and the effects of sampling time and animal were con-

sidered to be random. Hormone release, milk yield, milk composition and quality were subjected to an ANOVA by the mixed procedure in the SAS software, which separated the treatment (ACTH or placebo), day, sampling time and animal as causes of variation. In the model, the treatment effect was considered fixed, and the effects of the day, sampling time and animal were considered random. Gene expression in vivo and in vitro were subjected to an ANOVA using the general linear model procedure in the SAS software, which separated the treatments (0, 10, 100, or 1,000 $\mu\text{g/mL}$ of cortisol together with 0 or 100 ng/mL of IGF-1) and animal as causes of variation. In the model, the treatment effect was considered fixed, and the effects of the animals were considered random. When there was a significant effect, the means were compared using Fisher's test with the threshold for significance defined as $P \leq 0.05$.

RESULTS

Experiment 1

Goats that received the placebo maintained the basal concentration of cortisol during all experimental sampling. However, there were significant effects of first milking, vaccination, vermifugation, hoof trimming, and ACTH administration on cortisol release when compared with cortisol release in the placebo treatment (Figure 1). The maximum concentration of cortisol was observed 60 min after first milking, vaccination, vermifugation, hoof trimming, and ACTH administration, and the cortisol level remained significantly higher 120 min after first milking, hoof trimming and ACTH treatments than that following the placebo treatment. However, cortisol returned to the basal concentration 360 min after first milking, vaccination, vermifugation, hoof trimming, and ACTH administration. Twenty-four hours (1,440 min) after first milking, vaccination, vermifugation, hoof trimming, or ACTH or placebo administration, cortisol remained at the basal concentration. Finally, cortisol profiles after first milking, vaccination, vermifugation, preventive hoof trimming, and ACTH administration were similar, showing that the ACTH administration tested was adequate to promote an immediate cortisol increase in dairy goats.

Experiment 2

There was no effect of ACTH or placebo administration in vivo on milk yield. There was no effect of the treatments or any significant interaction between treatment and sampling time on plasma IGF-1, on fat,

Figure 1. Cortisol (ng/mL) release in Saanen goats before, during, and after first milking, vaccination, vermifugation, hoof trimming, and ACTH or placebo administration ($n = 4$). Data are presented as the mean $\pm$ SEM. Means with different letters exhibit statistically significant differences ($P \leq 0.05$).

Table 2. Milk yield; protein, fat, and lactose percentages; SCC; and total counts of bacteria, *Enterobacteriaceae*, and *Staphylococcus* sp. measured from 1 to 90 d of lactation in goats subjected to ACTH or placebo treatment (n = 15)¹

Item	In vivo treatment		SEM	P-value
	ACTH	Placebo		
Milk yield (kg)	2.19	2.18	0.35	0.79
Fat (%)	3.98	3.82	0.34	0.29
Protein (%)	3.17	3.19	0.01	0.37
Lactose (%)	4.31	4.34	0.02	0.34
SCC ² (cells/mL)	1,403.55	1,323.22	185.54	0.77
Total count of bacteria ² (cfu/mL)	16.87	19.92	5.09	0.08
<i>Enterobacteriaceae</i> ³ (cfu/mL)	—	—	—	—
<i>Staphylococcus</i> sp. ² (cfu/mL)	17.25	24.37	6.66	0.09

¹Data are presented as the mean $\pm$ SEM.²Values $\times 10^3$ /mL.³The count was insufficient for statistical analysis.

protein, and lactose percentage, SCC, or the number of colony-forming units in the plate count agar, MacConkey agar, and Baird Parker agar media (Table 2). There was a significant interaction between treatment and sampling time with regard to cortisol release, because in samples taken at 60 and 120 min, cortisol concentrations were higher after ACTH administration than those following the placebo treatment. The cortisol concentration returned to baseline levels 360 min after ACTH treatment and achieved similar values to those after the placebo administration (Figure 2). There was no effect of ACTH or placebo administration on the expression of the *GHR*, *IGF1*, *IGF1R*, *IGFBP3*, *IGFBP5*, *BAX*, or *BCL2* genes, nor were there any effects of ACTH or placebo treatment on the percentage of cell proliferation (PCNA) and cell apoptosis (TUNEL) in mammary tissue (Table 3).

Experiment 3

There was no interaction between the supplementation of cortisol and IGF-1 treatments in culture on the expression of the *GHR*, *IGF1*, *IGF1R*, *IGFBP3*, *IGFBP5*, *BAX*, or *BCL2* genes by epithelial cells, nor was there an effect of IGF-1 treatment on the expression of the same genes (Table 4). However, there was a significant in vitro effect of the cortisol treatment on the expression of the *GHR*, *IGF1R*, and *BAX* genes, without changing the expression of the *IGF1*, *IGFBP3*, and *IGFBP5* genes. In fact, the highest expression of the *GHR*, *IGF1R*, and *BAX* genes was observed in the treatment with 1,000 μ g/mL of cortisol (Table 5).

DISCUSSION

In the present study, cortisol release after ACTH challenge was similar to that measured after first milk-

ing, vaccination, vermifugation and hoof trimming. Furthermore, the cortisol increase was temporary, and the rapid return to the basal concentration was similar to that observed after first milking, vaccination, vermifugation, hoof trimming and ACTH administration. In general, the duration of a stressful event is used to class the stressor as acute or chronic. In this context, cortisol release is fundamental to the study of the response of the hypothalamic-pituitary-adrenal (HPA) axis to both types of stressors. Acute stress is established when a stressor occurs over few minutes or hours, and cortisol concentration returns to baseline between 4 and 6 h after to its basal concentration and homeostasis (Mormede et al., 2007; Trevisi and Bertoni, 2009; Chen et al., 2015). As in other studies (Verkerk et al., 1994; Jensen et al., 1996; Negrao and Marnet, 2003; Larzul et al., 2015), in the present study, ACTH administration caused a cortisol release similar to other acute stressors related to other farm management practices performed during goat lactation. Therefore, the ACTH dose used was considered adequate to study the immediate effect of cortisol on the mammary gland of dairy goats.

In contrast, chronic stress is established when a continuous or cumulative stressor is maintained. In that case, the cortisol concentration remains significantly higher than the basal concentration (Mormede et al., 2007; Kassahn et al., 2009; Brown and Vosloo, 2017), causing cumulative and negative effects for animals. In general, large amounts of exogenous glucocorticoids reduce milk yield (van der Kolk, 1990; Ollier et al., 2016; Ponchon et al., 2017). Indeed, other studies have shown that glucocorticoid administration was also associated with decreases in the immune response and failure of the HPA axis (Kassahn et al., 2009; Caroprese et al., 2010; Larzul et al., 2015). However, in the present study, no effect was observed of ACTH administration and cortisol increase on IGF-1 release in plasma, milk yield,

Figure 2. Cortisol (ng/mL) and IGF-1 (ng/mL) released in Saanen goats on d 60 of lactation before, during, and after ACTH or placebo administration 4 (n = 15). Data are presented as the mean $\pm$ SEM. Means with different letters exhibit statistically significant differences ($P \leq 0.05$).

or milk quality. Similarly, other studies that also used ACTH or glucocorticoid administration did not observe any negative effect on either milk quality or milk yield in dairy goats (Stewart and Thompson, 1984; Anderson et al., 1991; Shamay et al., 2000). For these reasons, we proposed to study the effect of high levels of cortisol in mammary tissue in vitro to verify the possible local effect of cortisol without affecting other mechanisms, tissues or glands involved in complex responses to stress.

In fact, the majority of studies that used large amounts of glucocorticoids or ACTH by a single or repeated administration observed a significant decrease in IGF-1 release and milk yield in cows (van der Kolk, 1990; Ollier et al., 2016; Ponchon et al., 2017) and ewes (Caroprese et al., 2010). Furthermore, previous studies with lactating ewes also reported that higher cortisol release due to stress decreased milk yield and increased SCC (Sevi et al., 2001a,b; Caroprese et al., 2010). However, in the present study, the ACTH challenge did not change the SCC or total counts of *Enterobacteriaceae* and *Staphylococcus* sp. in milk compared with those in control goats. Indeed, the SCC and bacteria counts in the milk following both ACTH and placebo treatments were similar to those in control goats (Paape et al., 2001, 2007; Barron-Bravo et al., 2013). These results confirmed that the immediate increase in cortisol after ACTH administration did not influence milk yield, milk quality or the susceptibility of experimental goats to microorganisms that may cause mastitis.

At the same time, no effect was observed of ACTH administration on IGF-1 release in plasma, or on the expression of the *IGF1*, *IGF1R*, *GHR*, *IGFBP3*, *IGFBP5*, *BAX*, and *BCL2* genes in the mammary tissue of the experimental goats. Thus, there was no in vivo effect of the immediate increase in cortisol on proliferation and apoptosis rates in mammary tissue, nor was there any effect on milk yield measured before or after both treatments. Consequently, it is possible to argue that the ACTH dose used in the present study was not sufficiently high to have a negative effect on the mammary gland. However, as discussed above, the ACTH dose tested caused an increase in cortisol release similar to that observed during first milking, vaccination, vermifugation and preventive hoof trimming. Furthermore, the cortisol concentration measured after ACTH treatment was similar to that observed by other authors who reported a decrease in milk yield when working with other stressors (Sevi et al., 2001a,b; Negro and Marnet, 2003; Canaes et al., 2009; Caroprese et al., 2010).

In this context, it is possible to argue that the negative effect of the increase in cortisol on mammary glands following an acute stressor is overestimated for healthy animals during early lactation, because HPA responses,

Table 3. Relative expression of target genes and the percentages of cell proliferation and cell apoptosis in fresh mammary tissue of goats subjected to ACTH or placebo treatment (n = 15)¹

Item ²	In vivo treatment		SEM	P-value
	ACTH	Placebo		
<i>GHR</i>	1.42	1.21	0.16	0.14
<i>IGF1</i>	1.44	1.40	0.31	0.94
<i>IGF1R</i>	1.17	1.48	0.26	0.46
<i>IGFBP3</i>	0.85	1.09	0.13	0.28
<i>IGFBP5</i>	2.44	1.69	0.88	0.62
<i>BAX</i>	1.20	1.48	0.29	0.51
<i>BCL2</i>	1.70	2.39	0.67	0.55
Cell proliferation (%)	20.15	22.04	1.58	0.63
Cell apoptosis (%)	10.26	11.21	1.81	0.78

¹Mammary biopsies were obtained at 60 d of lactation. Data are presented as the mean $\pm$ SEM.

²Genes were growth hormone receptor (*GHR*), insulin-like growth factor type 1 (*IGF1*), insulin-like growth factor type 1 receptor (*IGF1R*), insulin-like growth factor binding proteins 3 and 5 (*IGFBP3* and *IGFBP5*), *BAX*, and *BCL2*.

mainly cortisol release, are considered necessary for the return of that animal to homeostasis (Mormede et al., 2007; Brown and Vosloo, 2017). Although improvements in farm management practices are necessary to promote animal production and welfare (Chen et al., 2015; Brown and Vosloo, 2017), physiological responses to acute stressors promote animal adaptation. However, in the in vitro study, there was also no effect of IGF-1 treatment, nor was there any interaction between IGF-1 and cortisol treatment on the expression of the *IGF1*, *IGF1R*, *GHR*, *IGFBP3*, *IGFBP5*, *BAX*, and *BCL2* genes by mammary cells in vitro. Taking into account these results, we cannot speculate about the negative effect of cortisol on the action of IGF-1, nor can we speculate about the positive effect of IGF-1 on cell proliferation and the survival of epithelial cells in vitro. In fact, our initial argument that IGF-1 plays

a local role in conjunction with IGF-1R, IGFBP-3 and IGFBP-5 in inhibiting apoptosis and minimizing the action of cortisol on epithelial cells was not confirmed.

In vitro, the addition of cortisol did not change the expression of the *IGF1*, *IGFBP3*, or *IGFBP5* genes, or proliferation and apoptosis in the mammary tissue of the experimental goats. The action of IGF-1 on target cells is influenced by its bond to BPs, some studies have shown that the interaction between IGF-1 and IGFBP-3 is related to cell proliferation and that the interaction between IGF-1 and IGFBP-5 is related to cell apoptosis on mammary gland (Sakamoto et al., 2007; Flint et al., 2008; Murney et al., 2015). These different relationships between IGF-1 and IGFBP-3 and -5 can explain the effect of IGF-1 on both cell proliferation and apoptosis. As in the present study, cortisol supplementation in vitro did not significantly influence the expression of *IGF1*, *IGFBP3*, or *IGFBP5* in epithelial cells, and cortisol did not change proliferation or apoptosis rates in epithelial cells. These results in vitro show that our initial hypothesis that cortisol influences apoptosis and proliferation rates through the expression of *IGF1*, *IGFBP3*, and *IGFBP5* in mammary epithelial cells was not confirmed.

Conversely, in vitro cortisol supplementation significantly increased the expression of *GHR* and *IGF1R* in epithelial cells. Other authors have reported that GH and IGF-1 can modulate the survival of epithelial cells (Capuco et al., 2001; Flint et al., 2005; Rosenfeld and Hwa, 2009), and the increase in expression of *GHR* and *IGF1R* observed in vitro in the study can explain why GH and IGF-1 were previously associated with cell proliferation (Flint et al., 2008; Murney et al., 2015). As cortisol did not change the expression of *IGF1*, *IGFBP3*, or *IGFBP5*, our results demonstrated the direct effect of cortisol on the expression of *GHR*

Table 4. Relative expression of target genes by goat primary mammary epithelial cells cultured with 0 and 100 ng/mL of IGF-1 (n = 8)¹

Item ²	In vitro IGF-1 treatment (ng/mL)		SEM	P-value
	0	100		
<i>GHR</i>	1.32	1.14	0.23	0.15
<i>IGF1</i>	0.20	0.13	0.06	0.21
<i>IGF1R</i>	1.53	1.54	0.18	0.84
<i>IGFBP3</i>	1.48	1.13	0.20	0.33
<i>IGFBP5</i>	1.41	1.37	0.34	0.96
<i>BAX</i>	1.52	1.38	0.16	0.23
<i>BCL2</i>	1.14	1.01	0.14	0.35

¹Mammary biopsies were obtained at 60 d of lactation. Data are presented as the mean $\pm$ SEM.

²Genes were growth hormone receptor (*GHR*), insulin-like growth factor type 1 (*IGF1*), insulin-like growth factor type 1 receptor (*IGF1R*), insulin-like growth factor-binding proteins 3 and 5 (*IGFBP3* and *IGFBP5*), *BAX*, and *BCL2*.

Table 5. Relative expression of target genes, cell number, and percentage of apoptosis in goat primary mammary epithelial cells cultured with 0, 10, 100, and 1,000 µg/mL of cortisol (n = 8)¹

Item ²	In vitro cortisol treatment (µg/mL)				SEM	P-value
	0	10	100	1,000		
<i>GHR</i>	0.59 ^c	0.99 ^{bc}	1.06 ^b	2.42 ^a	0.25	0.01
<i>IGF1</i>	0.22	0.14	0.27	0.36	0.09	0.17
<i>IGF1R</i>	1.02 ^b	1.43 ^b	1.38 ^b	2.30 ^a	0.21	0.01
<i>IGFBP3</i>	1.42	1.53	1.23	1.03	0.19	0.44
<i>IGFBP5</i>	1.51	1.46	1.29	1.30	0.27	0.90
<i>BAX</i>	0.85 ^b	1.08 ^b	1.22 ^b	2.56 ^a	0.46	0.01
<i>BCL2</i>	1.46	0.64	0.94	1.25	0.11	0.35
Cell number ³ (mL)	1.02	0.91	0.93	0.89	0.02	0.85
Cell apoptosis (%)	10.31	10.54	11.02	11.57	0.60	0.88

^{a-c}Means within a row with different superscripts exhibit statistically significant differences ($P \leq 0.05$).

¹Mammary biopsies were obtained at 60 d of lactation, and epithelial cells were cultured for 5 d. Data are presented as the mean $\pm$ SEM.

²Genes were growth hormone receptor (*GHR*), insulin-like growth factor type 1 (*IGF1*), insulin-like growth factor type 1 receptor (*IGF1R*), insulin-like growth factor-binding proteins 3 and 5 (*IGFBP3* and *IGFBP5*), *BAX*, and *BCL2*.

³Values $\times 10^5$ /mL.

and *IGF1R* in mammary tissue, further suggesting that large amounts of cortisol can improve the actions of GH and IGF-1 in mammary cells, as reported in other studies that worked with large amounts by long-term exposure to glucocorticoid and other cells (Savage et al., 2011; Vakili and Cattini, 2012). In fact, the over-expression of *GHR* and *IGF1R* genes protects different cells against apoptosis (Mueller et al., 2012; Feng et al., 2013; Murney et al., 2015).

In contrast, cortisol supplementation also significantly increased the expression of the *BAX* gene (pro-apoptotic) without changing the expression of the *BCL2* gene (anti-apoptotic) in epithelial cells in vitro. This imbalance in the expression of *BAX* and *BCL2* can induce the expression of proteins which initiates apoptosis in mammary epithelial cells, because *BAX* is involved in the cascade of proteins that promote the cleavages that cause cell apoptosis (Valentijn and Gilmore, 2004; Jin and El-Deiry, 2005; Katz and Streuli, 2007). Other authors have suggested that long-term exposure to glucocorticoid can negatively regulate the expression of GH and *IGF1* genes in other cell models (Lembessis et al., 2004; Feng et al., 2013). However, in the present study, the cell number and percentage of apoptosis was not changed by cortisol supplementation in vitro, showing that the survival effects promoted by higher expression of *GHR* and *IGF1R* genes have overlapping apoptotic effects of higher expression of *BAX* gene in epithelial cells.

Consequently, in the present study, cortisol supplementation significantly increased the expression of the *GHR*, *IGF1R*, and *BAX* genes by epithelial cells in vitro. These results were unexpected, because cortisol

has been shown to have opposing effects on epithelial cells: (1) increasing the expression of *GHR* and *IGF1R*, which stimulates the survival of epithelial cells, and (2) increasing the expression of *BAX*, which induces apoptosis. Taking into account the fact that cortisol supplementation did not change the cell number or apoptotic rate of epithelial cells maintained in culture for 5 d, it is possible to argue that cortisol did not change the survival of epithelial cells, showing that large amounts of cortisol in vitro had no negative effects on mammary glands during early lactation in Saanen goats. However, further studies need to be performed to understand how cortisol can modulate the expression of *GHR*, *IGF1R*, and *BAX* and to elucidate their roles in milk synthesis.

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