Glucocorticoid effects on sheep peripheral blood mononuclear cell proliferation and cytokine production under in vitro hyperthermia

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ABSTRACT

The present experiment aimed at understanding the effects of cortisol levels on sheep peripheral blood mononuclear cell (PBMC) proliferation and cytokine production during hyperthermia. To mimic stress related to the exposition of high ambient temperatures, PBMC were cultured at 43°C for 12 h, and subsequently at 39°C for additional 12 h. Cells in normothermia were cultured at 39°C for 24 h. Phytohemagglutinin-stimulated PBMC were cultured with different cortisol levels: 0 ng/mL; 100 ng/mL, representing the physiological cortisol concentration simulating stress condition (Cort100); and 1,000 ng/mL, representing the hyperactivated hypothalamic-pituitary-adrenal axis (Cort1000). Phytohemagglutinin-stimulated PBMC with 0 ng/mL of cortisol concentration represented the positive control, whereas nonstimulated PBMC without cortisol represented the negative control (NC). The free cell supernatants were collected for the determination of IL-6, IL-1β, and IL-10 by ELISA. Bromodeoxyuridine assay was performed on cells to determine cell proliferation. Exposition to hyperthermia negatively affected cell proliferation, IL-6, IL-1β, and IL-10 concentrations in cell supernatants. The interaction of hyperthermia and cortisol level affected both cell proliferation and IL-10 production. Both PBMC proliferation and IL-10 production in positive control, Cort100, and Cort1000 decreased at 43°C as compared with 39°C NC. On average, the Cort100 treatment displayed higher concentrations of IL-6 than NC. The present experiment demonstrated that the action of cortisol concentration simulating stress condition on cell proliferation and cytokine production was a permissive/stimulatory action during normothermia, whereas it was a suppressive action during hyperthermia. These data confirmed that cortisol concentration simulating stress condition could have a role in the immune system of sheep via medi-
ating cellular homeostasis in the condition of hyperthermia. The negative effects of hyperthermia on sheep immune responses were apparent when performing an immunological challenge.
Key words: hyperthermia, glucocorticoid, sheep, noninfectious stressor

INTRODUCTION

Noninfectious stressors are defined disrupters of homeostasis of the innate immune response, leading to unbalanced cellular, immunological, and physiological responses (Le Grand and Day, 2016). Among the different types of noninfectious stressors faced by sheep, heat stress seems to deeply threaten production efficiency of the animals. Further, climate change leads to several vector-borne diseases affecting sheep welfare and health (Gaughan et al., 2009). From this point of view, it becomes crucial to study the effects of thermal stress on the immune efficiency of sheep. Hyperthermia, responsible for heat shock or cellular stress response activation, can alter cells' metabolism and gene expression (Collier et al., 2006), and the timing and amplitude of the heat shock response can determine cell survival, acclimation, or cell death (Collier et al., 2006).

Glucocorticoids (**GC**) are produced at high levels during the initial phase of thermal exposure; subsequently, prolonging the exposure to hyperthermia, a decrement of these hormones is observed (Collier et al., 2008). Furthermore, the increase in circulating hypothalamic-pituitary-adrenal (**HPA**) axis mediated GC levels is a key factor in the adaptive stress-inflammatory response (Cooper and Stewart, 2003). The HPA axis activates slowly taking 10 to 60 min from stressor onset for cortisol to reach peak levels, depending on the type of stressor acting, and an hour or more to return to pre-stress levels (Caroprese et al., 2010, 2014). The activation of HPA axis that is caused by stress exposure influences cytokine expression (Uchoa et al., 2014). On the other hand, cytokines, such as IL-1, IL-6, and tumor necrosis factor-α via negative feedback, can increase the HPA axis sensitivity to later stress chal-

lenges (Uchoa et al., 2014). The effects of GC on immune system are classified into suppressive, permissive, and anti-inflammatory actions, which were also found during hyperthermia conditions (Sapolsky et al., 2000; Coutinho and Chapman, 2011). It has been proposed that GC might affect the magnitude or direction of the cytokine-induced response by increasing the rate at which the response proceeds (Wiegers et al., 1995). Previous studies demonstrated that as cytokine actions were regulated by GC, also cytokines may have a regulatory role in GC actions (Arzt et al., 2000; Biola et al., 2001; Heiniger et al., 2001). The precise mechanisms controlling cytokine production and cell proliferation during stress in relation to GC action have not been fully delineated, but different key mechanisms have been emerging. In a previous experiment in sheep, the possible bi-directional effect of cortisol on the immune system in relation to the magnitude of the stressor and the duration of the stress was discussed (Ciliberti et al., 2017).

Our study focused on the comprehension of the GC effects on sheep immune system when exposed to hyperthermia; therefore, the aim of the present study was to evaluate the effects of in vitro incremental GC levels on sheep peripheral blood mononuclear cell (**PBMC**) proliferation and cytokine production in the condition of hyperthermia.

MATERIALS AND METHODS

Animals and Experimental Treatments

Dairy sheep used in the study were allocated at the Segezia research station of the Council for Research and Experimentation in Agriculture. The sheep involved in the experiment were Comisana dairy ewes, with a white coat, aged 3 ± 0.5 yr, reared in external pens of 5×12 m. In the experimental design 8 sheep were chosen with a random design, and an in vitro experiment was performed, with 2 cortisol concentration and 2 different temperature of incubation after mitogen stimulation for a total of 64 samples. Cortisol concentrations were chosen to represent a cortisol concentration simulating stress condition (100 ng/mL, **Cort100**), and level from an hyperactivated HPA axis status (1,000 ng/mL, **Cort1000**), according to results from in vivo studies on sheep (Caroprese et al., 2010) and also previously reported in an in vitro study (Ciliberti et al., 2017). Positive control (**PC**) was represented by PHA-stimulated PBMC without cortisol (0 ng/mL of cortisol), whereas nonstimulated PBMC without cortisol represented the negative control (**NC**). To mimic stress related to the exposition of high ambient temperatures, the experi-

ment was conducted at 2 different temperatures: 39°C to simulate normothermia and 43°C to mimic severe hyperthermia, according to Lacetera et al. (2006). To mimic stress related to the exposition of high ambient temperatures, cells (PC, NC, Cort100, and Cort1000) were treated for 12 h at 43°C and subsequently treated for additional 12 h at 39°C. Cells in normothermia (PC, NC, Cort100, and Cort1000) were cultured for 24 h at 39°C. The in vitro study was performed by the split-plot component involving the isolation of sheep PBMC, and the evaluation of their proliferative response and cytokines production after stimulation with the mitogen phytohemagglutinin (**PHA**) and in presence of 2 different concentrations of cortisol under hyperthermia or not. All procedures were conducted according to the guidelines of the EU Directive (2010) on the protection of animals used for experimental and other scientific purposes. The sheep were healthy and their conditions were carefully examined by veterinarians throughout the trial to exclude the presence of any signs of disease.

Isolation of PBMC

Blood samples (15 mL) were collected in vacuum tubes from the jugular vein of the sheep. Isolation of PBMC was performed by density gradient centrifugation according to Wattegedera et al. (2004). Peripheral blood mononuclear cells were finally resuspended at a final concentration of 2×10^5 cells/mL in Iscove's modified Dulbecco's medium (Sigma-Aldrich, Milan, Italy) supplemented with 10% FBS and 50 μg/mL of gentamicin.

Lymphocyte Stimulation Assay and Cytokines Measurement

Lymphocyte proliferation assays were performed adding 100 μL of cell suspension into quadruplicate wells of 96-well U-bottom plates. Peripheral blood mononuclear cells were activated with 50 μL of PHA (Sigma-Aldrich) at a final concentration of 10 μg/mL, or not activated with mitogen. Sheep PBMC stimulated with PHA were treated with 2 concentrations of cortisol (Sigma-Aldrich; 100, 1,000 ng/mL at final concentration). The plates were incubated at 5% CO_2 in a humidified incubator for 24 h. After incubation time, plates were centrifuged at $400 \times g$ for 10 min, and cell-free supernatants from each well were collected and stored at −80°C until ELISA was performed to measure cytokine production. After cell-free supernatant collection, cells were incubated with bromodeoxyuridine (Exalpha Biologicals Inc., Shirley, MA) to test cell proliferation. After 18 h of incubation, bromodeoxyuridine

incorporation during DNA synthesis was measured by determining optical density with a titer-ELISA spectrophotometer (Power Wave XS, BioTek, Swindon, UK) at 450 nm. During lymphocyte proliferation assay a total number of 256 samples were analyzed (64 samples × 4); for cytokine measurement a total number of 128 samples were analyzed (64 samples × 2).

Measurement of Cytokines in Culture Supernatants by ELISA

The assays were optimized in our laboratory for concentrations of mouse monoclonal antibodies (**mAb**), supernatants, polyclonal detecting Ab, and secondary conjugate Ab. The levels of IL-6, IL-1β, and IL-10 in cell-free supernatants were determined by capture ELISA performed on 96-well microtiter plates, according to Ciliberti et al. (2017). Both IL-6 and IL-1β incubations were at 37°C for 1 h, and after each step the plates were washed 4 times. All culture supernatants were read in duplicate against a standard curve-fitting 4-parameter logistic curve. The standard curve for IL-6 was obtained using scalar dilution of recombinant ovine IL-6 (Cusabio Biotech Co., Wuhan, P.R. China) in a range from 1,750 to 0 ng/mL (R^2 = 99.9%). The recombinant ovine IL-1β (Kingfisher Biotech Inc., St. Paul, MN) for IL-1 β standard curve in a range of 250 to 0 ng/mL was used (R^2 = 100%). Interassay and intraassay coefficients of variation were 2.8 and 3.9% for IL-1β, and 3.1 and 4.2% for IL-6, respectively.

All the incubations for the measurement of IL-10 in plasma were at room temperature, and after each step the plates were washed 4 times. The levels of IL-10 were measured colorimetrically at 450 nm in duplicate (by subtracting absorbance at 540 nm) and quantified by interpolation from standard curve fitted by 4-parameter logistic curve in a range of 500 to 0 ng/mL (R^2 = 99.7%). The IL-10 interassay and intraassay coefficients of variation were 3.7 and 4.1%, respectively.

Statistical Analysis

All variables were tested for normality using the Shapiro-Wilk test (Shapiro and Wilk, 1965). Orthogonal contrasts were constructed using the CONTRAST statement procedure of SAS (2013, version 6.1, SAS Institute Inc., Cary, NC). To determine the effect of PHA stimulation, the average of the 3 PHA+ treatment was compared with the NC. To determine the effect of cortisol treatment, the average of the cortisol treatment was compared with the PC. To determine the effect of cortisol dosage, the average of cortisol treatment at 100 ng/mL was compared with the average of cortisol treat-

ment at 1,000 ng/mL. To determine the temperature effect, the average of PHA stimulation, cortisol effect, and cortisol dosage at 39°C were compared with the average of PHA stimulation, cortisol effect, and cortisol dosage at 43°C. To determine the effect of the interaction among the temperatures tested, and PC, NC, Cort100, and Cort1000, the ANOVA for mixed models using the MIXED procedure of SAS was performed. The interaction between the temperatures tested and the different treatments were used as a fixed effect, and the animal as a random factor nested in the treatment. Proliferation, IL-6, IL-1β, and IL-10 were the dependent variables analyzed. When significant effects were found ($P < 0.05$), the Fisher's least significant difference test was used to locate significant differences between means for MIXED procedure, whereas the Scheffe test was used to locate the differences among orthogonal contrasts tested.

RESULTS

Cell Proliferation

Contrast analysis of proliferation data showed a significant effect of the temperature on Cort100 ($P < 0.0001$), Cort1000 ($P = 0.015$), and PC ($P = 0.0004$), with a decreasing proliferation at 43°C as compared with 39°C. The PHA stimulation showed a significant effect on cell proliferation both at 39°C ($P = 0.018$) and 43°C ($P = 0.035$) as compared with NC. In particular, PHA stimulation resulted in an increase in cell proliferation when cells were cultured at 39°C; conversely, PHA stimulation resulted in a decrease in cell proliferation when cells were cultured at 43°C. An interaction between hyperthermia and treatments was detected ($P = 0.0008$, Figure 1). At 39°C, the treatment Cort100 showed an increase of PBMC proliferation compared with NC ($P = 0.002$). On the contrary, at 43°C a reduction of PBMC proliferation in Cort100 compared with NC was detected ($P = 0.006$).

Cytokine Production

The results of the effects of the 3 orthogonal contrasts related to PHA stimulation, cortisol effect, and cortisol dosage on cytokine production by PBMC, at both 39 and 43°C, are reported in Table 1. Orthogonal contrasts showed that at 39°C PHA stimulation affected IL-6 production as compared with NC ($P = 0.044$), whereas cortisol effect and cortisol dosage affected IL-6 production at 43°C ($P = 0.0009$ and $P = 0.0036$, respectively). The average of PC, Cort100, and Cort1000 treatment displayed a reduction in IL-6 production during hyper-

Table 1. Effects of the orthogonal contrasts [phytohemagglutinin (PHA) stimulation, cortisol effect, and cortisol dosage] on in vitro IL-6, IL-1β, and IL-10 secretion (mean ± SEM) by sheep peripheral blood mononuclear cells (PBMC) following PHA stimulation at 39 and 43°C temperatures

Item	Temperature (°C)	Treatment[1]			SEM	Effect, P-value		
		PC	Cort100	Cort1000		PHA stimulation	Cortisol effect	Cortisol dosage
IL-6	39	188.26	191.09	161.68	34.34	0.04	0.81	0.48
	43	64.85[b]	125.08[a]	57.04[b]	10.75	0.37	<0.001	0.003
IL-1β	39	1.59	1.77	1.62	0.10	0.004	0.47	0.36
	43	1.14[ab]	1.35[a]	1.08[b]	0.08	<0.001	0.04	0.04
IL-10	39	1.57	1.45	1.45	0.06	0.002	0.45	0.93
	43	1.08	0.99	0.92	0.10	0.53	0.23	0.32

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

[1]Treatments: PC = positive control, PHA+/cortisol−; Cort100 = PHA-stimulated PBMC cultured with 100 ng/mL of cortisol; Cort1000 = PHA-stimulated PBMC cultured with 100 ng/mL of cortisol.

thermia as compared with 39°C ($P = 0.027$, $P = 0.043$, $P = 0.009$, respectively, Figure 2). Production of IL-6 at 39°C was higher in Cort100 ($P = 0.03$) and PC ($P = 0.03$) than in NC (Figure 2).

Orthogonal contrasts showed that PHA stimulation increased IL-1β production at 39°C as compared with NC ($P = 0.004$, Table 1), whereas PHA stimulation decreased IL-1β production at 43°C as compared with NC ($P = 0.0004$). Furthermore, cortisol treatment and cortisol dosage affected IL-1β production at 43°C ($P =$

0.043 and $P = 0.044$; respectively). The average of PC, Cort100, and Cort1000 treatment displayed a reduction in IL-1β production during hyperthermia as compared with 39°C ($P < 0.001$, $P = 0.025$, and $P < 0.001$, respectively, Figure 3). At 39°C, IL-1β production increased in cells cultured with Cort100 ($P = 0.005$) and Cort1000 ($P = 0.01$) as compared with NC, whereas at 43°C, IL-1β production was higher in PBMC with Cort100 treatment than NC ($P = 0.001$) and Cort1000 treatment ($P = 0.04$, Figure 3).

Orthogonal contrasts showed that PHA stimulation increased IL-10 production at 39°C as compared with NC ($P = 0.002$, Table 1); cortisol treatment and cortisol
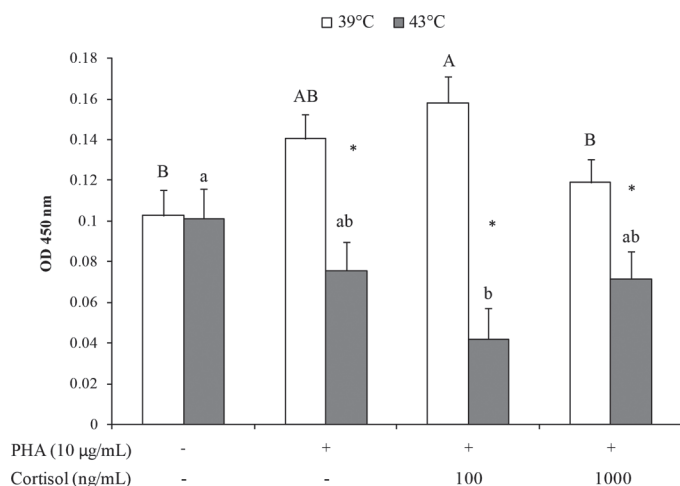

Figure 1. Proliferation of sheep peripheral blood mononuclear cells (PBMC; LSM ± SEM) following in vitro stimulation. Phytohemagglutinin (PHA)-stimulated PBMC were treated with 2 concentrations of cortisol [100 ng/mL, as physiological cortisol concentration simulating stress condition (Cort100), and 1,000 ng/mL, as hyperactivated hypothalamic-pituitary-adrenal axis concentration (Cort1000)], and cultured for 24 h at 39°C (normothermia) or 43°C (hyperthermia). PHA+/cortisol− represented positive control; PHA−/cortisol− represented negative control. A, B indicate differences among experimental treatments at 39°C; a, b indicate differences among experimental treatments at 43°C; * indicates significant differences between 39 and 43°C within each experimental treatment, $P < 0.05$. OD = optical density.
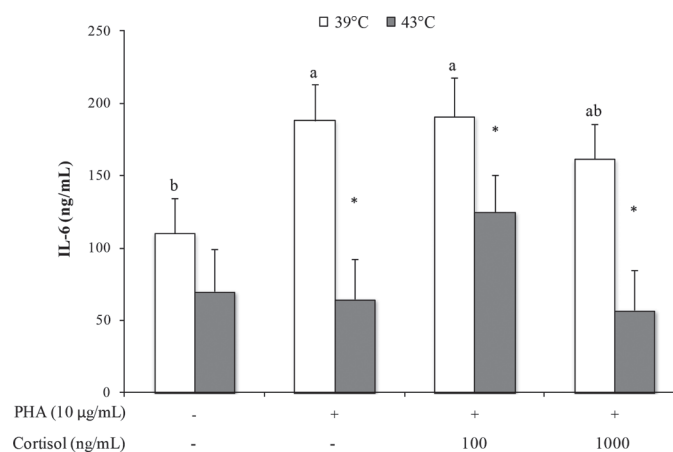

Figure 2. Interleukin-6 secretion by sheep peripheral blood mononuclear cells (PBMC; LSM ± SEM) following in vitro stimulation. Phytohemagglutinin (PHA)-stimulated PBMC were treated with 2 concentrations of cortisol [100 ng/mL, as physiological cortisol concentration simulating stress condition (Cort100), and 1,000 ng/mL, as hyperactivated hypothalamic-pituitary-adrenal axis concentration (Cort1000)], and cultured for 24 h at 39°C (normothermia) or 43°C (hyperthermia). PHA+/cortisol− represented positive control; PHA−/cortisol− represented negative control. a, b indicate differences among experimental treatments at 39°C; * indicates significant differences between 39 and 43°C for each experimental treatment, $P < 0.05$.
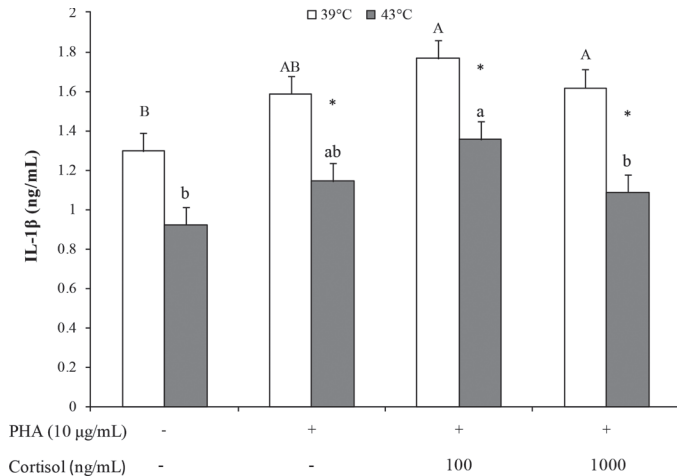
Figure 3. Interleukin-1β secretion by sheep peripheral blood mononuclear cells (PBMC; LSM ± SEM) following in vitro stimulation. Phytohemagglutinin (PHA)-stimulated PBMC were treated with 2 concentrations of cortisol [100 ng/mL, as physiological cortisol concentration simulating stress condition (Cort100), and 1,000 ng/mL, as hyperactivated hypothalamic-pituitary-adrenal axis concentration (Cort1000)], and cultured for 24 h at 39°C (normothermia) or 43°C (hyperthermia). PHA+/cortisol− represented positive control; PHA−/cortisol− represented negative control. A, B indicate differences among experimental treatments at 39°C; a, b indicate differences among experimental treatments at 43°C; * indicates significant differences between 39 and 43°C for each experimental treatment, $P < 0.05$.

dosage did not affect IL-10 at 39 or 43°C. The average of PC, Cort100, and Cort1000 treatment displayed a reduction in IL-10 production during hyperthermia as compared with 39°C ($P = 0.04$, $P < 0.001$, $P < 0.001$, respectively, Figure 4). Furthermore, at 39°C PC ($P = 0.002$) and cells cultured with both cortisol levels ($P = 0.04$) displayed higher IL-10 concentrations than NC (Figure 4). However, at 43°C PBMC with Cort1000 treatment displayed lower IL-10 production than NC ($P = 0.03$).

DISCUSSION

In our study, hyperthermia condition deeply influenced cell proliferation and cytokine production; furthermore, during hyperthermia physiological cortisol concentrations simulating stress condition increased IL-6 and IL-1β production. Moreover, the inhibitory effect of hyperthermia on immune responses was apparent in the presence of PHA acting as an immune challenge.

In a previous study, cortisol, when at concentrations simulating stress condition released in vivo after a challenge, exerted a stimulatory action on cell proliferation (Ciliberti et al., 2017). In the present study, during cell stimulation in normothermia conditions,

a comparable nonsignificant trend was observed. In particular, the physiological cortisol concentrations simulating stress condition resulted in increasing by 13% of cell proliferation as compared with PC, and by 35% as compared with NC. Such a stimulatory action of cortisol on cell proliferation has been found by other authors and attributed to an increased sensitivity to IL-2 action (Wiegers et al., 1995). However, during hyperthermia the physiological cortisol concentrations simulating stress condition showed a decreasing by 50% of cell proliferation as compared with PC, and by 60% as compared with NC. One of the first vital protective strategy the host has to implement when coping with a noninfectious stressor is the delay of growth and replication in order both to reduce energetic costs and to be less vulnerable (Le Grand and Day, 2016). As a consequence, the cortisol response after a stressor evolved to downregulate the enhancement of inflammatory responses activated by the prompt activation of the sympathetic nervous system during the first wave of stress responses. From this point of view the cortisol response has the aim to downregulate the inflammatory response and to control cytokine production (Sapolsky et al., 2000). The GC may exert different specific actions, such as permissive, stimulatory, or suppressive actions, in response to the nature, the magnitude, and the duration of the stressors experienced by most or-
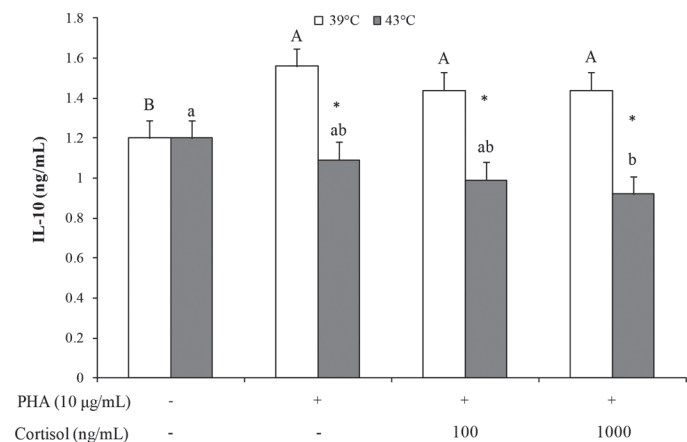

Figure 4. Interleukin-10 secretion by sheep peripheral blood mononuclear cells (PBMC; LSM ± SEM) following in vitro stimulation. Phytohemagglutinin (PHA)-stimulated PBMC were treated with 2 concentrations of cortisol [100 ng/mL, as physiological cortisol concentration simulating stress condition (Cort100), and 1,000 ng/mL, as hyperactivated hypothalamic-pituitary-adrenal axis concentration (Cort1000)], and cultured for 24 h at 39°C (normothermia) or 43°C (hyperthermia). PHA+/cortisol− represented positive control; PHA−/cortisol− represented negative control. A, B indicate differences among experimental treatments at 39°C; a, b indicate differences among experimental treatments at 43°C; * indicates significant differences between 39 and 43°C for each experimental treatment, $P < 0.05$.

ganisms, and to the adaptations needed to survive them by restoring homeostasis (Sapolsky et al., 2000). In the present experiment, the action of physiological cortisol concentrations simulating stress condition on cell proliferation and cytokine production was a permissive/stimulatory action in normothermia, whereas it was a suppressive action during hyperthermia. The present results suggested that in the sheep model a further increase of cortisol concentration from 100 to 1,000 ng/mL failed to support an additional effect on cell proliferation, both in normothermia and in hyperthermia. Therefore, the increase of cortisol level did not correspond to the further suppression of immune responses during hyperthermia. The suppressive action of cortisol on cell proliferation could be attributed to the biological mechanisms hypothesized by Van Laethem et al. (2001) in which GC were found able to inhibit T cell responses through early phosphorylating events induced after TCR ligation. The findings of the present experiment further demonstrated that a main role in defining the type of action of cortisol on immune cells is exerted by the nature of the acting stressor, which in this case was hyperthermia. Nevertheless, as suggested by Collier et al. (2008), when the stressor acting on cells is the heat stress, cellular responses involved a controlled network of genes coordinated to minimize the negative effects of the hyperthermia on cellular functions. Bovine mammary epithelial cells exposed to hyperthermia (42°C) exhibited morphological changes, reduced cell growth, and downregulation of gene transcripts associated with protein synthesis and cellular metabolism (Collier et al., 2006). It is well known from in vivo studies that during heat stress HPA axis is activated and cortisol concentration increased to mediating the ability of sheep to cope with high environmental temperatures (Sejian et al., 2013; Caroprese et al., 2014). The experimental data underlined that in the sheep model the role of cortisol, when at physiological cortisol concentrations simulating stress condition, could be considered as a paramount mediator in restoring cellular homeostasis in the case of heat stress. However, based on the absence of effects, it could be hypothesized that the level of cortisol simulating a hyperstimulation of HPA axis activated a mechanism similar to that of GC resistance, which is found in chronic diseases including asthma, major depression, and cardiovascular conditions (Rodriguez et al., 2016). However, further research should be done to confirm this also in sheep model.

Hyperthermia is suggested to regulate cell proliferation and inflammatory responses by changing cytokine production. Among proinflammatory cytokines, IL-6 is one of the most powerful cytokines with pleiotropic effects, being involved in hematopoiesis, B cell differentiation, primary antigen receptor-dependent T cell activation, and their subsequent proliferation (Kishimoto, 2006; Dienz and Rincon, 2009; Tanaka et al., 2014). Interleukin-1β was the first cytokine to be related to HPA activation, as a critical regulator of the adaptive response to stress by the control of stress-related alterations involving behavior, cognition, and emotions (Goshen and Yirmiya, 2009). In sheep, GC have the ability to influence proinflammatory cytokines as demonstrated by both in vivo and in vitro studies (Caroprese et al., 2010; Ciliberti et al., 2017). Accordingly, in the present study, both IL-6 and IL-1β production by cells cultured with cortisol, when at physiological concentrations simulating stress condition, were altered, and decreased during hyperthermia. The further increase in cortisol concentration to 1,000 ng/mL did not change proinflammatory cytokine production as compared with PC, according to proliferation results. Elevated hyperthermia in mice reduced proinflammatory cytokine production as a result of induced expression of activating transcription factor 3, a negative regulator of the IL-6 gene (Hagiwara et al., 2007; Takii et al., 2010). Heat shock protein-72 produced during heat stress has been demonstrated to inhibit the nuclear factor-κB signaling cascade activated by LPS, thus reducing IL-6 production (Chen et al., 2006). The observed alteration in cytokine production induced by GC during hyperthermia could be considered a further mechanism to restore homeostasis after a noninfectious stressor to reduce metabolic and health costs connected to cytokine production (Le Grand and Day, 2016). Interleukin-10 has an immunosuppressive role in maintenance of the balance in the immune response against invading pathogens (Ma et al., 2015). Interleukin-10 secretion by T cells can be upregulated by glucocorticoids (Elenkov and Chrousos, 1999). In an in vivo study on sheep, an increase in plasma IL-10 production was found when under hyperthermia (Caroprese et al., 2014). On the contrary, in the present in vitro experiment, hyperthermia lead to decreased IL-10 production by sheep cells when stimulated with PHA, acting as an immune challenge, regardless of cortisol level. Moreover, the lower IL-10 production than NC registered by the highest cortisol level straightened the probably suppressive action of GC also during hyperthermia as a model of a chronic stress. Consequently, the main suppressive effects of hyperthermia on cell proliferation and proinflammatory response could be ascribed to the inhibition of anti-inflammatory cytokines such as IL-10 as well.

The suppression of PBMC proliferation and of cytokines observed in the present experiment during in vitro hyperthermia may account for the impairment in

immune responses of sheep under heat stress and help to explain the increased incidence of udder health problems found during summer (Sevi and Caroprese, 2012).

CONCLUSIONS

In the present experiment, the effects of hyperthermia on cell proliferation and inflammatory responses were evaluated. Hyperthermia, followed by restoring of normothermia, simulated heat waves experienced by heat stressed sheep in livestock production systems. The action of physiological cortisol concentration simulating stress condition on cell proliferation and cytokine production was a permissive/stimulatory action during normothermia, whereas it was a suppressive action during hyperthermia. The findings of the present in vitro study suggested that the type of action of cortisol on immune cells depends on the nature of the stressor acting (i.e., normothermia vs. hyperthermia). The data lead to the hypothesis that cortisol, when at a physiological concentration simulating stress condition, can be considered a paramount mediator in restoring cellular homeostasis in the case of hyperthermia by increasing IL-6 and IL-1β production. Results from the present experiment suggested that the reduction in immune competence found in sheep under in vivo hyperthermia could be attributed to the reduction of cell proliferation as a result of alteration of cytokine production. The understanding of the mechanisms leading to the reduction of sheep immunological competences under hyperthermia could be crucial to develop new diagnostic and therapeutic tools to sustain disorders related to noninfectious stressors in animals.

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