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Dynamics of leukocytes and cytokines after moderate altitude exposure in pregnant ewes with low and high hematocrit levels

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Abstract

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The object of the present study was to investigate leukocyte subsets distribution and the dynamics of some pro-inflammatory and anti-inflammatory cytokines in response to moderate altitude exposure in sheep having different hematocrit values. Thirty Ile De France ewes were selected from an experimental herd according to their hematocrit levels and were allocated into 3 groups as follows: low hematocrit (LHct) group (hematocrit range 19.7-27.9 %), high hematocrit (HHct) group (hematocrit range 32.0-36.9 %) and mean hematocrit (MHct) group (hematocrit range 28.3-29.8 %). Immediately after shearing, ewes were transported from the Institute farm (altitude 500 m) to a mountain pasture (altitude 1440 m). Blood samples were taken by jugular venepuncture at the following time points: before transportation (baseline level), on day 7, 20 and 42 after the transport. The traits investigated were leukocyte subsets (basophils, eosinophils, neutrophils, lymphocytes, monocytes, large immature cells) and cytokines (IL-2, Il-4, IL-6, IL-10, IL-17A, IFN-γ, TNF-α). Moderate altitude exposure elicited significant decrease in lymphocyte numbers in all the 3 groups at d 7 after transport to moderate altitude followed by a return to normal levels at d 20 after transport. Neutrophil numbers were not influenced by moderate altitude hypoxia. There was hematocrit associated changes in basophil, eosinophil and monocyte numbers suggesting different trafficking patterns of these leukocyte subsets. There was a tendency of slight increase in large immature cells in HHct ewes at d 7. There was a relation between N/L ratio and large immature cells as follow: baseline levels ($r = 0.627995$, $P < 0.01$); at 7 d ($r = 0.771221$, $P < 0.001$); at 20 d ($r = 0.606801$, $P < 0.01$); at 42 d ($r = 0.566646$, $P < 0.01$). IL-10 levels in HHct and MHct ewes tended to decrease at 7 d compared to basal levels. There was relation between IL-2 and IL-6 at 20 d ($r = 0.531371$, $P < 0.01$) and 42 d ($r = 0.586212$, $P < 0.01$). Investigated pro-inflammatory Th17 (IL-17A) and Th1 cytokines (IL-2, IL-6, IFN-γ, TNF-α), as well as anti-inflammatory Th2 cytokines (IL-4 and IL-10) were not influenced by moderate altitude exposure. The results are interpreted to suggest that exposure of pregnant ewes at ambient temperature below the lower critical temperature at moderate altitude prevents cytokines increase.

Keywords: leukocytes; cytokines; interleukins, hematocrit; stress; sheep

Introduction

Oxygen is the basis of life on Earth. A low-oxygen state (hypoxia) is associated with organ development, stem cell maintenance, inflammation, aging, lung disorder, cardiovascular disease, neuronal degeneration and cancer (Semenza, 2007). Maintaining oxygen homeostasis represents the essential cellular metabolic process for the structural integrity of tissues in different pathological conditions, including severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection (Hertzog et al., 2021).

At high altitude, in addition to hypoxia, there is also exposure to low environmental temperature. In this case, the respiratory system is influenced by conflicting factors, because of the respiratory heat loss associated with an increase in ventilation during hypoxia (Diesel et al., 1990; Mortola &

Frappell, 2000). Thus, increased maintenance requirements to maintain homeostasis could have negative effect on both animal health and production.

Chronic stress is known to have health-aversive effects, some of which are mediated via immune mechanisms (Glaser & Kiecolt-Glaser, 2005; Butts & Sternberg, 2008). It has been proposed that stress-induced changes in blood leukocyte distribution may represent an adaptive response (Dhabhar et al., 1994).

Numerous studies have demonstrated that short-term stress, unlike chronic stress, induces specific changes in blood leukocyte distribution and enhances innate and adaptive immune responses in organs, such as the skin, subcutaneous tissue, sentinel lymph nodes and other compartments (Dhabhar et al., 1995; Dhabhar & McEwen, 1997; Dhabhar, 2009). It has been hypothesized that psychophysiological stress response is nature's fundamental survival mechanism that could be therapeutically used to augment immune function (Dhabhar & Viswanathan, 2005).

Activation of stress system and thus increased levels of glucocorticoids may systemically cause a selective suppression of the Th1 cellular immunity axis and a shift toward Th2 –mediated humoral immunity (Elenkov, 2004).

Stress-induced changes in blood neutrophil, lymphocyte and monocyte distribution are mediated mainly by catecholamines and glucocorticoids (Dhabhar et al, 1996; Dhabhar, 1998; Viswanathan & Dhabhar, 2005).

The majority of previous studies have focused on immune cell distribution profiles following exposure to short-term psychological and physical stress in laboratory animals and humans (Dhabhar et al., 1995; Marsland et al., 1997; Dhabhar, 2009).

According to the optimal hematocrit hypothesis, blood viscosity increases with rising Hct levels, limiting the blood's O2 transport capacity (Schuler et al., 2010).

The reports on the effects of acute hypoxic exposure on leukocytes and cytokines are few and often contradictory.

This study was thus designed to investigate the dynamics of a variety of pro-inflammatory and anti-inflammatory cytokines and magnitude of hypoxia-induced changes in leukocyte distribution during the 42 days following moderate altitude exposure.

Materials and methods

Study site and environment data

The current study was conducted strictly in accordance with the guideline of the Institutional Animal Ethics Committee. Our investigation was carried out in 2021, at the Institute of Animal Science, Kostinbrod, Bulgaria.

The ewes were shorn in the end of May, and were immediately transported from the experimental base of the Institute of Animal Science, Kostinbrod (500 m above sea level) to the Petrohan Pass region (Balkan Mountains), located at 1440 m above sea level. Minimum and maximum air temperatures on the day of arrival at the mountain pasture were 12.9°C and 24.4°C for the region of Kostinbrod (low altitude) and 8.0°C-13,1°C for the region of Petrohan Pass (moderate altitude), respectively. The animals stayed at high altitude for 4 months, where they were on pasture for 10 h during the day. At night, they stayed in a barn. In addition to pasture, they were offered concentrate mixture once per day. The ewes had free access to a NaCl licking stone and water. Mean air temperature range in the region of Petrohan Pass during the summer months of 2021 was 11.9 to 20.1°C.

Ewes

Institute′s research flock of 110 Ile De France ewes was used to select ewes with low, normal, and high level of hematocrit. Because of hematocrit variation, all animals were bled three times at 10-day intervals, one month before the start of the experiment in order to get correct hematocrit (Hct) values. Ewes were deprived of food the night before blood collection. In the beginning of May all ewes of the flock were artificially inseminated following estrus synchronization.

Thirty, clinically healthy Ile de France ewes were divided into 3 groups of 10 subjects, each according to their hematocrit level, i.e. ewes with mean Hct level (hematocrit range of 28.3-29.8%), ewes with low Hct level (hematocrit range of 19.7-27.9%) and ewes with high hematocrit level (hematocrit range of 32.0-36.9%). The age-matched groups consisted of 3 to 5 years old ewes. During the day, the animals grazed on natural pasture and were kept in a barn at night. They received supplemental concentrate and meadow hay twice daily with free access to water.

Plasma collection

Blood samples were taken by direct jugular venepuncture at the following time points: before transportation (baseline level), on day 7, 20 and 42 after the transport to moderate altitude. All blood samples were centrifuged at 5000 x g for 5 min at 10 °C, aliquoted and stored at – 20°C until assayed.

Estimation of blood variables

White blood cell differential test was performed with whole blood samples with 5-part differential using automated hematology analyzer (URIT-5160 Vet, URIT Medical Electronic Co., Ltd, China). The levels of white blood cells

(WBCs), and leukocyte differentials (basophils, eosinophils, neutrophils, lymphocytes, monocytes, and large immature cells, were determined via WBCs optical count.

Interleukins measurements

The concentrations of IL-2, IL-4, IL-10, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), IL-17α (Affymetric, eBioscience, USA) and IL-6 (Invitrogen, ThermoFisherscientific, USA) were determined using ELISA assay kits according to manufacturer's protocol. The optical density was measured at 450 nm with microplate reader (Biotek, USA).

Statistical analysis

Statistical significance was analyzed using one-way ANOVA. All data are presented as arithmetic means ± standard error of the mean (mean ± SEM). Results were considered significant when probability values (P) were less than 0.05.

Results

Leukocytes

The changes in WBC subpopulations are shown in Fig. 1-8. Moderate altitude exposure did not change significantly leukocyte numbers (Fig. 1) in all the tree groups of ewes. WBC tended to decrease at 7d in LHct ewes, while in HHct ewes WBC tended to decrease at 7d and 20 d compared to baseline levels. There was a tendency of higher WBC in LHct ewes on d 20 and d 42 compared to HHct and MHct ewes.

Moderate altitude exposure had no significant effect on neutrophil numbers throughout the experimental period. However, neutrophil count tended to increase from baseline to 7 d levels in HHct ewes following by significant decrease on d 20 compared to 7 d (Fig. 2).

Lymphocyte numbers declined significantly in all the 3 groups at 7d after altitude exposure relative to baseline values, followed by a trend of slight increase at 20 d after moderate altitude exposure. Lymphocyte numbers were significantly higher at 42 d in LHct ewes compared to MHct ewes (Fig. 3).

Neutrophil (N) to lymphocyte (L) ratio tended to increase in HHct group at 7 d after transport to moderate altitude and then declined significantly on d 20 compared to 7 d (Fig. 4).

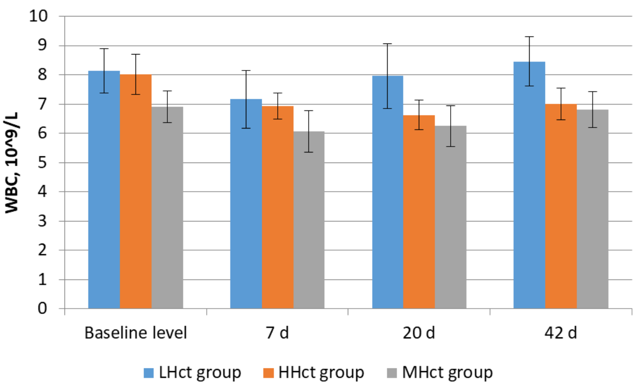

Fig. 1. WBC following moderate altitude exposure in pregnant ewes with low and high hematocrit levels

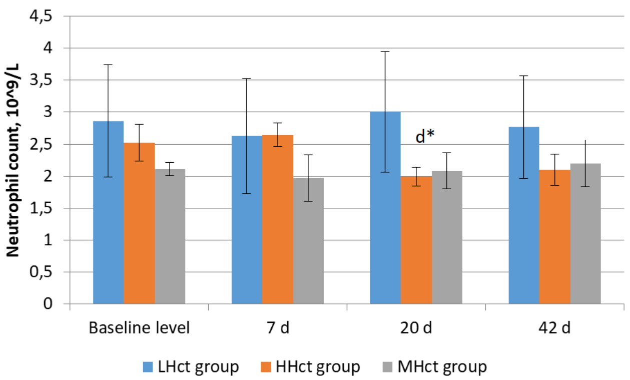

Fig. 2. Neutrophil count following moderate altitude exposure in pregnant ewes with low and high hematocrit levels

*P < 0.05

d – significantly different versus 7 d

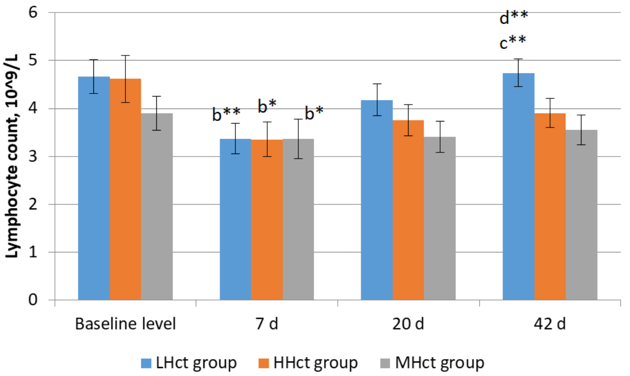

Fig. 3 Lymphocyte count following moderate altitude exposure in pregnant ewes with low and high hematocrit levels

* – P < 0.05; ** – P < 0.01;

b – significantly different versus baseline level;

c – significantly different versus MHct group;

d – significantly different versus 7 d

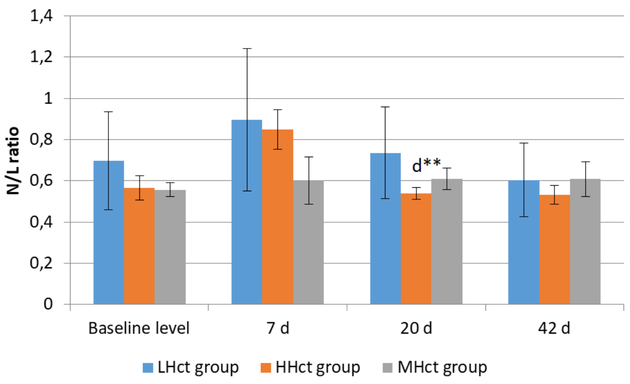

Fig. 4. N/L ratio following moderate altitude exposure in pregnant ewes with low and high hematocrit levels
* – P < 0.05;
d – significantly different versus 7 d

Baseline monocyte numbers in LHct ewes was significantly lower compared to HHct ewes (Fig. 5). Monocyte numbers in LHct ewes increased significantly at 7 d compared to baseline level. Monocyte numbers tended to increase in HHct ewes at 7d, while in MHct ewes tended to decrease. Monocyte numbers declined at 20 d in LHct and HHct ewes compared to d 7. On d 42 monocyte numbers increased in all groups compared to d 20, but statistical significance was reached in LHct ewes only compared to basal level. (Fig. 5).

Large immature cell values tended to increase in HHct ewes at 7 d compared to baseline levels and then declined significantly compared to d 7 (Fig. 6).

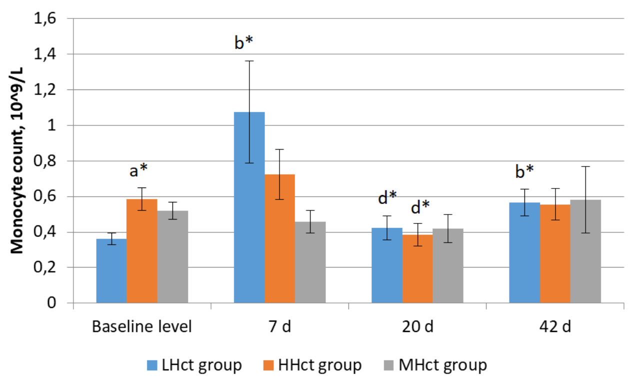

Fig. 5. Monocyte count following moderate altitude exposure in pregnant ewes with low and high hematocrit levels
* – P < 0.05;
a – significantly different versus LHct group;
b – significantly different versus baseline level;
d – significantly different versus 7 d

Eosinophil numbers in LHct ewes were significantly lower compared to MHct and HHct ewes at 7d after moderate altitude exposure. Also, eosinophil numbers in LHct ewes observed at 20 d after transport to moderate altitude were lower compared to HHct ewes (Fig. 7).

Eosinophil numbers in HHct ewes was significantly higher at 20 d compared to basal level. Eosinophil number in HHct ewes were significantly higher on d 20 compared to baseline levels.

Basophil numbers in LHct ewes were significantly higher before transport to moderate altitude (basal level) compared

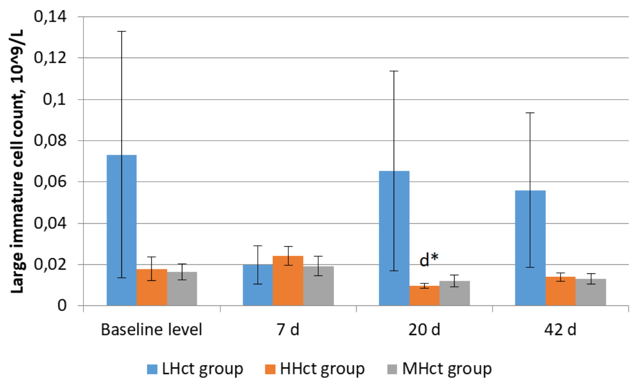

Fig. 6. Large immature cell count following moderate altitude exposure in pregnant ewes with low and high hematocrit levels
* – P < 0.05;
d – significantly different versus 7 d

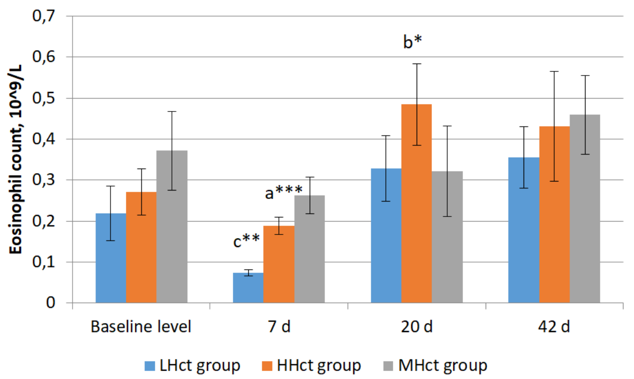

Fig. 7. Eosinophil count following moderate altitude exposure in pregnant ewes with low and high hematocrit levels
* – P < 0.05; ** – P < 0.01; *** – P < 0.001;
a – significantly different versus LHct group;
b – significantly different versus baseline level;
c – significantly different versus MHct group

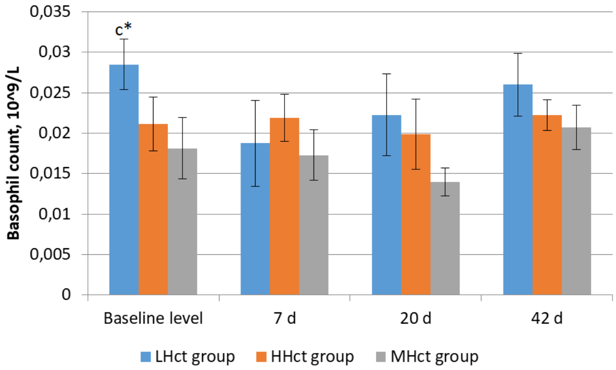

Fig. 8. Basophil count following moderate altitude exposure in pregnant ewes with low and high hematocrit levels

* – $P < 0.05$;

c – significantly different versus MHct group

to MHct ewes. Basophil numbers tended to decrease in LHct at 7 d (Fig. 8).

There was a trend of higher leukocyte numbers in LHct compared to other two groups.

Dynamics of cytokines

Interleukin 2 in LHct ewes tended to be higher compared to HHct and MHct ewes throughout the experimental period. (Fig. 9). Baseline IL-2 levels in LHct ewes were significantly higher compared to MHct ewes.

Interleukin 4 levels in all groups were not significantly influenced by exposure to moderate altitude. HHct ewes had a trend of higher IL-4 levels compared to other two groups at baseline, 7d, and 20 d (Fig. 10).

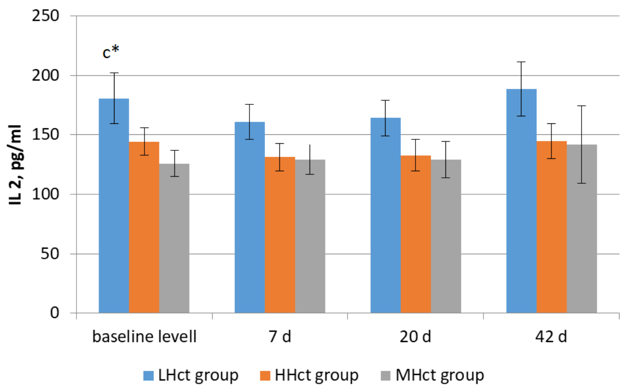

Fig. 9. IL-2 following moderate altitude exposure in pregnant ewes with low and high hematocrit levels

* – $P < 0.05$;

c – significantly different versus MHct group

There was a trend of decrease in IL-6 levels in HHct ewes (Fig. 11). There was no significant effect on IL-6 after transport to moderate altitude.

Both, baseline and 7 d levels of IL-10 tended to be higher in MHct ewes compared to LHct ewes (Fig. 12). IL-10 tend-

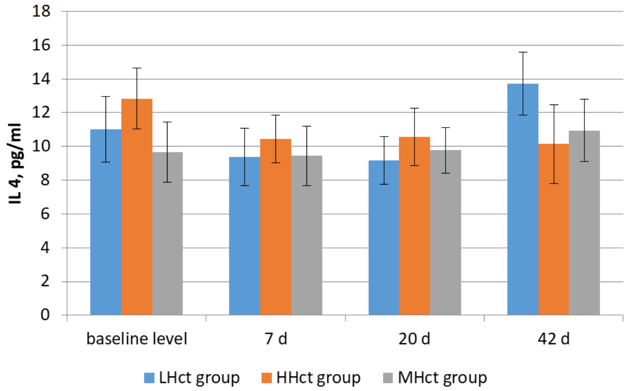

Fig. 10. IL-4 following moderate altitude exposure in pregnant ewes with low and high hematocrit levels

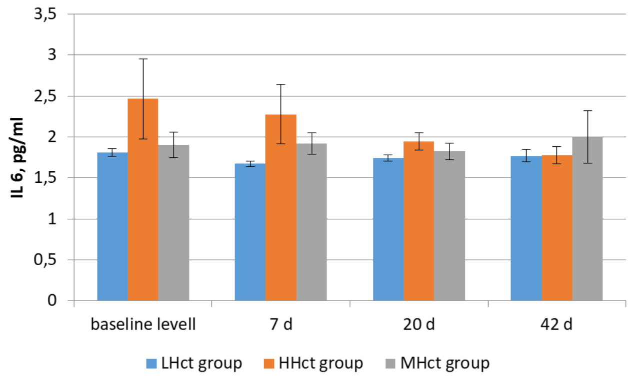

Fig. 11. IL-6 following moderate altitude exposure in pregnant ewes with low and high hematocrit levels

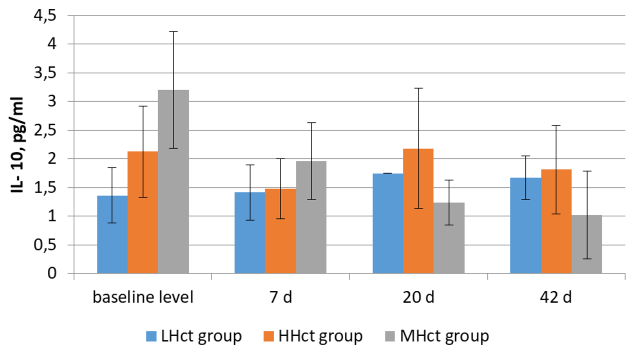

Fig. 12. IL-10 following moderate altitude exposure in pregnant ewes with low and high hematocrit levels

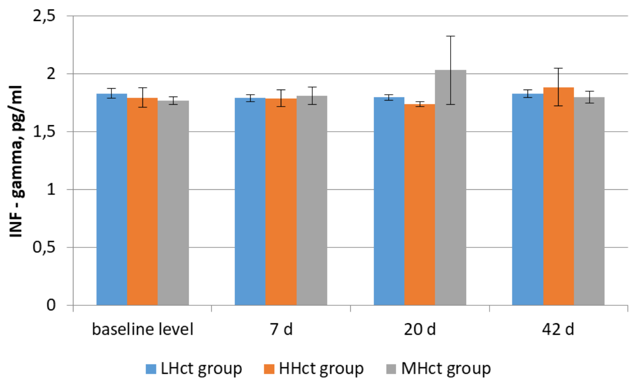

Fig. 13. INF-gamma following moderate altitude exposure in pregnant ewes with low and high hematocrit levels

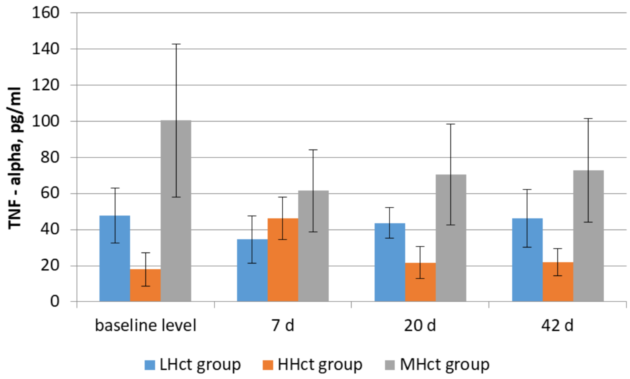

Fig. 15. TNF-alpha following moderate altitude exposure in pregnant ewes with low and high hematocrit levels

ed to decrease on d 7 compared to baseline levels in HHct and MHct ewes, while in LHct ewes remained unchanged. On d 20, Il-10 levels in HHct ewes tended to be higher compared to other two groups.

Interferon gamma levels in MHct ewes tended to be higher at 20 d (Fig. 13). There was a tendency of lower INF-γ at 20 d in HHct ewes compared LHct ewes. Exposure to moderate altitude did not influence significantly IFN-γ levels (Fig. 13).

Interleukin 17A (IL-17A) levels in LHct ewes was significantly higher at 7 d compared to HHct and MHct ewes. IL-17A was significantly higher at 20 d in LHct ewes compared to HHct ewes (Fig. 14).

There was no significant effect of moderate altitude exposure on TNF-α levels (Fig. 15).

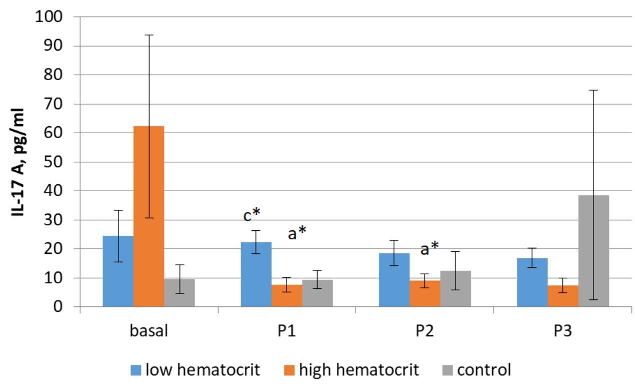

Fig. 14. IL-17A following moderate altitude exposure in pregnant ewes with low and high hematocrit levels.
* – $P < 0.05$;
a – significantly different versus LHct group;
c – significantly different versus MHct group

Discussion

Lymphocyte count declined significantly in the three groups of ewes ($P < 0.05$) on d 7 compared to baseline levels (Fig. 3). According to Dhabhar et al. (2012) the kinetics of leukocyte subpopulations in response to stress at early time points would mainly reflect mobilization of cells into the blood from certain compartments (e.g. spleen, bone marrow, lung, lymph nodes), while late time points would mainly reflect trafficking of cells out of the blood to target organs (decreased number). The decline of lymphocyte count at 7 d after the transport to moderate altitude is consistent with Dhabhar's view. Stress-induced reduction in circulating lymphocytes is due to glucocorticoid-induced alterations in the trafficking or redistribution of lymphocytes from the blood to other body compartments (Dhabhar, 2002). In our previous work we suggested that newly shorn sheep experienced mild-cold stress immediately after transport to moderate altitude. Given that cold stimulates sympathetic nervous system and secretion of catecholamines, it can be assumed that cold-induced increase in catecholamines elicits further increase in lymphocyte mobilization from the marginal pool and the spleen and stimulate an influx of lymphocytes into the blood.

Neutrophil (N) count was not influenced significantly by moderate altitude exposure.

There was a trend of increased neutrophil count in HHct ewes only at 7 d ($P > 0.05$) after transport to moderate altitude (Fig. 2) which is consistent with the reported increase in neutrophil numbers in response to cold air exposure (Castellani et al., 2002). The slight rise in circulating neutrophils following moderate altitude exposure in HHct ewes may be attributed to cortisol mediated demargination of neutrophils. Numerous studies have shown that glucocorticoids stimulate an influx of neutrophils into the blood from bone marrow, marginal pool and lung and attenuate the egress of neutrophils from the blood to other compartments (Dhabhar et al., 1994; Dhabhar et al., 1996). It has been suggested that either cortisol regulate blood neutrophils independently of the adrenergic system or that cortisol have permissive role in mobilization of granulocytes after adrenergic activation (Landmann et al., 1984).

The tendency of increased N/L ratio at 7d in HHct ewes after transport is consistent with the tendency of increased cortisol levels at that time, and represent a leukocyte response to moderate altitude exposure (Fig. 4). Also, cold exposure has been reported to increase norepinephrine concentration (Castellani et al., 2002). Therefore, it seems that both norepinephrine and cortisol mediate the observed trend of increase in N/L ratio on d 7 after altitude exposure. The lack of association between N/L ratio and plasma level of cortisol at 20 d may be due to decreased leukocyte glucocorticoid receptors

Although the baseline monocyte numbers in MHct and HHct ewes were significantly higher than in LHct ewes, they still were within the reference range. Monocyte numbers increased significantly at 7 d in LHct ewes ($P < 0.05$) compared to baseline levels. Monocyte numbers, similar to lymphocyte numbers, showed a trend towards decline at 7 d after transport in MHct ewes only compared to baseline levels (Fig. 5). Castellani et al. (2002) reported variable changes in monocyte counts and concluded that norepinephrine accounted for most of the variance in leukocyte subsets during cold exposure. The observed decline in MHct ewes at 7 d after transport to moderate altitude may indicate higher rate of transmigration from circulation to other tissues and better immune surveillance compared to the other groups. Stress is supposed to enhance monocytes traffic to sites of wounding, antigen/ pathogen entry or ongoing inflammation (Dhabhar et al., 2012). Consequently, it can be supposed that the main target site of monocytes in our study is skin which is exposed to both cold and direct solar radiation. However, there are yet no data available about monocyte subsets migration. It has been suggested that some subsets contribute to tissue damage, whereas others promote tissue repair (Wolf et al., 2019).

The observed trend towards a decline in eosinophil numbers in all the 3 groups at 7 d (Fig. 7) is consistent with the generally accepted view that eosinopenia in acute stress is mediated by adrenal glucocorticoids (Ohkaru et al., 2010). Our data are consistent with the reported decline in eosinophil numbers in response to mental stress (Karpoor et al., 2011) and swimming in cold water (Hess, 1963). The significantly lower eosinophil numbers in LHct ewes at 7 d compared to MHct ($P < 0.01$) and HHct ewes ($P < 0.001$) respectively may be explained with a more pronounced activation of the sympathetic nervous response (Johnson et al., 1977). This view is further supported by the suggested higher sympathetic activation in LHct ewes (Moneva et al. 2021). Also, baseline levels of IFN-γ level which is one of the major products of eosinophils (Spencer et al., 2009) tended to be higher in LHct compared to the other groups. (Fig. 13). The observed lower level of eosinophils in MHct ewes compared to other 2 groups on d 20, accompanied by increased IFN-γ concentration suggest a possible relation between IFN-γ concentration and eosinophil trafficking.

Unchanged basophil numbers at 7 d compared to baseline values (Fig. 8) are not in agreement with the reported inhibition of the rapid CD 63 upregulation on the membrane of Ig E-positive basophils in response to mental stress. It has been concluded that acute mental stress and sympathetic activation inhibit the functional activity of basophil granulocytes. This effect was mediated by B-2 adrenergic pathway (Raap et al., 2008. Therefore, the observed lack of significant change in basophil numbers at 7 d as well as the trend of decreased basophil numbers in HHct ewes compared to LHct ewes ($P > 0.05$) at 20 d may reflect differences in sympathetic responses and basophils migration from blood to tissues., The trend of decrease in basophil numbers observed in LHct ewes at 7d compared to baseline levels corresponded to significantly increased cortisol levels at that time. It is worth noting that the higher basophil numbers in HHct ewes at 20 d coincided with the observed higher levels of IL-4 (Fig. 10) and IL-10 (Fig. 12) compared to MHct ewes at that time. Given that, basophils are implicated in the Th2 cytokines (Il-4 and IL-10) differentiation, it can be assumed that these variables are functionally associated.

Increased values of the large immature cells at 7 d after exposure to moderate altitude in HHct ewes (Fig. 6) can be due to increased neutrophil numbers at that time, since neutrophilia is characterized by an increased presence of immature neutrophils in the blood (Suzuki et al., 2003). We found a significant Pearson correlation between N/L ratio and LIC as follow: baseline levels ($r = 0.627995$, $P < 0.01$); at 7 d ($r = 0.771221$, $P < 0.001$); at 20 d ($r = 0.606801$, $P < 0.01$); at 42 d ($r = 0.566646$, $P < 0.01$).

In our previous study we suggested that hematocrit was closely related to the metabolic energy supply. High hematocrit sheep was proposed to have predominantly anaerobic energy supply as compared to low hematocrit sheep (aerobic energy supply). Consequently, hematocrit associated response to stress found in this study may be attributed to the specific metabolic pathway for energy supply inherent in each hematocrit type.

Recently, leukocyte subpopulations are considered in the context of their bioenergetics. Neutrophils are known to rely on anaerobic glycolysis for energy supply, while lymphocytes in quiescent state use primarily oxidative phosphorylation to meet their energetic needs. However, lymphocyte activation is associated with an increase in both glycolytic function and mitochondrial oxygen consumption (Kramer et al., 2014).

Taken together, these findings suggest that distribution profiles of peripheral basophils, eosinophils and monocytes in LHct ewes differ from those of MHct and HHct ewes and may reflect different levels of immune surveillance.

No significant effect of altitude exposure was observed on either of the investigated cytokines in LHct, MHct and HHct ewes throughout the entire experimental period (Fig. 9-15). It is worth noting that three animals showed 2-3 fold higher levels of IL-10 and IFN-γ than the rest during the whole experimental period. The increased levels of IFN-γ in these animals can be associated with a possible local inflammation since IFN-γ acts as both an inducer and regulator of inflammation. Besides, IFN-γ is important for immune system homeostasis (Zhang, 2007; Wilke et al., 2011). Therefore, it may be assumed that these animals have probably developed mild local inflammation before the onset of this study. Increased level of IFN-γ may reflect an imbalance between Th1-type and Th2- type cytokines as judged by the increased levels of the anti-inflammatory cytokines IL-4 and IL-10 (Hu & Ivashkiv, 2009). An alternative explanation for this finding, however, is that these ewes probably had more pronounced inflammatory propagations to enable successful implantation (Dutta & Sengupta, 2017). Also, increased concentrations of the anti-inflammatory cytokines (IL-4 and IL-10) may reflect a transition towards a systemic anti-inflammatory innate phenotype aimed at preventing rejection of the semi-allogenic fetus (Graham et al., 2017).

There was a tendency of decline in IL-10 levels at 7 d in HHct and MHct ewes, while in LHct ewes remained unchanged (Fig. 12). IL-10 signaling in adipocytes was shown to limit thermogenesis and energy expenditure in mice (Rajbhandari et al., 2018). The observed differences in the dynamics of IL-10 between the experimental groups in our study may relate to the different acclimatization strategy in animals with low and high hematocrit. Our previous data show that the acclimatization strategy of animals with lower hematocrit is associated with lower energy consumption. In sheep with high hematocrit, the supply of tissues with oxygen allows these animals to increase heat production in response to cold stress by stimulating metabolism (oxygen consumption) and not by reducing oxygen consumption and metabolism, as is likely to be the case in sheep with low hematocrit. IL-10 signaling may function as a mechanism to conserve energy under moderate altitude hypoxia

The observed trend of decrease in IL-6 levels in HHct ewes at 20 d and 42 d may be an advantage by preventing hypoxia-induced chronic low-grade systemic inflammation (Fig. 11).

There was significant correlation (Pearson) coefficients as follow: between IL-2 and IL-6 at 20 d ($r = 0.531371$, $P < 0.01$) and at 42 d ($r = 0.586212$, $P < 0.01$); between Il- 2 and IL-17A at d 7 ($r = 0.53106$, $P < 0.01$); between IL-2 and TNF-α at d 42 ($r = 0.756791$, $P < 0.05$) between; IL- 2 and INF-γ at 42 d ($r = 0.445463$, $P < 0.05$). These data indicate that cytokines work in a concert in the adjustment to moderate altitude hypoxia, where IL-2 appears to play an essential role.

There was a weak but significant negative relation also between baseline lactate (data not shown) and IL-2 levels ($r = -0.36588$, $P < 0.05$), suggesting a role of the lactate as an IL-2 suppressor. In our previous study we found significantly lower baseline lactate levels in LHct ewes compared to HHct ewes (unpublished data). The higher baseline IL-2 levels in LHct ewes compared to HHct ewes may be explained by their higher dependence on oxidative phosphorylation in LHct ewes. The lower baseline IL-2 was probably due to the preferential reliance on glycolysis and therefore to higher capacity for quick supply of energy in HHct ewes.

Interestingly, our findings of unchanged cytokine levels in response to moderate altitude exposure are not in agreement with previous studies. Acute psychological and physical stress stimuli have been shown to elicit an increase in the proinflammatory and anti-inflammatory cytokines levels (Maes et al., 1998; Marsland et al., 2017; Suzuki, 2018). Hypoxia was shown to cause inflammation, including increase in transvascular leakage and oxidative stress with increased NF-*κ*B expression in lungs followed by significant increase in proinflammatory cytokines IL-1, IL-6, and TNF-*α* (Mazzeo*,* 2005). The observed discrepancy may be associated with the notion that body temperature modulates cytokine release. Clamping of body temperature during exercise has been found to decrease (Laing et al., 1985; Gagnon et al., 2014) or completely abolish increases in plasma cytokine concentrations (Rhind et al., 2004; Mestre-Alfaro et al., 2012). Furthermore, it has been suggested that exercise associated elevation of core temperature mediates increases of circulating stress hormones, which in turn contribute to induction of cytokine release (Rhind et al., 2004). Therefore, it can be assumed that the lack of cytokines increases in response to moderate altitude exposure in our study is due to the loss of sheep's insulating fleece accompanied by an increase in lower critical temperature which may ultimately result in increased sensitivity to cold. Lower critical temperature in shorn sheep at maintenance feeding has been estimated to be 25˚C (NRC, 1981). Moreover, pregnancy can also influence cytokine production. It has been reported that pregnancy influences the cytokine response to exogenous pyrogen with a predominant antipyretic/cryogenic cytokine response relative to pyrogenic response (Fofie et al., 2005).

Interestingly, our findings are not in agreement with the reported increase in IL-6 and TNF-α in domesticated camelids during the dry season in the Andean region of Perú (Arias et al., 2016). This discrepancy may be explained by the large environmental temperature differences (-3 – +20°C) in the reported study, accompanied by *Pasteurella multocida* challenge. Besides, the above mentioned study was carried out with unshorn, not pregnant llamas and alpacas.

Our findings suggest that exposure to moderate altitude of pregnant sheep at ambient temperature below the lower critical temperature prevents the production of pro-inflammatory and anti-inflammatory cytokines. Also, increased levels of IL-4 in three ewes support the view that anti-inflammatory bias becomes increasingly intense with increasing gestational age.

Conclusions

Moderate altitude exposure of newly shorn pregnant ewes at ambient temperature (8.0°C–13.1°C) decreased lymphocyte numbers. Exposure to moderate altitude did not influence neutrophil distribution. There was hematocrit associated differences in basophil, eosinophil and monocyte distribution profiles in response to moderate altitude exposure.

Moderate altitude exposure of newly shorn pregnant ewes at ambient temperature below the lower critical temperature did not elicit an increase in the measured pro-inflammatory and anti-inflammatory cytokines in MHct and HHct ewes.

There was hematocrit associated differences in cytokines concentrations. These results suggest that pregnancy and ambient temperature modulate cytokine production and leukocyte subsets mobilization and trafficking under moderate altitude hypoxia.

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