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Dynamics of leukocytes and cytokines after shearing 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 shearing 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%). 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). The experiment was conducted at the end of the first month after artificial insemination. Daily minimum and maximum ambient temperatures during the whole experimental period were 13.4 and 24.2˚C, respectively. Blood samples were taken by jugular venipuncture before shearing, immediately after shearing, at 3 h and 48 h after shearing. Shearing elicited significant increase in neutrophil numbers in all the 3 groups at 3h after shearing followed by a return to normal levels at 48 h after shearing. Lymphocyte numbers were not influenced by shearing. There were hematocrit associated changes in basophil, eosinophil and monocyte numbers suggesting different trafficking patterns of these leukocyte subsets. Large immature cells increased significantly in all experimental animals at 3 h after shearing. Three ewes in LHct group had 3-8 fold higher levels of IFN-γ, IL-4 and IL-10 compared to the other animals in the same group suggesting pregnancy associated transition towards Th2-type cytokines. 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 shearing. The results are interpreted to suggest that shearing of pregnant ewes at ambient temperature below the lower critical temperature prevents cytokines increase.

Keywords: leukocytes; cytokines; interferon; hematocrit; stress; sheep

Introduction

Wool removal is more stressful than any of the other manipulations involved in conventional shearing (Grandin, 2014). Shearing can act as both a psychological and physical stimulus. Shearing-induced acute stress is followed by a chronic heat (Titto et al., 2016) or cold stress (Aleksiev, 2008) depending on the ambient temperature. 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).

This study was thus designed to investigate the dynamics of a variety of pro-inflammatory and anti-inflammatory cytokines and magnitude of shearing-induced changes in leukocyte distribution during the first 48 h following shearing of sheep.

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 at the end of May, 2019 at the Institute of Animal Science, Kostinbrod, Bulgaria located at an altitude of 540 m above sea level. Mean, minimum and maximum daily temperatures during the experimental period were 19.8°C, 13.4°C and 24.2°C respectively. Relative humidity range was 39-68 %. Wind speed was in the range of 1-3 m/s.

Ewes

Institute′s research flock of 110 Ile De France ewes was used to select ewes with low, mean or 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. 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 (Hct) levels, 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.

Shearing and plasma collection

On the day of shearing, sheep were penned in a shearing shed within easy access of the shearers who removed them individually from the pen to be shorn. Sheep were shorn by professional shearers who handled the sheep in a low-stress manner.

Blood samples were collected by direct jugular venipuncture before shearing (baseline level), immediately after shearing, 3 h and 48 h after shearing. 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 Figures 1-8. Stress-induced changes in leukocyte numbers (Figure 1) were apparent at 3 h after shearing, only when the cal-

culation of statistical significance was based on all animals included in the experiment (n=30). Neutrophil count in all the 3 groups increased significantly at 3 h following shearing compared to baseline count then declined significantly at 48 h following shearing (Figure 2). However, the rate of neutrophil count increase from baseline to shearing levels tended to be higher in LHct (225.6%) and MHct (226.38%) ewes compared to HHct (207.47%).

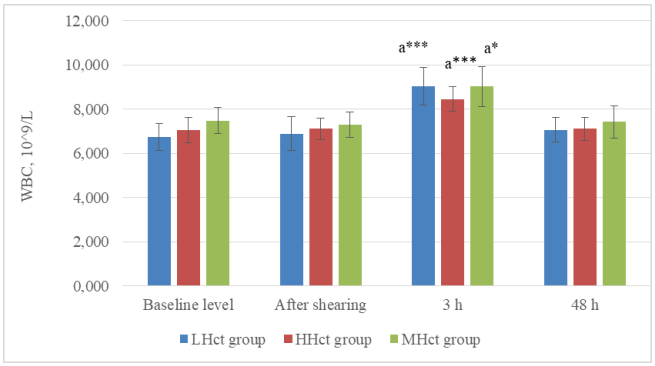

Fig. 1. WBC after shearing in pregnant ewes with low and high hematocrit levels
* – P < 0.01, *** – P < 0.001;
a – significantly different versus respective baseline level

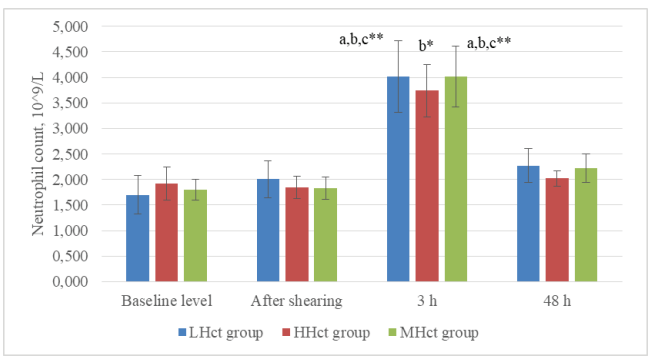

Fig. 2. Neutrophil count after shearing in pregnant ewes with low and high hematocrit levels
* – P < 0.01, *** – P < 0.001;
a – significantly different versus respective baseline level,
b – significantly different versus after shearing,
c – significantly different versus 48 h

Shearing had no significant effect on lymphocyte numbers throughout the experimental period. There was a trend of lymphocyte numbers decline in all the 3 groups at 3 h after shearing relative to baseline values, followed by a trend of slight increase at 48 h after shearing. There were no differences between the groups (Figure 3).

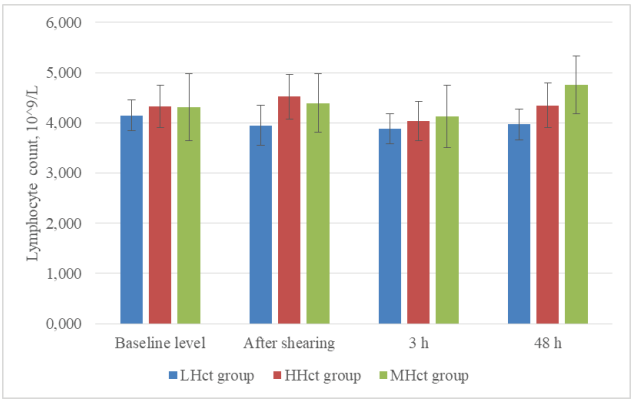

Fig. 3. Lymphocyte count after shearing in pregnant ewes with low and high hematocrit levels

Neutrophil (N) to lymphocyte (L) ratio in all the 3 groups increased significantly at 3 h after shearing (Figure 4).

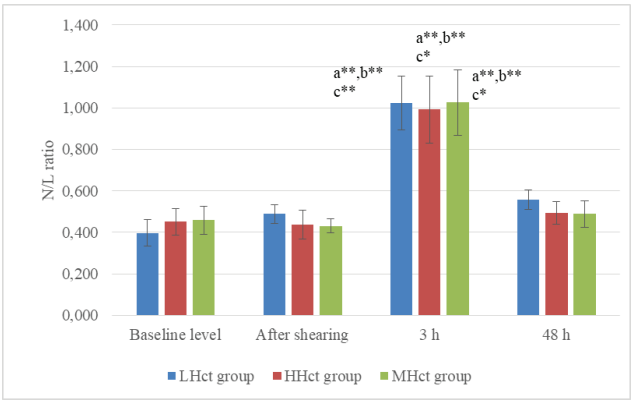

Fig. 4. N/L ratio after shearing in pregnant ewes with low and high hematocrit levels
* – P < 0.05; ** – P < 0.01;
a – significantly different versus respective baseline level;
b – significantly different versus after shearing;
c – significantly different versus 48 h

Baseline monocyte numbers in MHct ewes were significantly higher compared to LHct ewes (Figure 5). There was a trend of decline in all the 3 groups at 3 h and 48 h after shearing, but statistical significance was reached at 48 h in MHct ewes only compared to baseline levels (Figure 5).

Large immature cell values increased significantly in all the groups at 3 h following shearing compared to baseline values (Figure 6).

Eosinophil numbers in LHct ewes were significantly lower compared to MHct ewes immediately after shearing. Also, eosinophil numbers in LHct ewes observed at 48 h

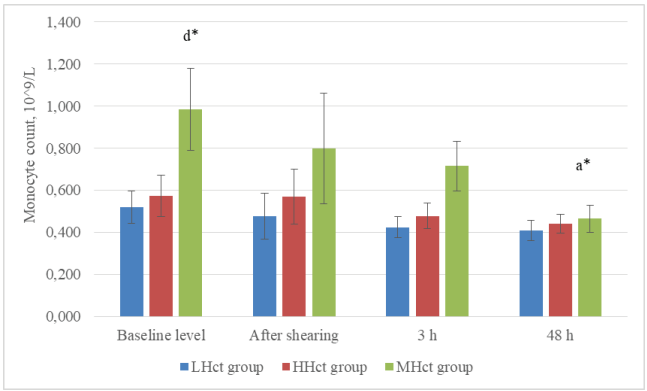

Fig. 5. Monocyte count after shearing in pregnant ewes with low and high hematocrit levels
* – P < 0.05;
a – significantly different versus respective baseline level;
d – significantly different versus LHct group

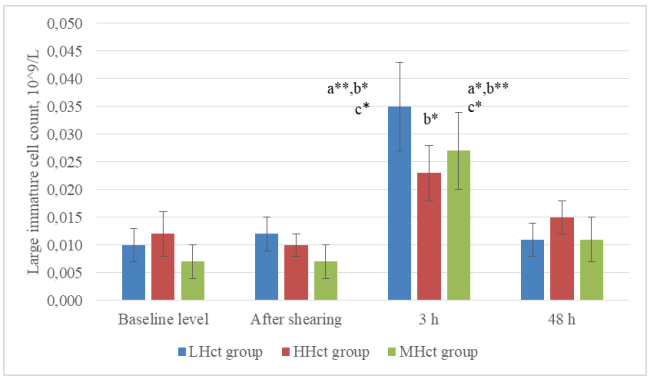

Fig. 6. Large immature cell count after shearing in pregnant ewes with low and high hematocrit levels
* – P < 0.05; ** – P < 0.01;
a – significantly different versus respective baseline level;
b – significantly different versus after shearing;
c – significantly different versus 48 h

after shearing were significantly lower compared to HHct ewes (Figure 7). Basophil numbers in LHct ewes increased significantly at 3 h after shearing relative to shearing level and were significantly higher at that time compared to HHct ewes (Figure 8).

There was a trend of higher leukocyte numbers in all the 3 groups at 3 h after shearing.

Dynamics of cytokines

Interleukin 2 in all the 3 groups exhibited a trend of increase immediately after shearing (Figure 9). Interleukin 4 levels in all groups were not significantly influenced by

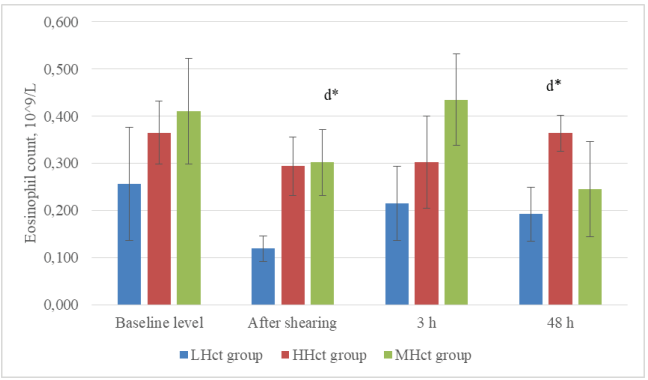

Fig. 7. Eosinophil count after shearing in pregnant ewes with low and high hematocrit levels
* – P < 0.05;
d – significantly different versus LHct group

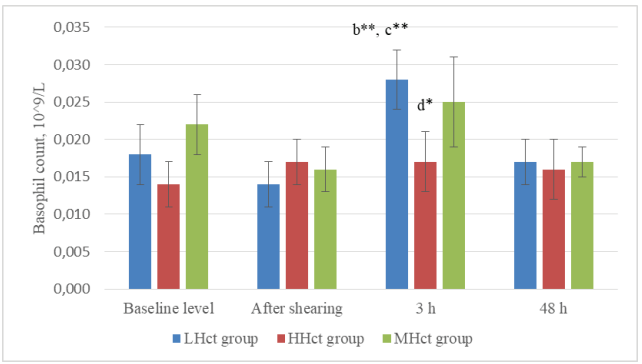

Fig. 8. Basophil count after shearing in pregnant ewes with low and high hematocrit levels
* – P < 0.05; ** – P < 0.01;
b – significantly different versus after shearing;
c – significantly different versus 48 h;
d – significantly different versus LHct group

shearing stress. However, IL-4 levels in LHct ewes tended to be higher compared to the other two groups in all measurements after shearing (Figure 10). Both baseline and shearing levels of IL-10 were significantly higher in LHct ewes compared to MHct ewes (Figure 11). Interleukin 17A levels in LHct and MHct ewes tended to be lower immediately after shearing and at 3 h after shearing compared to their respective baseline levels (Figure 12). Interferon gamma levels in MHct ewes were significantly lower at 48 h following shearing compared to LHct ewes (Figure 13). There was a trend of higher IFN-γ in LHct ewes throughout the whole experimental period. Shearing did not influence IFN-γ levels (Figure 13). There was no significant effect of shearing on TNF-α levels throughout the entire experimen-

tal period (Figure 14). Interleukin 6 levels fell in the lower range of the standard curve.

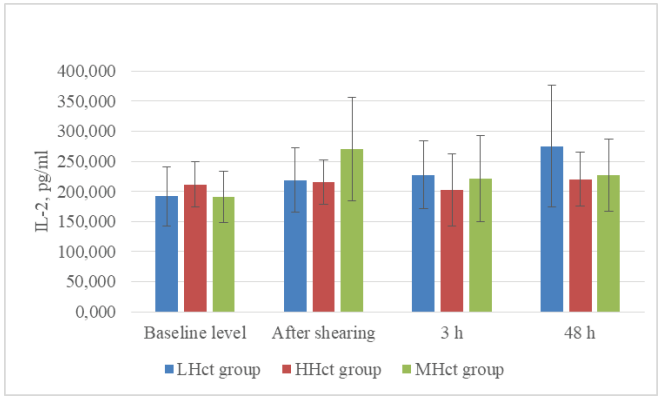

Fig. 9. IL-2 after shearing in pregnant ewes with low and high hematocrit levels

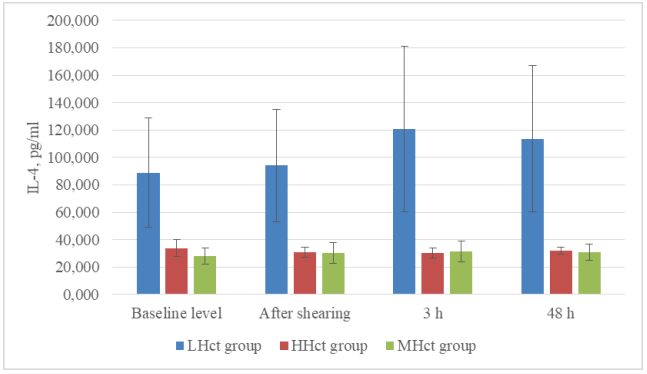

Fig. 10. IL-4 after shearing in pregnant ewes with low and high hematocrit levels

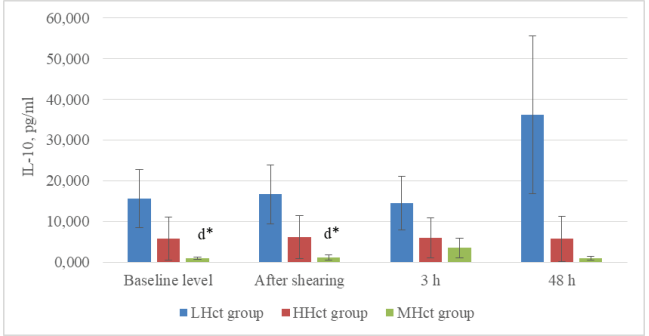

Fig. 11. IL-10 after shearing in pregnant ewes with low and high hematocrit levels.
* – P < 0.05;
d – significantly different versus LHct group

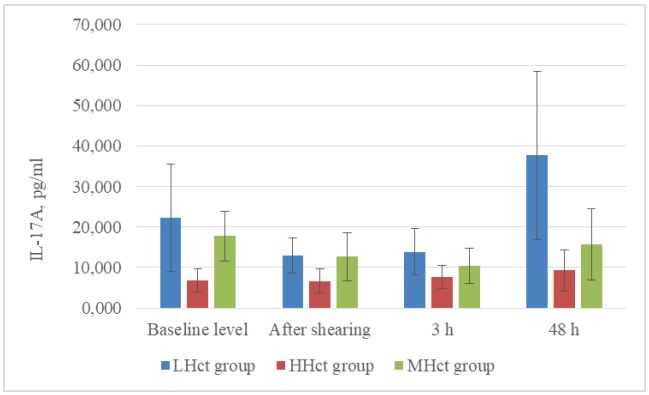

Fig. 12. IL-17A after shearing in pregnant ewes with low and high hematocrit levels

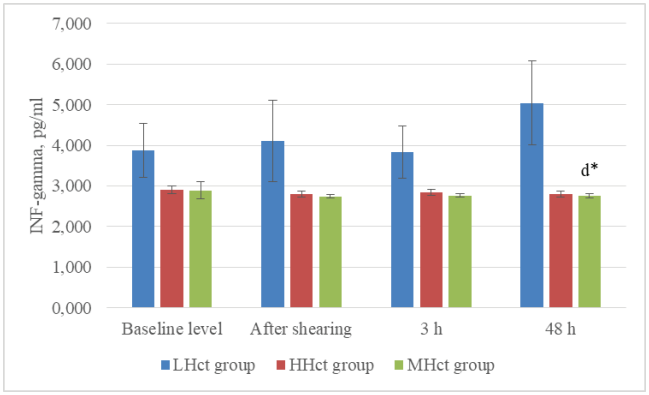

Fig. 13. INF-gamma after shearing in pregnant ewes with low and high hematocrit levels
* – P < 0.05;
d – significantly different versus LHct group

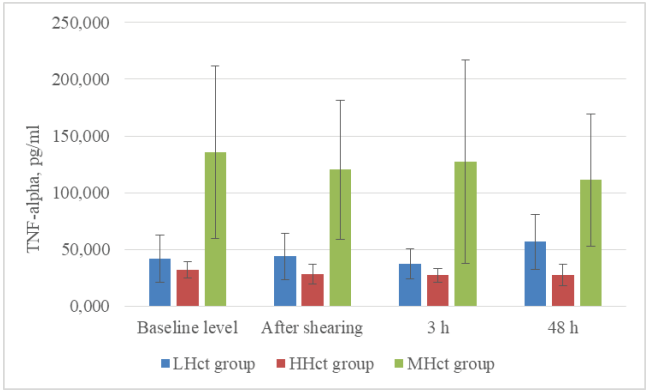

Fig. 14. TNF-alpha after shearing in pregnant ewes with low and high hematocrit levels

Discussion

Lymphocyte count (L) dynamics in our study is not consistent with the reported quick mobilization (less than 15 min) in lymphocyte numbers, followed by a decrease within 60-120 min in response to short-term acute stress. Lymphocyte mobilization from the marginal pool and the spleen is mediated via catecholamine activation of lymphocyte beta 2- adrenoreceptors (Landmann et al., 1984; Benschop et al., 1996; Pedersen & Hoffman-Goetz, 2000). 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). Besides, lymphocyte numbers may be influenced by the receptor subtype involved since the effect of adrenal steroids on immune cell distribution is exerted through type I and type II receptors (Miller et al., 1994). Cold stress, unlike psychological stress was found not to influence hippocampal mineralocorticoid (Type I) receptors density (Gesing et al., 2001). In our previous work we suggested that sheep experienced mild-cold stress immediately after shearing at the same ambient temperature (Moneva et al., 2021b). 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. Alternatively, increased catecholamines may decrease the egress of lymphocytes from the blood to other compartments. According to Landmann and al. (1984) increased blood lymphocyte numbers might indicate that catecholamines prevent the cells from residing in the site of antigen or target recognition and processing. Besides, cold is found to suppress lymphocyte functional activity (Beilin et al., 1998). Our results indicate that shearing-induced mild cold stress prevents the lymphocyte numbers decrease (Figure 3).The unchanged lymphocyte numbers throughout the whole study is consistent with the reported lack of lymphocyte change during the recovery period (30-120 min) following exercise in the cold (Kim et al., 2014).

Neutrophil (N) count increase at 3 h after shearing (Figure 2) is consistent with the reported increase in neutrophil numbers in response to cold air exposure (Castellani et al., 2002). It is widely believed that the movement of neutrophils from marginal pools located intravascularly and from extravascular storage pools contributes to exercise-related neutrophilia (Pedersen & Hoffman-Goetz, 2000). The rise in circulating neutrophils following shearing 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). Increased N/L ratio at 3 h after shearing is consistent with the increased cortisol levels in all the 3 groups immediately after shearing presented in our previous paper (Moneva et al., 2021b), and represent a delayed leukocyte response to shearing (Figure 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 increase in N/L ratio at 3 h after shearing.

Although the baseline monocyte numbers in MHct were high, they still were within the reference range. Monocyte numbers, unlike lymphocyte numbers, showed a trend towards decline at 48 h after shearing, and reached level of significance in MHct ewes only compared to baseline levels (Figure 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 at 48 h after shearing 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 immediately after shearing (Figure 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 lack of significance is probably due to the relatively short duration of shearing procedure followed by exposure to mild cold stress. The lower eosinophil numbers in LHct ewes immediately after shearing and 48 h later compared to MHct and HHct ewes 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., 2021a). Also, IFN-γ which is one of the major products of eosinophils (Spencer et al., 2009) tended to be higher in LHct compared

to the other groups and reached level of significance at 48 h following shearing (Figure 13). Therefore, the observed lower level of eosinophils in LHct ewes, accompanied by increased IFN-γ concentration suggests a possible relation between IFN-γ concentration and eosinophil trafficking.

Unchanged basophil numbers immediately after shearing compared to baseline values (Figure 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 change in basophil numbers immediately after shearing as well as decreased basophil numbers in HHct ewes compared to LHct ewes at 3 h after shearing may reflect differences in sympathetic responses and basophils migration from blood to tissues. It is worth noting that the higher basophil numbers in LHct ewes at 3 h after shearing coincided with the observed higher levels of IL-4 (Fig. 10) and IL-10 (Figure 11). 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 3 h after shearing (Figure 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)

Elevated WBC count at 3 h following shearing was primarily due to increased neutrophil numbers.

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 shearing was observed on either of the investigated cytokines in LHct, MHct and HHct ewes throughout the entire experimental period (Figures 9-14). It is worth noting that three animals in LHct group showed 2-3 fold higher levels of IFN-γ than the rest during the whole experimental period. The same animals had 3-8 fold higher levels of IL-10 and IL-4 compared to the other animals in this group. 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).

Interestingly, our findings of unchanged cytokine levels in response to shearing are not in agreement with previous studies. Acute psychological and physical stress stimuli have been shown to elicit an increase in the pro-inflammatory and anti-inflammatory cytokines levels (Maes et al., 1998; Marsland et al., 2017; Suzuki, 2018). 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 shearing 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). Besides, pregnancy is reported to attenuate adrenal response to shearing in ewes (Ungerfeld & Freitas-de-Melo, 2019). Our findings are not in agreement with the reported 2 fold increases in IL-2, IL-6 and TNF-α during the 1st day after shearing (Hefnawy et al., 2018). This discrepancy can be explained by the higher environmental temperature (31˚C) accompanied with increased rectal temperature and respiratory rate in the reported study, which indicate shearing-induced heat stress. Besides, the above mentioned study was carried out with rams.

Our findings suggest that shearing 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 and IL-10 in three LHct ewes support the view that anti-inflammatory bias becomes increasingly intense with increasing gestational age.

Conclusions

Shearing at ambient temperature (13.4°C – 24.2°C) did not influence lymphocyte distribution. There were hematocrit associated differences in basophil, eosinophil and monocyte distribution profiles in response to shearing.

Shearing of 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 were hematocrit associated differences in IFN-γ and IL-10 concentrations. These results suggest that pregnancy and ambient temperature modulate cytokine production and leukocyte subsets mobilization and trafficking.

Acknowledgements

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