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LYMPHOCYTE TRAFFICKING FOLLOWING ACUTE STRESS AND ALTITUDE HYPOXIA IN LOW AND HIGH HEMATOCRIT SHEEP

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ABSTRACT

The object of the present study was to investigate small and large lymphocytes trafficking in sheep with low and high hematocrit values following shearing, exposure to moderate altitude and transport to low altitude. Twenty out of 101 Ile de France ewes (1-7 years old) were used in the present experiment. All ewes of the flock were artificially inseminated in May 2015 following estrus synchronization. The animals were allocated into two groups following threefold measurements of hematocrit in all ewes as follows: I- low hematocrit group (n=10) and II - high hematocrit group (n=10). The ewes were transported to the Petrohan Pass (1440 m above sea level) in June 2015 immediately after shearing, conducted at the experimental farm of the Institute of Animal Science, Kostinbrod (500 m above sea level). Blood samples were collected before shearing, immediately after shearing, 3 h after shearing, at 14 d following exposure to moderate altitude, immediately after transport to low altitude and following 7d of stay at low altitude. All leukocyte subpopulations were counted microscopically. In the current study we presented the percentage of lymphocytes only, including small and large (reactive) lymphocytes. High and low hematocrit ewes had different percentage of small lymphocytes when exposed to various acute and chronic stress stimuli. There were significant differences in the percentage of large (reactive) lymphocytes between low and high hematocrit ewes following blood collection and immediately after shearing. The observed difference in small lymphocyte dynamics among the groups in response to different stress stimuli was attributed to hematocrit related differences in the time course and magnitude of lymphocyte distribution at early and late phases of stress. The results were interpreted to mean that the differences in lymphocyte trafficking between the two groups of sheep in response to stress were related to possible difference in the share of aerobic and glycolytic pathways for energy supply.

Key words: *small lymphocytes, large lymphocytes, hematocrit, sheep, stress*.

INTRODUCTION

There is now substantial evidence that immune function may be altered after exposure to acute or chronic stress. Phenotyping analysis, based on the size of lymphocytes revealed that both, small and large lymphocytes are present in sheep. The mobilization of leukocytes is selective and primarily affects, among others, effector CD8+ T cells and NK cells (Benschop et al., 1996, Kruger and Mooren, 2007). There is evidence that acute immunologic reactions may be mediated by concomitant sympathoadrenal activation, as evidenced by acute rises in heart rate, blood pressure, and plasma catecholamine concentrations (Bachen et al., 1992; Manuck et al., 1991; Zakowski et al., 1992).

Stress hormones have been identified as the major endocrine mediators in leukocyte distribution during acute or chronic stress (Dhabhar, 2006). The endocrine and autonomous nervous systems regulate immune functions not only directly via hormones and neural innervation, but also indirectly via influences on blood flow, blood pressure, lymph flow (Ottaway and Husband, 1992; Maestroni, 2001) and the supply of substrates like glucose, fatty acids and oxygen (Fox et al., 2005; Straub et al., 2010; Besedovsky and Rey, 2011).

There is evidence that the basal values of hematocrit are closely related to the adrenal response and the metabolic pathway for energy supply (aerobic, anaerobic) (Evans and Whitlock, 1964; Jones et al., 1967; Grace et al., 1992, Mason, 2000; Stark and Schuster, 2012; Shirasawa et al., 2013). In the recent years immunologists have accentuated on the possibility of using bioenergetic profiles of leukocytes, including lymphocytes, with the realization that leukocytes metabolism is closely tied to immunity (Kramer et al., 2014). Recent findings support the concept that circulating leukocytes can serve as early sensors of mitochondrial function under conditions of metabolic stress (Chacko et al., 2014).

The object of the present study was to investigate small and large lymphocytes trafficking in sheep with low and high hematocrit values following shearing, exposure to moderate altitude and transport to low altitude.

MATERIALS AND METHODS

One hundred one Ile de France ewes (1-7 years old) were used in the present experiment. All ewes of the flock were artificially inseminated in May following estrus synchronization. The animals were allocated into two groups following hematocrit measurement in all ewes. Group I comprised individuals with low level of hematocrit (low hematocrit group; n=10) and group II comprised individuals which had high level of hematocrit (high hematocrit group; n=10). Two additional measurement of baseline hematocrit at intervals of 10 days were performed in the sheep of both groups to verify hematocrit values of both groups, since hematocrit is known to be influenced by many factors and fluctuates from day to day. The average age of the ewes in groups I and II was 3.9± 0.795 and 3.1± 0.745 years respectively. The ewes were shorn on June 2nd 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 temperatures on that day were 13.9 and 25^{o}C for the region of Kostinbrod (low altitude) and 8.2- 13.6^{o}C for the region of Petrohan Pass (high altitude) respectively. The animals remained at high altitude for 4 months where they were on pasture for 10 h during the day. At night they stayed in a barn. The ewes had free access to a NaCl licking stone and water. In addition to pasture, they were offered concentrate once per day. Mean air temperature range in the region of Petrohan pass during the summer months of 2015 was 12 to 20^{o}C. At the end of the grazing season the ewes were transported back to low altitude. At that time the ewes in groups I and II were at 131±6.652 and 140± 4.015 d of gestation respectively as estimated by the day of parturition.

Blood samples were collected before shearing, immediately after shearing, 3 h after shearing, at 14 d following exposure to moderate altitude, immediately after transport to low altitude and following 7d of stay at low altitude.

All samples were taken via jugular venipuncture within 3 min in the morning before feeding in order to minimize handling stress and avoid possible interference caused by cortisol diurnal variation. Differential white blood cell count was performed. All leukocyte subpopulations were counted microscopically in smears made after staining with Giemsa-Romanovsky. In the current study we presented the percentage of lymphocytes only, including small and large (reactive) lymphocytes. The results of one factor analysis are expressed as means ± S.E.M. and were analyzed by ANOVA.

RESULTS AND DISCUSSION

Baseline number of small lymphocytes was significantly higher in low hematocrit ewes as compared to that in high hematocrit ewes (Fig.1). In contrast, the number of large lymphocytes was significantly higher in high hematocrit ewes relative to low hematocrit ewes (Fig.2). It is well known that small, mature lymphocytes are the most common lymphocyte in peripheral blood (EclinPath, 2017). Large lymphocytes have been found to be infrequent in healthy animal blood (Cotter, 2015). Consequently, the increased baseline number of large lymphocytes in the high hematocrit ewes could be due to the act of sheep handling and restraint during blood collection. This view is consistent with the reported significant increase in T cell and natural killer cells immediately after the jump in parachutists (Dhabhar, 2006). Similar results have been reported by Rinner et al. (1992) who found that short stressor (1-min handling) caused an increase in mitogen – induced proliferation of T and B cells obtained from peripheral blood. The authors suggested that the modulation of immune cell distribution by acute stress may be an adaptive response designed to enhance immune surveillance and increase the capacity of immune system to respond to challenge in immune compartments. We are prone to believe that the above mentioned increase in blood T and B lymphocytes following short stress involved mainly large (reactive) cells, since the presence of reactive cells is considered to be an immune response in progress to stress (Cotter, 2015). Our view is consistent with the reported change in the percentage of large CD4 and CD8 cells 6 weeks before written examination, one

day before the examination day and 12-14 days after the examination (Halvorsen and Vassend, 1987). Also, this view is supported by the reported significant increase in the diameter of activated CD8-T cells relative to resting cells diameter suggesting that activation was accompanied by an increase in cell volume (Du et al., 2017). Our results suggest that stress, caused by blood sampling, may modulate both small and large (activated) lymphocyte trafficking to their respective compartments. However, low hematocrit ewes, unlike high hematocrit ewes, had relatively low baseline percent of large lymphocytes which could be due to delayed immune response or higher stress threshold in these animals.

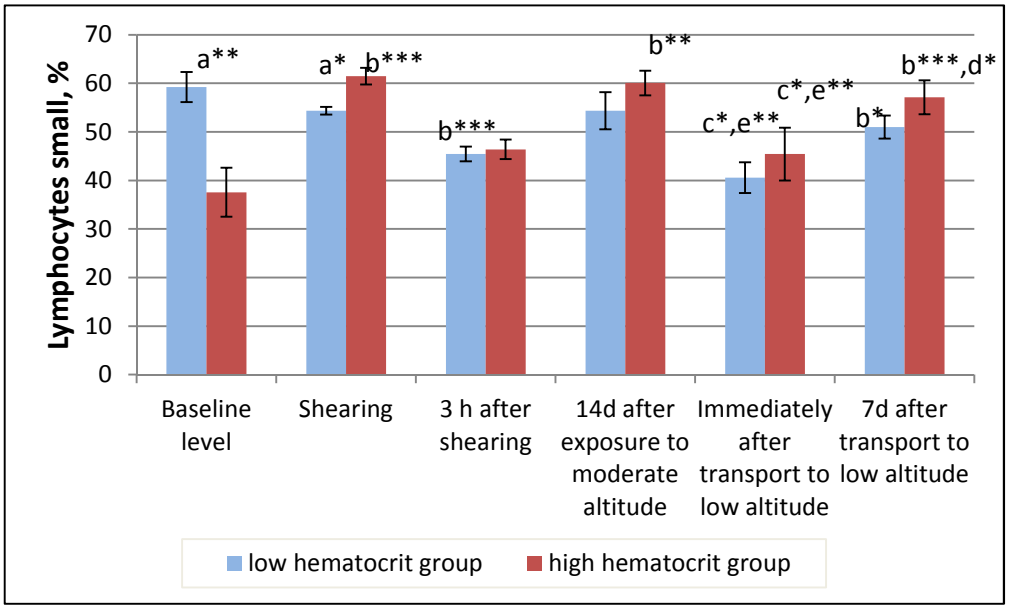

Fig. 1 Lymphocytes (small) in sheep with low and high hematocrit values following shearing, exposure to moderate altitude and transport to low altitude. P*<0.5, ** P<0.01,***P<0.001

a- significantly different among the groups
b- significantly different versus baseline level
c- significantly different versus 14 d after exposure to moderate altitude
d- siginificantly different versus immediately after transport to low altitide
e- significantly different versus shearing

The observed opposite dynamics of large lymphocytes during shearing, which is considered to be an acute stress, confirms once again the established difference in lymphocytes trafficking among the groups, caused by blood sampling. Although less prominent the difference in small lymphocyte percentages between the two groups caused by shearing was still significant. Furthermore, small lymphocyte percentage increased in high hematocrit ewes whereas it was unchanged in low hematocrit ewes as compared to the respective blood collection values. Besides, the percentage of large lymphocytes in low hematocrit ewes in response to shearing

(Fig.2) was significantly lower as compared to that observed in high hematocrit ewes during blood sampling. Shearing stress, unlike the stress caused by blood collection, caused further increase in the percent of small lymphocytes in high hematocrit ewes, while the percentage of small lymphocytes in low hematocrit ewes declined insignificantly (Fig.1). The observed decline in the percentage of large (reactive) lymphocytes in high hematocrit ewes when exposed to shearing stress versus blood sampling stress could be due to faster mobilization and trafficking of lymphocytes and their distribution among different body compartments. If we assume that the effect of shearing stress is more severe compared to the effect of blood collection stress than it is more plausible to accept that the observed decline of large lymphocytes in high hematocrit ewes was due to higher speed in large lymphocyte mobilization to specific target organs which leads to decrease in blood large lymphocyte numbers that occurs later during acute stress (Dhabhar et al., 2012). These results suggest that immune response in low hematocrit ewes was less pronounced than that in high hematocrit ewes which could be due to higher stress threshold in low hematocrit ewes.

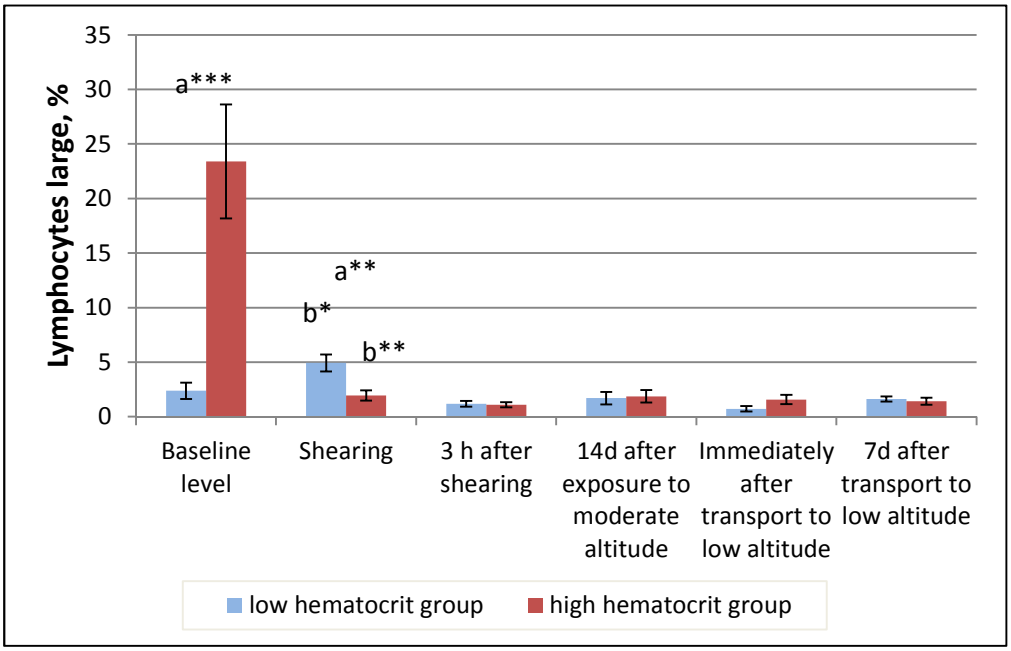

Fig. 2 Lymphocytes (large) in sheep with low and high hematocrit values following shearing, exposure to moderate altitude and transport to low altitude. *P<0.05, **P<0.01, ***P<0.001

a- significantly different among the groups

b- significantly different versus baseline level

Small lymphocyte numbers in both groups were similar at 3 hrs following shearing stress (Fig.1). However the percentage of small lymphocytes in low hematocrit ewes, unlike that in high hematocrit ewes, was significantly lower relative to the respective baseline levels. 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 small lymphocyte percentage at 3 hrs after shearing is consistent with Dhabhar's view. The percentage of large lymphocytes at that time was similar in both groups. It is interesting to note that large lymphocyte percentage in high hematocrit ewes (Fig.2) was significantly lower than the respective baseline value (Fig.2). These results are in agreement with the reported decline in total lymphocyte percentage and lymphocyte subpopulations (helper T cells, cytolytic cells, B cells, natural killer cells) in restraint rats following an initial increase of these lymphocytes (Dhabhar et al, 2012).

Small lymphocyte percentage in high hematocrit ewes, unlike that in low hematocrit ewes was significantly higher relative to the baseline value following 14 d exposure to moderate altitude (Fig.1). We assume that the observed percentage of small lymphocytes at that time displayed lymphocyte trafficking caused by adaptation to altitude. This view is in agreement with the results of our earlier finding showing that cortisol level at 14 d following exposure to moderate altitude is similar to baseline cortisol levels (Moneva et al., 2016). Also, the difference in baseline cortisol levels among the groups persisted following 14 d adaptation to moderate altitude. The principal stress hormones have been found to be the major endocrine mediators of a stress-induced leukocyte distribution (Dhabhar et al., 2012.). Therefore, the established differences in small and large lymphocytes percentage between the groups is in agreement with the difference in cortisol levels among the groups following blood collection and exposure to moderate altitude, found in our earlier study.

The percentage of small lymphocytes in both groups declined significantly immediately after transport to low altitude when compared to lymphocyte percentage at 14 d following exposure to moderate altitude (Fig.1) while the percentage of large lymphocytes remained unchanged (Fig.2). There was no difference between the groups in the percentage of small and large lymphocytes at that time despite the higher cortisol level immediately after transport in low hematocrit ewes as compared to cortisol level at14d following exposure to moderate altitude, found in our earlier study (Moneva et al., 2016). The observed discrepancy between lymphocyte numbers and cortisol levels is consistent with Dhabhar's hypothesis (Dhabhar et al, 2012) that specific combinations of stress hormones (epinephrine, norepinephrine and cortisol) would mediate distinct aspects of stress-induced leukocyte redistribution.

There were no significant differences in the percentage of both small and large lymphocytes among the groups at 7 d following transport from moderate to low

altitude (Fig.1,2). However the percentage of small lymphocytes in low hematocrit ewes declined while it increased in high hematocrit ewes relative to the respective baseline levels (Fig.1). The observed difference in small lymphocyte dynamics among the groups in response to different stress stimuli once again indicate that baseline hematocrit level is most probably related to the time course and magnitude of stress-induced lymphocyte distribution at early and late phases of stress as well as during exposure to acute and chronic stress episodes.

Sheep exposed to confinement and isolation stress were found to change their complex behavioral pattern, cortisol secretion and the numbers of various types of lymphocytes (Degabriele and Fell, 2001). In their animal model the authors found that the severity of the stressor in behavioural (and possibly physiological) terms tended to parallel the ability of CD5 components of the immune system to recover from an adverse reaction to stress.

In our earlier study (Moneva et al., 2016) we suggested that hematocrit level depends on hemoglobin type (hemoglobin's affinity for oxygen) which on its turn is related to the ratio between glycolytic and oxidative muscle fibers. It is well known that oxygen demand exceeds oxygen supply during stress. Also, the fast glycolytic system is a key contributor to the total energy requirements for moderate to high intensity stress (Baker et al., 2010). It is consequently reasonable to expect different energy contribution of the two metabolic pathways in response to stress between high and low hematocrit ewes.

Resting lymphocytes do not have large internal glycogen stores and are highly dependent on the import of extracellular glucose and glycolysis for the production of ATP (Fiorucci et al., 2004). Therefore, the observed difference in lymphocyte trafficking between the two groups of sheep in response to stress could be due to the expected difference in blood glucose levels between low and high hematocrit ewes that is crucial for extracellular glucose uptake by lymphocytes.

CONCLUSIONS

High and low hematocrit ewes had different percentage of small lymphocytes when exposed to various acute and chronic stress stimuli.

There were significant differences in the percentage of large (reactive) lymphocytes between low and high hematocrit ewes following blood collection and immediately after shearing.

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