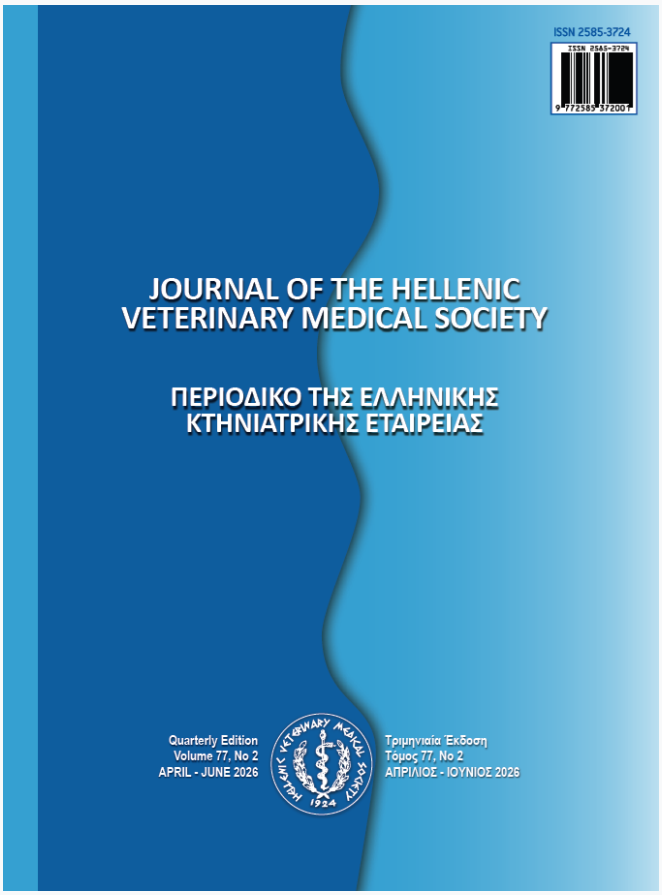
Journal of the Hellenic Veterinary Medical Society

Vol 77, No 2 (2026)



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EG Katsogiannou , EV Bouroutzika , PD Katsoulos , EK Theodosiadou, MC Naskou , LV Athanasiou , I Valasi

doi: 10.12681/jhvms.38049

To cite this article:

Katsogiannou , E., Bouroutzika , E., Katsoulos , P., Theodosiadou, E., Naskou , M., Athanasiou , L., & Valasi , I. (2026). Mitigating Heat Stress Effects:Impact of Melatonin on Blood Cell Morphology in Ewes . *Journal of the Hellenic Veterinary Medical Society*, *77*(2), 10345–10350. https://doi.org/10.12681/jhvms.38049

J HELLENIC VET MED SOC 2026, 77 (2): 10345-10350
ΠΕΚΕ 2026, 77 (2): 10345-10350

Research article
Ερευνητικό άρθρο

Mitigating Heat Stress Effects: Impact of Melatonin on Blood Cell Morphology in Ewes

E.G. Katsogiannou,[1] E.V. Bouroutzika,[1] P.D. Katsoulos,[2] E.K. Theodosiadou,[1] M.C. Naskou,[3] L.V. Athanasiou,[1*] I. Valasi[1*]

[1]*Faculty of Veterinary Medicine, University of Thessaly, Karditsa, Greece*

[2]*Clinic of Farm Animals, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece*

[3]*Department of Pathobiology, College of Veterinary Medicine, Auburn University, AL, USA*

ABSTRACT: Sheep exposed to excessive heat can suffer various adverse effects, particularly on their hematological system, including blood cell integrity and function. Melatonin has a protective role by mediating antioxidant pathways and acts as a regulator of apoptosis. The objective of this study was to evaluate the impact of heat stress on blood cell morphology and hematological variables in ewes and whether melatonin administration could mitigate it. Seventy-four ewes of the Karagouniko breed participated in the study and were divided into three groups; the heat stress-melatonin group (HM, n = 20), the heat stress-control group (HC, n = 20) and the control group (C, n=34). The heat stress-melatonin group as well as the heat-stress control group were evaluated during the summer period, whereas the control group was evaluated in autumn. All the animals in the HM and HC groups experienced heat stress, while those in group C did not. Following the administration of melatonin implants to the HM group (D0), blood samples were collected forty days later (D40) as well as from those in the HC and C groups. Blood smears were prepared and Giemsa stained, and thereafter, the packed cell volume (PCV), hemoglobin (Hgb) were measured, and mean corpuscular hemoglobin concentrations (MCHC) was calculated in all blood samples. Lower values of MCHC ($P<0.05$) were observed in the HC group compared to the HM or C group. The percentage of ewes exhibiting echinocytes, polychromatophils, red blood cell (RBC) and platelet (PLT) aggregates was significantly higher ($P<0.05$) in the HC group than in the HM or C group. This study represents the first report documenting blood cell morphological changes and highlights the potential benefits of melatonin supplementation in preserving blood cell integrity under heat-stress conditions.

Keyword: ewes; heat stress; melatonin; morphological changes; PCV

Correspondence author:
L.V. Athanasiou, I. Valasi,
Clinic of Medicine, Faculty of Veterinary Medicine,
University of Thessaly, Trikalon 224, GR-43100 Karditsa, Greece
E-mail address: elkatsog@uth.gr

Date of submission: **18-4-2024**
Date of acceptance: **19-2-2026**

INTRODUCTION

Heat stress poses significant challenges for sheep, especially in hot and humid regions such as the Mediterranean area, which affects their thermoregulatory mechanisms that can be disrupted. Excessive heat exposure triggers a cascade of negative effects on sheep physiology by reducing feed intake, heightening water loss through sweating and panting, increasing respiratory rate, and diminishing reproductive performance, lactation, and lamb growth rates (Marai et al., 2007). Moreover, heat stress is responsible for the elevated reactive oxygen species (ROS) in blood, leading to oxidative stress and immune function dysregulation, which increases susceptibility to diseases (Mutinati et al., 2013).

Furthermore, heat stress induces profound alterations in the hematological system, impacting blood cell composition and function. A study found that prolonged exposure to high temperatures in sheep caused hemolysis, leading to the rupture of red blood cells (RBCs) as their membranes weakened, releasing hemoglobin into the bloodstream (Autukaitė et al., 2020). Consequently, the influence of heat stress can be imprinted on packed cell volume (PCV), hemoglobin (Hgb) and mean corpuscular hemoglobin concentration (MCHC). It has been reported that extreme ambient temperatures also alter RBCs' morphology leading to abnormal shapes (poikilocytes) and increased RBC aggregation strength (Waltz et al., 2014). Changes in white blood cell (WBC) count and granulocyte function, as well as slight variations in platelet (PLT) numbers during heat stroke in rats, further amplify the systemic impact of heat stress on the hematological profile (Iba et al., 2023; Iba et al., 2022).

Melatonin, known primarily for its role in regulating the circadian cycle of sleep, emerges as a potential protective agent against heat-induced cellular damage. Melatonin's antioxidant activity mitigates the harmful effects of reactive oxygen species (ROS) and reduces oxidative stress through direct and indirect mechanisms (Banerjee et al., 2020). In this respect, we have recently proved higher glutathione levels and reduced lipid peroxidation after the administration of melatonin in ewes under heat stress conditions (Bouroutzika et al., 2022). Additionally, melatonin exerts anti-inflammatory properties, which regulate the acute and chronic inflammation processes (Chitimus et al., 2020). The amine seems to optimize mitochondrial function as it is involved in mitochondrial electron transport, playing a key role in the respiratory chain and interfering with proteins that regulate the apoptosis under heat stress conditions in ewes (Bouroutzika et al., 2024).

In a recent study, the antioxidant effect of melatonin implants on hematological and redox status parameters and reproductive performance was evaluated in ewes exposed to high ambient temperatures during the summer period (Bouroutzika et al., 2022). In that study, variables such as PCV, WBC differential count, and PLT counts were measured in the ovine blood smears, as previously described (Katsogiannou et al., 2020). The present study further investigates the impact of heat stress on blood cell morphology in ewes and the potential association between variations in blood cell morphology induced by heat stress, with or without melatonin implants, and the effect of melatonin administration on additional hematological variables. This study aims to provide a comprehensive understanding of the physiological responses of blood cells of ewes to heat stress and the potential protective effects of melatonin supplementation.

MATERIALS AND METHODS

Animals and experimental protocol

All animal procedures regarding animal care and handling were approved by the Institutional Ethical Committee (University of Thessaly, approval number: 123, date of the approval: 10/08/2021).

This research is a complementary investigation to a previously published study (Bouroutzika et al., 2022) performed during the summer period in the region of Thessaly, Greece (21° 55′ 17″ N/ 39° 21′ 50″ E). Seventy-four ewes of Karagkouniko breed participated in the study. The forty ewes were studied during the summer period and the remaining thirty-four in late autumn. During the summer period, the 40 ewes were equally divided into two groups: the melatonin group (HM, n = 20), where melatonin implants [1 implant per ewe; Regulin, Ceva, Lisbourne, France; (Chemineau et al., 1996)] were administered on day 0 (D0) and the control group (HC, n = 20). Over a forty-day period (D0 to D40), all animals experienced heat stress, as was previously demonstrated (Bouroutzika et al., 2022); with the mean Thermal Humidity Index (THI) ranging from 27.5 to 32.7 (Marai et al., 2007). Blood samples were collected on D40 by jugular venipuncture with 18-gauge needles in vacuum tubes containing EDTA. Day 40 was chosen because 40 days after the insertion of implants, melatonin reaches its maxi-

mum concentration in the bloodstream (Chemineau et al., 1996).

The remaining 34 ewes (C group) were blood sampled in late autumn [THI < 22.2 (Marai et al., 2007)]. This group was used as a control group for the comparison with the groups of ewes that underwent heat stress.

Blood samples analysis

The packed cell volume (PCV) value for each blood sample was measured by the microhematocrit method (Bull et al., 2000).

The measurement of hemoglobin (Hgb) concentration in blood samples was performed by the Cyanmethemoglobin (HiCN) method using a commercial kit (Dutch Diagnostics, Zutphen, Holland). Briefly, the blood samples were centrifuged at 1370× g for 10 min at 4 °C to separate the packed erythrocytes from the plasma. Then, the packed erythrocytes were lysed with distilled water (1:1 v/v), inverted vigorously, centrifuged at 4000× g for 15 min at 4 °C, and the erythrocyte lysate was collected. A mixture of 5 μL of erythrocyte lysate and 1 mL of hemoglobin reagent was prepared for each sample, vortexed and incubated in a dark place for 10 min. The hemoglobin concentration was measured using a spectrophotometer (U-1900; Hitachi, Ltd., Tokyo, Japan) at the optical density of 540 nm wavelength.

Finally, the mean corpuscular hemoglobin concentration (MCHC) was calculated by dividing the concentration of Hgb by the PCV value and multiplying it by 100 for each blood sample, as previously determined by Sarma (Sarma, 1990).

Microscopic evaluation of blood smears

Blood smears were prepared immediately after blood collection and stained with Giemsa. Morphological changes in blood cells were evaluated using microscopic examination of stained blood smears (x10 ocular, x100 magnification) (Harvey, 2012; Weiss, 1984).

Statistical Analysis

The data were analyzed using the MedCalc Statistical Software version 14.8.1 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014). Mean values of PCV, Hgb, and MCHC among groups HM, HC, and C were compared by one-way ANOVA. The Shapiro-Wilk test was selected in residuals to test the normal distribution. For post-hoc comparisons, the Tukey-Kramer test was applied. The differences between groups in the percentages of ewes of each group presenting each of the morphological alternations in blood cells were assessed by the Comparison of proportions calculator. A p value of <0.05 was considered significant for all comparisons.

RESULTS

Mean PCV values were significantly higher ($p<0.05$) in group C compared to group HM. Mean Hgb concentration was significantly lower ($p<0.05$) in group HC compared to group C. However, the MCHC concentration was significantly lower ($p<0.05$) in group HC compared to the HM or C group. Values of PCV, Hgb and MCHC are presented in Table 1.

Microscopic evaluation of the blood smears revealed morphological changes in blood cells. More specifically, both echinocytes and polychromatophils were identified in groups HM and HC, with a significantly lower percentage observed in the HM group ($p=0.0013$ and $p=0.0052$, respectively). Likewise, the occurrence of RBC and PLT aggregations in blood smears was significantly lower in the HM group ($p=0.0021$ and $p=0.0036$, respectively) compared to the HC group. Reactive lymphocytes were observed in both groups HM and HC, with no significant difference ($p>0.05$). When comparisons were performed to the C group, a significantly lower percentage was observed in the C group compared to the HC group for all the aforementioned morphological changes (polychromatophils, $p=0.0052$; echinocytes and reactive lymphocytes, $p<0.0001$; RCB aggregation, $p=0.0021$; PLT aggregation, $p=0.0036$). However, when the C group was compared to the HM group, a significantly lower percentage of reactive lymphocytes was observed ($p=0.0002$). The lowest percentages of the observed morphological changes were found in the C group. The results are depicted in Figure 1.

Furthermore, the morphological changes are shown in the following figures (Figure 2-4).

DISCUSSION

To the authors' best knowledge, this is the first study presenting a primary investigation into the morphological changes in ovine blood cells resulting from heat stress following the administration of melatonin. These changes include the presence of polychromatophils, echinocytes, reactive lymphocytes, as well as RBC and PLT aggregations in a high percentage of ewes under heat stress. The

observed alterations in blood cells may be attributed to oxidative stress caused by heat stress. In fact, in a recent study (Bouroutzika et al., 2022), the redox status was meliorated in ewes forty days after the insertion of melatonin implants.

The higher PCV reported in the C group compared to those that underwent heat stress (HM and HC groups) might be season-related, due to the hemoconcentration or as a result of the high demand for oxygen that the body requires in the cold season, the hypoxemia and the high metabolic rate that are usually observed in winter compared to summer (Kuzmenko et al., 2021; Nathawat et al., 2023). In our previous study (Bouroutzika et al., 2022), a decrease in the hematocrit was observed over time as thermal stress developed, both in control and melatonin-treated ewes. In this study, the PCV value and the Hgb concentration were lower in the C group compared to the HM group, but the difference was statistically significant only in the PCV value. However, the hemoglobin concentration was significantly higher in the C group than in the HC group, which is in accordance with a previous study performed in heat-stressed Merino sheep (Srikandakumar et al., 2003). These findings may be attributed to the protective effects of melatonin against red blood cell damage caused by oxidative stress. Indeed, in a study conducted by Banerjee and colleagues (Banerjee et al., 2020), melatonin acted as a protective agent against oxidative stress-induced anemia, leading to increased levels of hemoglobin and the lifespan of RBCs in the bloodstream.

Furthermore, MCHC could be considered a reliable indicator of anemia in sheep, as it is a parameter calculated from Hgb concentration and PCV value and can contribute to the classification of anemia (Katsogiannou et al., 2018). In this study, lower values of MCHC were observed in the HC group compared to the C group, which is consistent with previous findings in heat-stressed Naimey sheep (Alhaidary, 2004). Notably, lower values of MCHC were calculated in the HC group compared to the HM while MCHC did not differ between the C and HM groups. The decrease in MCHC has been previously attributed to the increase of reticulocyte number in the peripheral circulation (Polizopoulou, 2010). In this study, the presence of polychromatophils was evaluated instead of reticulocytes, as polychromatophils are easily observed in routinely stained blood smears while reticulocytes require methylene blue staining for better identification. According to Harvey (2012), the percentage of reticulocytes directly correlates with the percentage of polychromatophils. Therefore, it can be assumed that an increase in polychromatophils indicates a corresponding increase in reticulocytes, which would result in a decreased MCHC. Nevertheless, the presence of polychromasia is indicative of regeneration since neither polychromatophils nor reticulocytes are normally encountered in the blood circulation of adult goats and sheep (Byers and Kramer, 2010). The higher concentration of hemoglobin and MCHC value found in the HM group, compared to the HC group, highlight the beneficial role of melatonin in maintaining the function of RBCs in simultaneously melatonin-treated and heat-stressed ewes, regardless of the reduced PCV.

Erythrocytes are highly sensitive indicators of elevated exposure to ROS. Melatonin concentration is abundant in RBCs and originates either from the bloodstream or *de novo* synthesis in RBCs (Banerjee et al., 2020). Our findings revealed higher levels of polychromatophils in the HC group compared to the HM and C groups. This observation aligns with a previous study in swine, demonstrating a significant rise in reticulocyte count during the initial stages of progressive heat stress exposure compared to thermoneutral conditions (Waltz et al., 2014). Similarly, rats experiencing heatstroke exhibited an increased release of immature RBCs into the bloodstream (Iba et al., 2022). The proportion of reticulocytes in circulation and RBCs deformability are contingent upon environmental conditions, particularly heat stress or heat stroke. These changes can impede smooth blood flow through vessels and hinder efficient oxygen delivery (Waltz et al., 2014). However, melatonin can reverse side effects caused by heat stress in cells and tissues mainly by mediating the release of proteins that regulate cell cycle, proliferation and differentiation (Bouroutzika et al., 2024). This could explain why the HM group showed low levels of polychromatophils in our study and consequently the levels of reticulocytes. Additionally, elevation in core body temperature can induce a transition from normal discocytes to echinocytes, characterised by crenation and subsequent assumption of a smaller spherical shape with rounded protrusions (Rudenko, 2010). A previous study reported a threefold increase in echinocyte percentage as core body temperature rose from 37.1 to 40.0°C (Moore et al., 2013), potentially explaining the presence of echinocytes in the blood samples of the HC group in our study. Specifically,

higher levels of echinocytes were observed in the HC group compared to HM and C groups. No distinction was detected between the HM and C groups, a finding that in the case of the HM group may be attributed to the antioxidant properties of melatonin.

Moreover, melatonin has been proposed to effectively protect the structure and function of impaired RBCs due to oxidative stress. In this respect, administrating melatonin to mice exposed to low-dose bacterial lipopolysaccharide not only reduced oxidative stress indices in blood plasma but also assisted stabilize RBCs membranes (Kurhaluk et al., 2018). Moreover, the increased RBC aggregation that was observed in the HC group is consistent with findings in swine during heat stress exposure compared to thermoneutral conditions (Waltz et al., 2014). The reduced RBCs damage in the HM group may be the result of melatonin's protective effect against oxidative injuries. When oxidative stress occurs, melatonin is actively utilized for cellular defence, delaying hemoglobin denaturation and hemin release (Tesoriere et al., 1999). Melatonin chelates free iron and upregulates antioxidant gene expression in RBCs under stress circumstances, inhibiting ROS generation. Consequently, melatonin is considered an optimal molecule for preserving RBCs' structural and functional integrity (Banerjee et al., 2020).

Regarding the WBCs, the only notable change observed in both groups was an increase in reactive lymphocytes. Reactive leukocytes are considered as B lymphocytes potential sources of immunoglobulins (Weiser, 2012). This finding aligns with a previous study in rats, where heat stroke in conditions of high temperature and humidity led to functional alterations in granulocytes, including the presence of reactive lymphocytes and neutrophils with hyper-segmented nuclei (Iba et al., 2022).

Concerning PLTs, there was an observed increase in PLT clumping in the HC group, consistent with findings from prior studies. These studies, also, reported the simultaneous appearance of large and giant PLTs alongside clot formation (Iba et al., 2023; Iba et al., 2022). Similarly, platelet adhesion, spreading, and fibrin clot retraction were observed in rats under heatstroke (Ke et al., 2024). Hyperthermia directly affects platelet function by altering their shape and leading to their aggregation or by activating heat shock proteins (HSPs), specifically HSP27 and HSP72 (Iba et al., 2023). Additionally, hyperthermia induces ROS production in platelets, which plays a crucial role in platelet activation, apoptosis, and aggregation (Freedman, 2008; Wang et al., 2013). The production of ROS in platelets amplifies their production and consequently enhances platelet activation, adhesion, and recruitment, creating a self-perpetuating cycle contributing to the heightened thrombotic risk associated with oxidative stress-related diseases (Masselli et al., 2020). A decreased number of observed PLT clots in the HM group may be attributed to melatonin's antithrombotic effect, which reduces PLT activation (Chitimus et al., 2020; NaveenKumar et al., 2019). Kornblihtt and co-workers (Kornblihtt et al., 1993) discovered that melatonin, apart from inhibiting cyclooxygenase, is involved in the receptors' activity and/or the signal transduction mechanisms. Another explanation for this effect of melatonin can rely on the findings of proteomics analysis conducted by Bouroutzika et al. (Bouroutzika et al., 2024). They revealed the upregulation of beta-2-glycoprotein 1, a protein that participates in blood coagulation and negatively regulates fibrinolysis, in melatonin-treated pregnant ewes forty days after the administration of melatonin implants. Beta-2-glycoprotein 1 can inhibit platelet aggregation via intricated processes involving multiple components, including ADP activation (Nimpf et al., 1987) and inhibition of von Willebrand factor (Hulstein et al., 2007). Interestingly, melatonin was suggested as a potent regulator of normal platelet function (Pashalieva et al., 2012).

CONCLUSIONS

In conclusion, heat stress impacts the PCV and MCHC values, the aggregation of PLTs and RBCs, as well as the morphology of RBCs and lymphocytes in ewes during the summer period, which can be alleviated by the administration of melatonin. Melatonin seems to protect blood cells from heat stress via redox mechanisms, suggesting a potential treatment for heat stress management in ewes. Additional research in various sheep breeds and seasons will shed light on melatonin-mediated processes.

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