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Research Article

Analysis of Effect of Hyperthermia on White Blood Cells of Experimental Rats Using Geometric Morphometrics Method

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Abstract

An elevated temperature in the environment increases the core body temperature which stimulates thermoregulatory mechanisms. The aim of this study was to examine the effect of elevated temperature on number and shape of leukocytes using descriptive statistics and geometric morphometrics. The study included 32 Wistar rats divided into two groups according to exposed temperature (41°C and 44°C) and more two subgroups in groups associated with death (antemortem and postmortem). Peripheral blood smears were performed before exposure to temperature and after exposure to temperature. The number of leukocytes was determined. Images of peripheral blood smears were taken, on which eight landmarks on the outside of leukocytes were marked in tpsDig program. In the MorphoJ program, we analyzed shape of leukocytes. The results showed significant differences in the number of neutrophils and lymphocytes in subgroup antemortem and in subgroup postmortem exposed to a temperature of 41°C. In group exposed to a temperature of 44°C, a significant difference was found in the number of all leukocytes, except monocytes in the antemortem subgroup, and except basophils and eosinophils in the postmortem subgroup. The results of geometric morphometrics shape analysis showed significant differences in the shape of lymphocytes between subgroups antemortem and postmortem.Exposure to elevated temperature resulted with number and shape changes of leukocytes.

Keywords: hyperthermia, white blood cells, geometric morphometrics, experimental rats

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Introduction

The negative effects of elevated air temperature on the body are numerous and are noticeable in both healthy and sick individuals. The body's internal temperature can reach 40.5°C, which can result in significant damage to all organ systems, and the most severe consequences will occur if the collapse of the central nervous system develops as a result of hyperthermia, which will eventually lead to an imbalance within the entire organism [1].

Precisely for this reason, there was a need to research the effects of hyperthermia on certain cells, organs, and organ systems. These studies can be performed on different cells, but due to the fact that changes in blood cells can be found very quickly after exposure to high temperatures, the hematological parameters play a very important role in the diagnosis and prognosis of the effects of heat stress on the body [2]. The established daily rhythm of body temperature is regulated by a complex thermoregulatory center, localized in the preoptic nucleus of the front part of the hypothalamus [3]. With constant exposure to high temperatures, the organism must invest a certain amount

of energy to activate various adaptation mechanisms in order to prevent its collapse or damage. The activity of these mechanisms depends on the preservation and functionality of the respiratory, circulatory, nervous, and endocrine systems, and on the function of certain body enzymes, which represent very important links that contribute to the complex process of adaptation of the organism to elevated temperatures, with the least possible harmful consequences on the organism itself [4]. However, thermoregulation can only be carried out within certain limits of ecological valence, below that limit hypothermia occurs, and above that limit hyperthermia occurs, i.e. overheating, which acts as a stressogenic factor on the animal's organism [5].

The increased temperature of the environment affects the general condition of the organism, which results in reduced physical activity, reflects on the motor and cognitive functions of the organism, and increases the risk of health problems, which has been proven by numerous scientific studies [6-8]. Taking into account global warming, it is necessary to examine how the increased temperature of the environment is reflected on the organs and organ systems, as well as on the cells of the organism exposed to the increased temperature. It is also necessary to investigate how the organism adapts to conditions of increased environmental temperature.

Disorders of the leukocyte lineage after exposure to heat stress are noticeable in the form of an increase in the number of granulocytes and the ratio of granulocytes to lymphocytes, which is a feature of inflammation [9]. Numerous cellular and physiological effects occur after long-term exposure to high temperature, which are used to improve temperature tolerance, but also to change the innate immunological response. Increased temperature causes activation of phagocytes, granulocytes and infiltration of lymphocytes, and expression of NK cells [10,11].

It has also been proven that heat can lead to disturbances at the molecular level, causing numerous changes in the leukocyte genes of the peripheral blood, which consequently leads to a series of other changes within the organism [12].

In the literature, there are studies where the authors examined the impact of increased environmental temperature on animals. In a study conducted by Rana et al.[13] in 2014, the authors examined the effect of exposure to increased sunlight on indigenous sheep from Bangladesh. The authors monitored how exposure to elevated temperature over a long period of time affects red blood cells (RBC), hemoglobin (Hb%), compacted cell volume (PCV%) and white blood cells (WBC). This research is of great importance considering that sheep are exposed to high sunlight when grazing grass, which has a great impact on the economy in the sheep industry. The results of the study showed that exposure to elevated temperature for a long time resulted in increased values of the number of RBC, Hb% and PCV% which wa statistically significant, while changes in WBC were not statistically significant.

In a study conducted in 2019, Mofizur and the authors examine the impact of increased water temperature on juvenile Red Spotted Grouper *Epinephelus akaara* by analyzing blood elements. The study included 180 tested fish that were exposed to water temperatures of 25°C, 28°C, 31°C and 34°C for 6 weeks. Blood analysis was performed after the second, seventh and forty-second days. An increase in the number of red blood cells and leukocytes was observed in the groups that were exposed to temperature 31°C and 34°C after six weeks, and there were abnormalities in erythrocyte cells. In the group exposed to temperature 34°C, the analysis of number of leukocytes showed an increase in the number of neutrophils and a decrease in the number of lymphocytes. The authors concluded that exposure to elevated water temperature 31°C and 34°C changes the morphology and number of blood cells in red spotted grouper, which may affect their immune system [14].

Exposure to elevated temperature that conditions hyperthermia was used for therapeutic purposes in cancer patients, treated with whole body hyperthermia and chemotherapy, before, during and one day after the treatment. Apoptosis rates of the entire lymphocytes and natural killer cells were determined. During treatment, there was a significant increase in apoptosis in the entire lymphocytes. In contrast, an increased rate of apoptosis in natural killer cells was observed 20 hours after the finished therapy [15]. Two treatment whole body hyperthermia protocols are most commonly used: long-term exposure to slightly elevated temperature and short-term exposure to very elevated temperature [16].

It was established that the number of blood cells cannot be a realistic indicator of the true effect of heat, and that morphological changes in blood cells should also be taken into account in order to reach a conclusion about the real effects of heat. The number of blood elements may be within the reference values, but their function and form are completely incorrect and ineffective [17].

The aim of this study was to examine the effect of elevated temperature on number and shape of leukocytes on experimental rats using descriptive statistics and geometric morphometrics method.

Material and Methods

Ethical Statement

The study was conducted at the Faculty of Veterinary Medicine of the University of Sarajevo, by valid ethical principles on biomedical research on animals and after obtaining approval from the Ethical Committee at the Faculty of Veterinary Medicine of the University of Sarajevo (number: 07-03-850-4/22).

In the study was included 32 albino Wistar rats of both sexes, six months old and weighing from 250 to 300 g. All animals were kept under the same laboratory conditions and for seven days before the experiment they were kept in a vivarium with a 12-h light regime day-night and at room temperature (20°C±2°C) for the purpose of acclimatization and adaptation. During the duration of the experiment, the animals received commercial feed for laboratory animals and running water ad libitum. Keeping and caring for animals, as well as conducting all experimental procedures, were carried out in compliance with international guidelines for biomedical research on animals - CIOMS (The Council for International Organizations of Medical Sciences) and ICLAS (The International Council for Laboratory Animal Science).

The state of hyperthermia was achieved by immersing the rats in water of temperature 41°C and 44°C. Before the experiment, the rats were anesthetized with 10 mg/kg xylazine HCl (Rompun® 2%, Bayer) and 50 mg/kg ketamine HCl (Ketaminol® 100 mg/mL, MSD Animal Health), by intramuscular injection into the thigh muscle (m. quadriceps). We filled the water bath with water and heated it to the target water temperature. The temperature of the water was continuously monitored on the display with an additional measurement with a probe immersed in the water and a reading on the thermometer. We immersed a previously anesthetized rat with its head above the water level in pre-heated water of the target temperature. The survival time was recorded, which included the time from the immersion of the rats in the water of the given temperature (41°C and 44°C) until the moment when death was established. We defined hyperthermia as an increase of 0.5°C in internal temperature, and heat stroke as an increase in internal temperature above 40.5°C.

Experimental rats was divided into two groups according to exposed temperature (41°C and 44°C) which were further divided into two subgroups (antemortem and postmortem). Before exposure to temperature, peripheral blood smears were performed on each experimental rat so that the animals themselves were the control.

Immediately before immersion in water, an esophageal probe for measuring the internal temperature (RET-4 Probe for mice and rats) was placed in the esophagus (5 cm) of an anesthetized rat, and the core temperature was continuously read on a thermometer (Physitemp Thermalert Model TH-8) and recorded before immersion, immediately after immersion, at the 20th min, and at the moment of death. The thermometer and probe for temperature measurement is manufactured by Physitemp, Instruments Clifton, USA.

Blood for obtaining serum was sampled on two occasions: 7 days before the planned experiment from the tail vein of anesthetized rats (control group) and another blood sampling from the abdominal aorta during the duration of the experiment. Peripheral blood smear with standard staining (Giemsa) was performed. On peripheral blood smears for each experimental rat in subgroups the number of leukocytes (neutrophils, lymphocytes, basophils, monocytes, eosinophils) was determined for analysis of changes of the numbers of leukocytes. For analysis of changes of morphology of leukocytes, images of leukocytes on peripheral blood smears were taken. On twodimensional models of leukocytes eight landmarks on the outside were marked in tpsDig program. Position of the landmarks is defined by values of x and y axis in coordinate system. This values for all landmarks and for all leukocytes we entered in the MorphoJ program where we analyzed shape of leukocytes and their difference between groups.

Statistical Analysis

All statistical analyses were performed using SPSS version 13.0 for Windows (Chicago, IL, USA).

The distribution of quantitative variables was tested using the Kolmogorov-Smirnov test. Results of descriptive statistics for continuous variables were expressed as mean and standard deviation (SD) for normally distributed variables or as median and interquartile range for variables with skewed distributions.

The categorical variables were reported as frequencies and percentages. An independent two-sample Student t-test assessed the significance of the mean differences between the two groups. The Mann-Whitney U-test assessed the difference in the values of parameters that showed a non-normal distribution. The differences between categorical variables were assessed using the Chi-square test. P values less than 0.05 were considered statistically significant.

For shape analysis of leukocytes we used geometric morfpmetrics method. On two-dimensional models of leukocytes we marked 8 landmarks on each leukocytes cells using tpsDig program. In MorphoJ program we analysed differences on the shape of leukocytes between experimental groups using Principal Component Analysis (PCA) and Discriminant Functional Analysis (DFA).

Table 1. *Mean values of the number of leukocytes in the control group and in the antemortem group of rats exposed to temperature 41°C*

Type of Leucocytes	Basal Values in Control Group Antemortem-41 (n=6)	Antemortem (n=6)	P
Neutrophils x 10^9/L	19.7±8.2	6.3±4.6	0.004
Lymphocytes x 10^9/L	73.3±8.4	86.0±6.6	0.009
Basophils x 10^9/L	1.17±2.4	1.3±3.3	0.931
Monocytes x 10^9/L	1.17±1.8	1.67±1.5	0.681
Eosinophils x 10^9/L	4.67±2.9	4.67±2.3	1.0

Table 2. *Mean values of the number of leukocytes in the control group and in the postmortem group of rats exposed to temperature 41°C*

Type of Leucocytes	Basal Values in Control Group Postmortem-41 (n=7)	Postmortem (n=7)	P
Neutrophils x 10^9/L	18.0±5.4	7.0±6.9	0.022
Lymphocytes x 10^9/L	76.0±5.9	81.3±6.4	0.183
Basophils x 10^9/L	0.29±0.49	1.0±1.53	0.220
Monocytes x 10^9/L	1.7±1.5	7.1±4.0	0.011
Eosinophils x 10^9/L	4.0±1.5	3.6±1.6	0.667

Table 3. *Mean values of the number of leukocytes in the control group and in the antemortem group of rats exposed to temperature 44°C*

Type of Leucocytes	Basal Values in Control Group Antemortem-44 (n=6)	Antemortem (n=6)	P
Neutrophils x 10^9/L	18.3±2.7	3.8±4.2	< 0.001
Lymphocytes x 10^9/L	73.4±4.4	93.7±5.7	< 0.001
Basophils x 10^9/L	0.67±0.52	0.0±0.0	0.025
Monocytes x 10^9/L	3.5±2.6	1.0±1.7	0.161
Eosinophils x 10^9/L	4.17±1.3	1.5±1.5	0.01

Results

The number of neutrophils decreased statistically significantly, and the number of lymphocytes increased statistically significantly in the group of rats exposed to temperature 41°C- antemortem *(Table 1)*.

The number of neutrophils decreased statistically significantly and the number of monocytes increased statistically significantly in the postmortem group of rats exposed to a temperature of 41°C *(Table 2)*.

The number of neutrophils, basophils, and eosinophils decreased statistically significantly and the number of lymphocytes increased statistically significantly in the antemortem group of rats exposed to a temperature of 44°C *(Table 3)*.

The number of neutrophils and eosinophils decreased statistically significantly and number of lymphocytes increased statistically significantly in the postmortem group of rats exposed to temperature of 44°C *(Table 4)*.

Table 4. *Mean values of the number of leukocytes in the control group and in the postmortem group of rats exposed to temperature 44°C*

Type of Leucocytes	Basal Values in Control Group Postmortem- 44 (n=7)	Postmortem (n=7)	P
Neutrophils x 10^9/L	15.6±1.99	3.6±2.8	< 0.001
Lymphocytes x 10^9/L	78.1±1.6	94.3±3.99	< 0.001
Basophils x 10^9/L	0.79±0.91	0.0±0.0	0.062
Monocytes x 10^9/L	2.1±1.6	1.4±1.6	0.526
Eosinophils x 10^9/L	3.4±1.3	1.0±1.4	0.001

Table 5. *Results of correct classification test of lymphocytes of rats using geometric morphometrics for external surface shape analysis*

True	Allocated to Lymphocytes-Antemortem	Allocated to Lymphocytes-Postmortem	Total Number
Number of lymphocytes-antemortem	**33**	16	49
Number of lymphocytes-postmortem	18	**39**	57

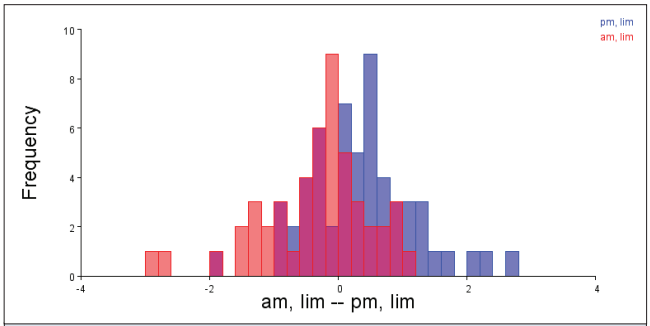

Fig 1. Results of discriminant functional analysis of the shape of lymphocytes between antemortem and postmortem groups of rats

Geometric morphometrics method is used for analysis of shape of leukocytes where provided general Procrustes analysis, test of correct classification and discriminant functional analysis.

Test of correct classification based on the shape of external surface of lymphocytes using geometric morphometrics correct classified 33 of 49 lymphocytes in the group antemortem (67.35% accuracy), and 39 of 57 lymphocytes in group of postmortem (68.42% accuracy). Results of test of correct classification showed in *Table 5*. These differences are statistically significant, P value was 0.0104.

Fig. 1 showed the results of discriminant functional analysis of external surface shape analysis of lymphocytes in rats exposed to temperature.

Discussion

After the target organism is exposed to long-term hyperthermia, a whole series of negative effects arise in its immune system. Long-term exposure to hyperthermia changes the immune response of organs and tissues, leading to the activation of neutrophils at the site of inflammation, infection or injury caused by elevated temperature [18].

The present study showed a statistically significant decrease in the number of neutrophils in the antemortem and postmortem groups after exposure to temperatures of 41 and 44 degrees, while in both groups there was an increase in the number of lymphocytes. Since neutrophils have the shortest lifespan of all leukocytes, it is believed that this could be the reason for the statistically significant decrease in their number, which was confirmed by the results of our study. The lifespan of neutrophils in the blood lasts an average of 8 hours, while in the tissues they can last from 1 to 4 days [19]. Exposure of neutrophils to high temperatures leads to the creation of mediators that accelerate their apoptosis, which results in a decrease in their number. There are many such mediators; among them, cytokines, chemokines, and some conditions such as acidosis and hyperthermia stand out [20]. Raheim et al.[21] stated that such changes in neutrophils may be the result of changes in the action of the Na/K pump, which results in a change in intracellular osmolarity, but it is not excluded that hyperthermia may lead to direct mechanical damage to the cell membrane.

Although a statistically significant change in the number of neutrophils was observed, no statistically significant difference in the shape of neutrophils was found using geometric morphometrics. The reason for this may be because hyperthermia led to the destruction of a larger number of neutrophils, which ultimately led to a low absolute number of relatively preserved neutrophils on which shape could be assessed by geometric morphometrics. In a pilot study on rats, Iba et al.[22] neutrophils were presented with a hypersegmented, clustered nucleus, but no changes in the shape of the outer surface of neutrophils were observed. In our study, in the geometric morphometrics program, we marked the landmark points on the outer surface of the cell membrane and not on the nuclei. Unlike neutrophils, lymphocytes showed statistically significant shape changes using geometric morphometrics. Exposure of rats to hyperthermia consequently led to an increase in the number of lymphocytes, which allowed us to initially have a higher absolute number of lymphocytes that we could subject to geometric morphometrics analysis.

In our study, eosinophils were more sensitive and showed a significant decrease in number compared to basophils, after exposure to a temperature of 44°C. A study by Edwards et al.[23] on pigs showed a special sensitivity of eosinophils to an increase in temperature, in which necrobiotic changes and extreme lobulation of the nucleus occurred after exposure to hyperthermia for one h. Such changes undoubtedly reduce their number in the peripheral blood smear, which agrees with our results. Salanova et al.[24] state that exposure to heat also affects the internal processes of neutrophils, interfering with signal transmission within the cell itself. This type of destabilization of cellular processes can occur even during short-term exposure to heat stress.

In the blood of rats under physiological conditions, the number of eosinophils, basophils and monocytes is low. Considering this, it is very difficult to determine the effect of elevated temperature on these cells. In our study, too, we only found a decrease in the number of eosinophils in two groups of rats after exposure to a temperature of 44°C, while an increase in the number of monocytes was recorded only in one group [25]. Our results correlate with the results of the study by Mahmutovic and associates, where it was found that eosinophils did not show statistically significant variations during the test, which is expected, according to their role in the body [26].

In vitro studies showed that an increase in temperature leads to increased production of lymphocytes and antibodies [27]. In our study, there was also a significant increase in the number of lymphocytes in all tested groups, regardless of the exposed temperature. An exception is the postmortem group after exposure to a temperature of 41°C. The results of the previous research showed a significant decrease in the number of T lymphocytes in organisms exposed to hyperthermia in a standardized hyperthermic spa with a mean increase in rectal temperature of 1.35°C, but without a significant change in the total number of lymphocytes [28].

In our study, we did not classify lymphocytes into B or T lymphocytes. Therefore, the unchanged number of lymphocytes in one group may be the reason for the missing additional classification. Also, Ashman et al.[29] state that blood T-lymphocytes incubated at 39°C show an increased response to the stimulatory effect of IL1 and IL2 on their proliferation, compared to those incubated at a temperature of 37°C. The same response to stimulation by the mentioned interleukins in B-lymphocytes was not recorded. In this study, it is stated that hyperthermia predominantly affects the proliferation of T-lymphocytes. Using the ELISPOT technique, Huang et al.[30] found that hyperthermia has a stimulating effect on B-lymphocytes, leading to increased production of IgG, IgA and IgM antibodies. This effect on B-lymphocytes refers to the effect of temperatures up to 42°C, because temperatures above 42°C oposite effect. Exposure to heat stress leads to an increase in the number of granulocytes and to an

increased ratio of granulocytes to lymphocytes, which is characteristic of inflammation [6].

An increased number of monocytes was found only in the postmortem group of rats exposed to a temperature of 41°C, without statistically significant changes in the shape of monocytes. Previous studies have shown that monocytes are most often attached to platelets, which indicates their interaction during heat stress [30]. This can ultimately explain such a small number of observed monocytes in our study has an inhibitory effect. Bouchama et al.[31] stated that hyperthermia is related to an increase in the number of total leukocytes as well as an increase of the percentage of its subpopulation. This increase in the number of leukocytes is mostly due to the increase in the number of suppressor T cells and natural killer cells. The study confirmed a significant correlation of the total number of circulating lymphocytes with the severity of hyperthermia.

When interpreting the results, it should be kept in mind that rats are much more sensitive to the effects of high temperature and develop a shock state much faster with a fatal outcome. This is very important to keep in mind when presenting and comparing the results of the study on the human heat stroke model. It is also important to note that lymphocytes are the most numerous type of leukocytes in this type of animal, and regardless of the fact that they are the smallest type of leukocytes, their importance in the body's defense against various pathogens is immeasurable.

In our study, rats were briefly exposed to hyperthermia. As a consequence of the organism's stress reaction, there was an increase in the number of lymphocytes.

Also, the results of our study showed a changed shape of lymphocytes between subgroups antemortem and postmortem *(Fig. 1)*, which indicates that altered lymphocytes may have altered function. The result is a changed reaction of the immune system in organisms exposed to elevated temperature.

Declarations

Availability of Data and Materials: The datasets generated during the current study are available from the corresponding author (Z. Ajanović) on reasonable request.

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Conflict of Interest: The authors declared that there is no conflict of interest.

Author Contributions: ZA, ED and LD conceived and designed the study. ED, MK and ZA executed the experiment and analyzed the serum and tissue samples. AD and ZA analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

Ethical Approval: The study was conducted at the Faculty of Veterinary Medicine of the University of Sarajevo, by valid ethical principles on biomedical research on animals and after obtaining approval from the Ethical Committee at the Faculty of Veterinary Medicine of the University of Sarajevo (Number: 07-03-850-4/22).

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