=========== **EXPERIMENTAL PAPERS** ===========

Indicators of Hypoxia Tolerance as Determined by Cellular Elements of Rat Blood

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Abstract—Although hypoxia tolerance is mainly determined genetically, it is important to study individual variability of animal organisms in order to identify the factors that underlie their tolerance to hypoxic exposure. We investigated blood cell counts and coagulograms in Wistar rats as predictors allowing the animal population to be split into hypoxia-tolerant and hypoxia-intolerant individuals. The validity of the specific predictors' choice was proved by a coincidence between the population split in accordance with the detected individual parameters and the results of testing animals in a decompression chamber at a rarefaction corresponding to the "rise to an altitude" of 11500 m above sea level. Circulating blood cells were quantitatively assessed by eighteen indicators before and after hypoxic exposure. The differences between animals low-tolerant (LT), high-tolerant (HT), and medium-tolerant (MT) to hypoxia were determined by five indicators: white blood cell count (WBC), granulocyte count (Gran#), red blood cell count (RBC), reticulocyte count/percent (RTC), and mean corpuscular hemoglobin (MCH). The RBC, RTC, and MCH values in HT rats were significantly higher than in LT animals (by 1.4, 1.9, and 1.1 times, respectively). The WBC and Gran# values in HT rats were lower than in LT individuals. The hypoxia tolerance indices (HTI) were calculated using the original formula. It was established that in LT rats, the HTI ≤ 0.203, in HT rats ≥ 0.335, and in MT rats < 0.335 but > 0.203. After testing in a decompression chamber, the activated partial thromboplastin time (APTT), thrombin time (TT), and prothrombin time (PT) decreased, but the fibrinogen level increased. LT rats were characterized by the lowest APTT, TT, and PT values and the highest values of the fibrinogen level. Our results indicate that one of the most important mechanisms underlying a high hypoxia tolerance in rats consists in sustaining reciprocal relationships between the complex of RBC indicators, which tend to increase under hypoxia, and Gran# indicators, which tend to decrease after hypoxic exposure.

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INTRODUCTION

It has long been known that virtually all diseases, no matter infectious or noninfectious, as well as in extreme stressful conditions, cause pathological endogenous hypoxia (low oxygen

content) [1, 2]. Specifically, hypoxia is one of the pathogenetic mechanisms, as well as a factor, that determines the resistance to standard methods of cancer therapy [3]. With a decrease, for whatever reason, in the efficiency of tissue oxygen supply, there are microvascular dysregulation, extravasation and activation of white blood cells (WBCs) in the zone of hypoxic microenvironment. There are many types of immune and pro-inflammatory resident cells, infiltrating this zone in a state of activation, which produce significant amounts of reactive oxygen species leading to oxidative stress, oxidative damage and mitochondrial dysfunction in most of the surrounding cells. The majority of the cells infiltrating the hypoxic zone is represented by activated neutrophils that release procoagulant bioactive substances into the extracellular space [4]. Generally, all processes caused by hypoxia, oxidative stress and mitochondrial dysfunction provoke the secretion of signaling molecules that additionally stimulate the inflammatory response [5, 6]. This situation is often accompanied by a damage to the endothelial glycocalyx, which performs the anticoagulant function. This results in a release of such procoagulant endothelial products as endothelin-1, superoxide anions, and thromboxane A2, as well as a decrease in the bioavailability of nitric oxide (NO) [6, 7]. Consolidation of the prothrombotic effects of neutrophils and endothelium leads to the formation of a "vicious circle" of the processes that enhance dysfunction of the anticoagulant system, causing the dominance of the blood coagulation system and the possibility of transition of a local inflammatory reaction to a systemic level [4].

The individual threshold of sensitivity to oxygen deficiency, directly related to genetic factors, largely determines the duration and severity of diseases [8, 9]. The study of the relationship between the tolerance to hypoxia and the peculiarities of biochemical, cellular, organ and phenotypic properties of the animal organism, aimed at extrapolating the obtained information to humans, is important for solving the senescence problems, as well as increasing the tolerance to hypoxia in various diseases and the working capacity of people engaged in professions associated with large psychophysiological and environmental loads. This is the most common cause of anthropogenic impacts associated with air pollution, one of the effects of which is a reduction in the oxygen concentration.

At present, the priority trend in experimental biology and medicine is searching for biomarkers of animal tolerance and receptivity to hypoxia in order to determine therapeutic targets for the modeling of various human diseases and implementation of an individual approach to treatment. In most cases, it becomes necessary to split the population of laboratory animals into low-tolerant (LT), medium-tolerant (MT), and high-tolerant (HT) to hypoxia before starting the experiment. Most often, researchers work with groups of animals that differ sharply in all parameters, i.e. with LT and HT. Currently, it is known that LT individuals are characterized by uneconomical consumption of oxygen—they consume a lot more oxygen per unit mass of tissue per unit time than HT individuals, whereas HT animals have developed the mechanisms for more effective adaptation to hypoxia, enabling them to withstand prolonged exposure to oxygen deficiency [10]. To divide the population of laboratory animals, they are tested individually in a decompression chamber, where conditions of acute hypoxia are created. Several types of hypoxia are reproduced in biomedical research, with the imitation of exogenous hypobaric hypoxia being the most commonly used [11]. This situation is artificially reproduced by a controlled pumping air out of a decompression chamber, thus a decrease in the environmental oxygen content leads to a decrease in the oxygen tension (pO_2) in the alveoli and arterial blood of the animal. Secondary tissue hypoxia develops in animals as a result of a decrease in the oxygen tension in blood and tissues to levels below critical, at which the rate of oxygen utilization (consumption) in tissues begins to go down. If the strength and/or duration of hypoxic exposure exceed the adaptive capabilities of the body, then irreversible changes will appear in organs and tissues and the animal will die.

Due to the fact that testing in a decompression chamber can damage the central nervous system and even cause death of animals, it is necessary a longer (at least 10-month) recovery period for the survived individuals to be involved in further

experiments. The topical task is to reveal the relationship between certain physiological indicators in the pre-hypoxic period and the level of hypoxia tolerance [9, 12]. Any hypoxic situation induces a complex of responses, which involves all the functional systems of the organism. The main and most well-known factor mediating this response is the HIF-1 transcription complex (HIF-1α and HIF-1β subunits) produced by most cells in response to oxygen deficiency. HIF-1β is a constitutively expressed subunit, whereas HIF-1α is an oxygen-regulated subunit [13]. It has been shown that the level of HIF-1α expression in human and animal WBCs varies, which indicates phenotypic differences in its regulation [14, 15]. However, HIF-1α is difficult to use as a predictor of hypoxia tolerance due to significant changes in its level depending on many factors. Since the contribution of HIF-1α to the pathogenesis of any diseases, as a rule, constantly changes, the issue of targeted pharmacological impacts on HIF-1α for the addressed regulation of the processes of urgent and long-term adaptation to hypoxia in animals and humans is quite ambiguous [16]. The same is true for many neuroimmunoendocrine indicators.

Undoubtedly, blood appears the most attractive object of studies aimed at identifying predictors of hypoxia, since it is the main conduit for transporting oxygen from the lungs to tissues and carrying carbon dioxide in the opposite direction. Under any environmental challenges, including hypoxia, erythroid cells continue to perform their specific functions, while changing their numbers, size, oxygen content, etc. Under hypoxic conditions, WBCs, in small laboratory rodents mainly represented by neutrophils, are activated. It was determined, that one of the activators is HIF-1α, which plays a crucial role in the regulation of cellular responses to hypoxia. Activated WBCs can affect coagulation directly by producing procoagulant and anticoagulant molecules, and/or indirectly by affecting platelets and endothelial cells. The appearance of a large number of activated WBCs can slow down the movement of red blood cells (RBCs), become a direct cause of microvessel occlusion, and reduce the efficacy of oxygen transportation by blood, thus provoking hypoxia in the microcirculation [17, 18].

We assumed that the analysis of blood cell parameters in the pre-hypoxic period would allow the identification of predictors of the tolerance to acute hypoxic hypoxia. Therefore, the objective of this work was to study the cellular composition and coagulation system of the peripheral blood in Wistar rats before and after testing in a decompression chamber, and then to distinguish a number of indicators as predictors allowing the rat population to be split on the basis of hypoxia tolerance.

MATERIALS AND METHODS

The study was carried out on 40 sexually mature male Wistar rats obtained from the "Stolbovaya" (a branch of the Scientific Center of Biomedical Technologies of the Federal Medical and Biological Agency of Russia) with a body weight of 180–200 g. Animals that had been quarantined for at least 14 days were kept in standard vivarium conditions (randomly allocated by 10 individuals per cage) under natural lighting at a temperature of 20–22°C, with ad libitum access to water and pelleted food (GOST 34566-2019). All experimental procedures were carried out in compliance with the Directive of the European Parliament 2010/63/EU "On the protection of Animals used for Experimental Purposes" (dated 22.09.2010). The study was approved the Bioethics Committee of the A.P. Avtsyn Research Institute of Human Morphology.

The personalized hypoxia tolerance of animals was determined by modeling acute hypoxic hypoxia in a decompression chamber. To achieve the similarity of recording conditions, testing was carried out in the morning (9–11 a.m.), considering the phase of infradian biorhythms, i.e. multiday (by to our data, 4-day), periodically recurring fluctuations in the intensity of many parameters in animals and humans [9, 19]. Namely, testing was performed between acrophase and bathyphase. i.e. the highest and lowest values of corticosterone levels, locomotor activity, etc.

Hypoxia tolerance was determined by measuring the time taken for the onset of gasping (gasping time). Rats were exposed, one at a time, to simulated hypobaric hypoxia caused by a rarefaction equivalent to the "rise to an altitude" of 11500 m above sea level, in a decompression

chamber coupled to a mercury barometer (equivalent to 180 mm Hg). All the decompression and recompression instances were achieved gradually at a rate of 80 m/s to prevent any tissue injury due to a sudden fall or rise in the ambient pressure. Rats that had an impaired postural reflex "at an altitude" for less than 3 min were considered low-tolerant (LT), more than 9 minutes—high-tolerant (HT), more than 3 minutes, but less than 9 minutes —medium-tolerant (MT) [20].

Peripheral blood was collected from the caudal vein under zoletil anesthesia (5 mg/100 g, Virbac Santй Animale, France) into test tubes with EDTA as an anticoagulant, a day before the simulation of acute hypoxic hypoxia and 5−10 min thereafter. The blood count was carried out for to 18 parameters, using a Mindray BC-2800 Vet Automatic Hematology Analyzer (China) with Rat software (WBC, Lymph#, Mon#, Gran#, Lymph%, Mon%, Gran%, RBC, HGB, HCT, MCV, MCH, MCHC, RDW, PLT, MPV, PDV, RTC). Parameters of hemostasis were determined by using a KC4 Delta Semi Automated Hemostasis Analyzer (Tcoag, Ireland). The serum corticosterone concentration was determined by Enzyme-Linked Immunosorbent Assay (ELISA, IBL, Germany).

Statistical data analysis was carried out using Statistica 8.0. Normal distribution was checked by the Shapiro−Wilk test. It was found that the empirical distribution of our data is different from the normal. For statistical processing, nonparametric methods for paired samples was used, the Mann−Whitney U-test and the Wilcoxon−Mann−Whitney t-test. The results were expressed as the median and interquartile range Me (25−75%). The differences were considered significant at $p < 0.05$.

RESULTS

The analysis of all blood parameters with categorization of blood cells into drement in blood indicators vs. the mean in a given group, bac—mean background value of blood indicators in a given group, RBC—absolute number of red blood cells, MCH—mean corpuscular hemoglobin level in the red blood cell, RTC—percent of reticulocytes vs. the total number of red blood cells, WBC—absolute number of white blood cells, Gran#—absolute number of granulocytes.

It was found that normal hypoxia tolerance indices are characterized by the values ≤ 0.203 (LT rats), ≥ 0.335 (HT rats), and < 0.335 but > 0.203 (MT rats).

The major evidence that such indicators as the absolute number of WBCs, granulocytes and RBCs, as well as the percent of reticulocytes vs. the total number of red blood cells, and mean corpuscular hemoglobin in red blood cells can be predictors of hypoxia tolerance in rats was obtained when testing animals in a decompression chamber. It was found that "at an altitude" LT rats had impaired postural reflex for less than 3 min. In almost all cases, LT animals began rushing about the decompression chamber, not reaching the maximum "altitude" of 11500 m; some of them showed signs of seizures; 3 rats died after testing. MT animals endured a maximum "altitude" for no longer than 9 min. The behavior of these rats was characterized by anxiety and vigorous running at the onset of reaching a maximum "altitude", calming down after 4−5 minutes. HT individuals were distinguished by a pronounced calmness and the ability to stay at an "altitude" of 11500 m for more than 9 min. Of the population of 40 rats, 30% turned out to be HT, 40% LT, and 30% MT. It should be emphasized that the individual values of the hypoxia tolerance index (HTI) in HT, MT, and LT rats, determined during testing in a decompression chamber, coincided in their HTI values obtained before testing.

Such cellular components of blood as WBCs, specifically granulocytes, make a significant contribution to hemostasis, which has a strong influence on oxygen transport and utilization in tissues and, accordingly, on hypoxia tolerance [22−24]. In this regard, we studied hemostasis in rats before and after testing in a decompression chamber.

It was found that values of hemostasis' indicators of naive HT, LT and MT rats did not have statistically significant differences, whereas after testifferent subpopulations allowed us to reveal statistically significant differences between rats low-tolerant (LT), high-tolerant (HT) and medium-tolerant (MT) to hypoxia only in five parameters: white blood cell counts (WBC), granulocyte counts (Gran#), red blood cell counts

Table 1. Hematological indicators of rats high-tolerant (HT) and low-tolerant (LT) to hypoxia before and after testing in a decompression chamber (DpCh), Me (25−75%)

Phenotypes	Condition	Absolute number of red blood cells (RBC) $\times10^{12}$/L	Reticulocyte (RTC), % of RBC	Mean corpuscular haemoglobin in RBC (MCH), pg	Absolute number of white blood cells (WBC) $\times10^9$/L	Absolute number of granulocytes (Gran#) $\times10^9$/L
HT (1)	before DpCh (3)	9.2 (4.7−11.7) $p1{-}2 < 0.001$ $p3{-}4 < 0.001$	3.2 (1.8−4.4) $p1{-}2 < 0.001$ $p3{-}4 < 0.001$	20.1 (19.7−21.2) $p1{-}2 < 0.001$ $p3{-}4 < 0.001$	8.8 (4.3−12.9) $p1{-}2 < 0.001$ $p3{-}4 < 0.001$	2.9 (2.3−3.6) $p1{-}2 < 0.001$ $p3{-}4 > 0.05$
	after DpCh (4)	12.3 (9.2−13.9) $p1{-}2 < 0.001$	9.2 (5.1−12.6) $p1{-}2 < 0.001$	22.1 (21.8−31.1) $p1{-}2 < 0.001$	13.1 (8.8−16.6) $p1{-}2 < 0.001$	5.1 (3.3−6.1) $p1{-}2 < 0.001$
LT (2)	before DpCh (5)	6.4 (3.9−9.3) $p5{-}6 < 0.001$	1.7 (0.8−2.6) $p5{-}6 < 0.001$	19.1 (18.5−19.7) $p5{-}6 > 0.05$	19.2 (11.9−29.8) $p5{-}6 < 0.001$	3.7 (3.8−4.6) $p5{-}6 < 0.001$
	after DpCh (6)	8.5 (5.9−11.9)	4.8 (1.6−5.2)	19.9 (18.9−21.5)	36.6 (22.9−54.7)	7.4 (5.9−8.9)

(RBC), % reticulocyte (RTC) and mean corpuscular hemoglobin (MCH). The RBC, RTC and MCH values were significantly higher in HT rats than in LT animals (by 1.4, 1.9 and 1.1 times, respectively) both before and after testing in a decompression chamber, as can be seen in Table 1. The Gran# and WBC indicators in HT individuals were much lower than in LT rats under the same conditions (Table 1). For instance, while in HT individuals the normal WBC was 2.2 times and the Gran# 1.3 times lower, after testing in a decompression chamber, the differences were 2.8 and 1.5 times, respectively (Table 1). Overall, immediately after testing

in a decompression chamber, all blood cell parameters were found to increase in all animals (Table 1). The values of the same indicators were higher in MT compared to LT rats, but lower than the HT individuals. Since many hematological parameters of MT rats were statistically indistinguishable from those in LT and HT animals, their values are not shown Tables 1, 2.

Since the amplitudes and units of measurement for blood parameters showed a great dispersion [21], we developed a formula to calculate the hypoxia tolerance index (HTI) based on the formula for the integrative assessment of functional reserves of an organism:

$$HTI = 1/ \sqrt{\left(\frac{\Delta RBC}{RBC_{bac}}\right)^2 + \left(\frac{\Delta MCH}{MCH_{bac}}\right)^2 + \left(\frac{\Delta RTC}{RTC_{bac}}\right)^2 + \left(\frac{\Delta WBC}{WBC_{bac}}\right)^2 + \left(\frac{\Delta Gran\#}{Gran\#_{bac}}\right)^2},$$

where Δ—incing in a decompression chamber, the APTT, TT and PT values decreased, and the levels of fibrinogen increased (Table 2). It was shown that LT animals were characterized by the lowest values of APTT, TT and PT and the highest levels of fibrinogen (Table 2).

Serum corticosterone levels did not differ significantly between animals with different hypoxia tolerance before and after testing in a decompression chamber (Table 2). The corticosterone content increased significantly after the hypoxia, as in any case of acute stress (Table 2) [25].

Table 2. Indicators of hemostasis and corticosterone levels in rats high-tolerant (HT) and low-tolerant (LT) to hypoxia before and after testing in a decompression chamber (DpCh), Me (25–75%)

Phenotypes	Condition	APTT, s	TT, s	PT, s	Fibrinogen level, g/L	Corticosterone level, ng/mL
HT (1)	before DpCh (3)	17.3	38.4	18.6	2.3	255.6
		(15.9–18.3)	(32.9–44.1)	(17.9–19.2)	(2.1–2.4)	(226.2–286.7)
		$p1–2 > 0.05$	$p1–2 > 0.05$	$p1–2 > 0.05$	$p1–2 > 0.05$	$p1–2 > 0.05$
		$p3–4 > 0.05$	$p3–4 > 0.05$	$p3–4 > 0.05$	$p3–4 < 0.001$	$p3–4 < 0.001$
	after DpCh (4)	16.4	34.6	18.3	2.5	425.3
		(12.7–17.5)	(33.3–37.1)	(17.6–19.3)	(2.2–2.7)	(361.4–489.2)
		$p1–2 < 0.001$	$p1–2 < 0.001$	$p1–2 < 0.001$	$p1–2 < 0.001$	$p1–2 > 0.05$
LT (2)	before DpCh (5)	15.6	36.7	17.9	2.3	250.3
		(11.5–18.4)	(31.8–40.9)	(16.5–19.1)	(2.1–2.7)	(227.5–267.9)
		$p5–6 < 0.001$	$p5–6 < 0.001$	$p5–6 < 0.001$	$p5–6 < 0.001$	$p5–6 < 0.001$
	after DpCh (6)	10.3	21.8	14.7	3.7	436.4
		(8.5–12.1)	(19.8–24.4)	(13.7–16.1)	(3.4–4.1)	(335.1–531.6)

DISCUSSION

In this study, it was found that such indicators of peripheral blood of Wistar rats as the white blood cell count (WBC), granulocyte count (Gran#), red blood cell count (RBC), reticulocyte count/percent (RTC) and mean corpuscular hemoglobin (MCH) can be used as predictors of hypoxia tolerance. The priority information obtained in the present study is that one of the most important mechanisms that underlie high hypoxia tolerance consists in sustaining reciprocal relationships between the specified set of indicators of the erythroid hemopoietic branch, which tend to increase, and those of the granulocytic branch, which tend to decrease under hypoxia. At the same time, compared to LT, HT animals are characterized by significantly higher values of the RBC, RTC and MCH, as well as by lower values of WBCs and Gran# in naive rats. The same ratio of all these cellular elements remains after testing rats in a decompression chamber. Our data echo the results by L.A. Gridin, who found that hypoxia, as a specific stimulator of erythropoiesis, activates the mechanisms that lead to a compensatory adaptation, i.e. a decrease in the reproduc-tion of white blood cells in the bone marrow [26]. In this study, we recorded, possibly genetically fixed, an increased mobilization readiness of the organism of HT rats to respond to hypoxia or other stressful exposures in the form of a mechanism that suppresses the reproduction of granulocytic cells and boosts the proliferation of erythroid cells. Perhaps this is due to the fact that, in the case of a significant predominance of WBC elements, counter to their protective function, a disaster may result, because granulocytes, which account for the majority of WBCs in animals and humans, instantly respond to an emergency situation by a release of large amounts of toxic substances that also have a negative impact on the host body. In this case, the vascular endothelium can be damaged, causing the activation of the coagulation system. These negative effects of neutrophils, representing a significant portion of the granulocytic elements of the WBC population, are now well documented when studying their action in COVID-19 [27, 28].

Increased blood coagulation plays a significant role in decreasing hypoxia tolerance [29, 30]. In the present work, we established that after being in conditions of acute hypoxic hypoxia, LT rats

developed a state of hypercoagulation associated with the intrinsic (shortening of the APTT) and extrinsic (shortening of the PT) pathways of activation of blood coagulation, which was aggravated by an increase in fibrinogen levels. This can be explained by a damage to the vascular wall, inflicted by reactive oxygen species, and an increased (vs. HT rats) number of hypoxia-activated granulocytes that release procoagulant factors [17, 24, 31]. Our data are consistent with those obtained by other authors [32−34]. An increase in the blood fibrinogen level in response to stress-induced corticosterone elevation may have been causative for the shortening of the TT indicator, which reflects a fibrinogen-to-fibrin conversion and depends on the blood fibrinogen level [35].

Testing of rats in a decompression chamber proved the fact that individual hypoxia tolerance in rats can be determined by HTI values. Besides hypoxic stimuli, other stress factors also act on living organisms in a decompression chamber. In physiology, at all stages of evolution, it is customary to distinguish two qualitatively different strategies of survival: the active, resident strategy characterized by an active overcoming of stress factors, and passive, tolerance strategy implying a quiet perception of an external stimulus' intervention (a freezing response). These adaptation strategies are provided by different neuroimmunoendocrine mechanisms [36, 37]. As follows from our data, in a decompression chamber, LT rats demonstrated a distinct resident strategy, due to which oxygen reserves were overspent, and the animals quickly lost the ability to sustain postural reflex. While HT rats held to a tolerance strategy that promoted long-term saving in oxygen consumption, MT rats demonstrated an alteration of strategies, which determined the intermediate values retention time of postural reflex in HT vs. LT rats. The manifestation of behavioral strategies, likewise hypoxia tolerance, is believed to be genetically fixed [37, 38]. As shown in our previous studies, moderate physical loads (swimming) allowed tolerant behavior conditioning in most of Wistar rats in the population. At the same time, it was established that animals adopted to tolerant strategy, became HT, while unlearned rats turned out to be LT [39]. Given that tolerance to hypoxia is genetically fixed and MT rats are probably the most tolerant to hypoxia, it can be assumed that MT rats, which learned the strategy of tolerance as the main principle of their behavior, became HT animals. Our studies give a reason to pose the problem of including behavioral mechanisms in the integrative response of animals and humans to acute hypoxia.

Thus, it has been demonstrated for the first time that HT rats are characterized by an increased mobilization readiness (probably fixed genetically) to respond to hypoxia in the form of suppressing the reproduction of WBCs and boosting erythropoiesis. We also identified the predictors of hypoxia tolerance, namely WBCs, granulocytes, RBCs, RTC, and MCH. We developed an original formula for calculating the hypoxia tolerance index (HTI), which allows splitting the rat population into HT, LT and MT individuals without testing them in a decompression chamber. It was established that in LT rats, the HTI values ≤ 0.203, in HT rats > 0.335, and in MT rats < 0.335 but > 0.203. Prediction of individual tolerance to hypoxia in naive animals is of great interest from both theoretical and practical points of view, since acute hypoxic hypoxia damages the central nervous system, can cause death of animals, and prolongs the recovery period of surviving individuals (up to 1 month) for the involvement in further experiments. The identification of the relationship between certain physiological indicators in naive animals or humans with the level of hypoxia tolerance makes it possible to improve the accuracy of predicting the outcome of acute hypoxic exposure, which is important for the professionals whose activities are associated with a high risk of hypoxia. Moreover, it is important to search for therapeutic targets when modeling various human diseases in order to develop a personalized approach to treatment.

AUTHORS' CONTRIBUTION

M.V.K.—idea, experimental design, data collection, writing of the article; K.A.A., V.V.A., N.B.T.—technical support during the experiments, participation in data processing and discussion; M.N.B.—data processing and discussion, editing of the article.

COMPLIANCE WITH ETHICAL STANDARDS

All applicable international, national and/or institutional principles for the care and use of animals have been observed.

This article did not contain the results of any research involving people as research objects.

CONFLICT OF INTEREST

The authors declare that they have neither evident nor potential conflict of interest related to the publication of this article.

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