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EFFECTS OF SHORT-TERM COLD EXPOSURES ON BLOOD LEUKOCYTE PARAMETERS IN RATS OF DIFFERENT AGES

The intermittent short-term cold exposures (STCE) at 10 and –12 °C on the ratio of leukocyte types in the blood of 6-, 12-, and 24-month-old rats, as well as the changes in integral leukocyte indices (ILIs) were studied. Under both STCE regimens, the total number of leukocytes in 6-month-old animals increased, while in 12- and 24-month-old ones it decreased, the percentage of segmented neutrophils in all age groups augmented, and that of lymphocytes reduced. The allergization and immunoreactivity indices decreased, and cellular immunity predominated (except for 24-month-old rats at –12 °C). After STCE 10 °C, the Garkavi adaptation index decreased in rats of all age groups, but at STCE –12 °C it reduced in 6-month-old ones only. The innate immunity was activated in rats of all age groups at 10 °C, but at –12 ° C it was only in 6- and 12-month-old animals. Under both STCE regimens, the 6- and 12-month-old rats showed decreased adaptive immunity and increased inflammation. Changes in ILIs after STCE –12 °C were the highest in 6-month-old animals and the lowest in 24-month-old ones.

Key words: *blood leukocytes, cold adaptation, short-term cold exposure, immune system, age.*

Cold is known to be one of the main adaptogenic factors. Under the active physiological cold adaptation, whose biological purpose is to maintain homeostasis, the specific and nonspecific responses develop, enabling the body to exist in altered environmental conditions. At the same time, the body is forced to change some constants of functional activity, *i. e.* homeostasis is adjusted to a level that is more adequate for certain conditions [15].

To simulate the cold exposures in the experiment, the animals are kept in cages under either short-term intermittent or long-term exposures to low temperatures. In our work, we used low positive (10 °C) and negative (–12 °C) temperature regimens. According to the universal temperature index, under 10 °C exposure, there is no thermal stress, while a temperature of –12 °C corresponds to moderate thermal stress [1].

The qualitative and quantitative ratios of leukocyte types in the blood is one of the highly informative criteria used to assess the body's adaptive responses. The task of studying the mechanisms of cold adaptation is of great practical importance, including the age-related aspects.

The aim of this study was to investigate the changes in blood leukocyte parameters after short-term intermittent cold exposures in rats of different ages.

MATERIALS AND METHODS

The experiments were carried during winter in 6-, 12-, and 24-month-old males of white outbreed rats in accordance with the Law of Ukraine "On the Protection of Animals Against Cruelty" (No. 3447-

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IV of February 21, 2006) and the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986). The Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine (Kharkiv) did not reveal any violations during experimental studies (protocol No. 2 of March 11, 2020).

Under intermittent short-term cold exposure (STCE), the rats were kept at 10 (STCE 10 °C) or –12 °C (STCE –12 °C) in a cold chamber during 15 min every hour, and then outside the cold chamber for 45 min at 22—24 °C. Thus, STCE procedures were performed nine times during daylight hours over two consecutive days. Rats of all ages were divided into the following groups: control (intact animals); animals after STCE 10 °C and STCE –12 °C.

Blood was collected after decapitation of animals as part of a comprehensive experiment. The total leukocyte number (TLN) was determined in Goryaev chamber. The qualitative and quantitative ratios of leukocyte types was counted in blood smears fixed with May-Grünwald solution and stained according to Romanowsky. Based on the data obtained, there were calculated the integral leukocyte indices (ILIs) [5, 11, 12], which significantly determine the changes in particular components of the immune system and in the whole state of the human and animal body. The following ILIs were used in the research:

index of neutrophil-to-monocyte ratio (N/M) characterizes the components of the micro- and macrophage systems;

index of lymphocyte-to-monocyte ratio (L/M) reflects the ratio of the affector and effector components of immunological process;

index of neutrophil-to-lymphocyte ratio (BLs+ +SLs/L) reflects the ratio of non-specific and specific defense cells;

lymphocyte-granulocyte index (10L/(MCs+ +MMCs+BLs+SLs+E+B)) allows differentiation between infectious and auto-intoxication;

nuclear shift index ((MCs+MMCs+BLs)/SLs) shows the ratio of the content of all young forms of neutrophils to their mature forms;

lymphocyte-to-eosinophil ratio (L/E) reflects the ratio of immediate and delayed hypersensitivity processes;

leukocyte shift index ((E+B+SLs+BLs)/L+M), its increase indicates an active inflammatory process and impaired immunoreactivity;

leukocyte index (L/N) reflects the relationship between the humoral and cellular components of the immune system;

leukocyte intoxication index by Kalf-Kalif (4MCs + 3MMCs + 2BLs + SLs) × (PCs + 1)/(L + + M) × (E + 1) characterizes the level of endogenous intoxication and activation of tissue breakdown;

neutrophil reactive response index (BLs × SLs/ (L + M) × E) is an index of endogenous intoxication;

as well as allergization index (L + 1 – (E + 1)/ BLs + SLs + M), Garkavi adaptation index (L/SLs), and index of immune reactivity (L+E/M).

The abbreviations in the above formulae refer to cell types such as: BLs — band and SLs — segmented leukocytes; L — lymphocytes; M — monocytes; N — neutrophils; E — eosinophils; B — basophils; MCs — myelocytes; MMCs — metamyelocytes (immature cells), PCs — plasm cells (expressed in percentage).

The data were analyzed using the Excel software (Microsoft, USA) and Social Science Statistics (https://www.socscistatistics.com/). The normal distribution of data was checked using the Kolmogorov-Smirnov test. The results were statistically processed using ANOVA, expressed as $M \pm SE$. The significance of the results was assessed at a level of at least 95% ($p \leq 0.05$).

RESULTS AND DISCUSSION

Age-related changes in the qualitative and quantitative ratios of leukocyte types in the blood of intact rats have been described by us previously [12].

Analysis of changes in blood leukocyte parameters after STCE in rats of different ages showed an increase in TLN under STCE –12 °C in 6-month-old rats, while 12- and 24-month-old rats, on the contrary, had leukopenia, *i. e.*, with age increase, cold stress resulted in TLN decrease.

The percentage of segmented neutrophils increased, that of lymphocytes decreased in rats of all age groups, the percentage of band cells augmented only in 12-month-old animals, and monocytes increased in 6- and 12-month-old ones (it did not change in 24-month-old rats); the percentage of eosinophils varied depending on age: it remained unchanged in 6-month-old rats, augmented in 12-month-old ones, and reduced in 24-month-old animals (Table 1).

The impact of STCE 10 °C resulted in changes in TLN and percentage of eosinophils, band and segmented neutrophils similar to those observed at STCE –12 °C, and the percentage of lymphocytes also decreased (but this time in rats of all age groups), while the percentage of monocytes changed (increased) only in 6-month-old rats (Table 1).

M. Eimonte *et al.* [7] have demonstrated that after short-term cooling (sharp immersion in cold water), the percentage of neutrophils also rises and that of lymphocytes diminishes.

As we have shown previously [13], the long-term cold exposure (LTCE) at a temperature of about 5 °C for a month in rats of different ages results (as after both studied STCE regimens) in leukopenia (except for 6-month-old rats), an increase in percentage of segmented neutrophils and monocytes (only in 24-month-old rats), a decrease in that of band neutrophils (only in 12- and 24-month-old animals), lymphocytes (mostly in 24-month-old rats), and eosinophils (only in 24-month-old ones).

Leukocytosis, which is a protective response of the system to stimuli, was observed only in 6-month-old rats after both STCE regimens (as well as after LTCE [13]). Physiological redistributive leukocytosis occurs after exposure to cold or heat, emotional or physical stress. Leukopenia, observed in 12- and 24-month-old rats after both STCE regimens, may be due to destruction and utilization of leukocytes, their redistribution when the ratio of circulating, near-wall and tissue neutrophils is disturbed.

Increase in percentage of only band neutrophils (degenerative shift to the right), observed in 12-month-old rats after both STCE regimens, suggests suppression of bone marrow leukopoietic function. The percentage of young band neutrophils may decrease under physical factors, when bone marrow leukopoietic activity is suppressed, resulting in impaired immune system function, decreased resistance to infectious diseases, *etc.* A decrease in the pool of band neutrophils was observed in 24-month-old animals after STCE 10 °C, which might also be due to the accelerated maturation of these cells [3, 10, 16].

An increased percentage of segmented neutrophils against a reduced percentage of lymphocytes (in all age groups after both STCE regimens) may indicate physiological changes associated with overexertion and stress. Since the rats have a lymphocytic type of hematopoiesis, the TLN in their blood is high. In addition, lymphocytes are divided into subtypes with different lifespan: short (capable of reproduction) and long (retaining immunological "memory") [3, 10, 16]. Lymphocytopenia may be caused by age, physiological reactions aimed at

Table 1. **Leukocyte counts in blood after STCE in rats of different ages**

Control			STCE –12 °C			STCE 10 °C		
Age, month								
6 (n = 15)	12 (n = 6)	24 (n = 6)	6 (n = 10)	12 (n = 6)	24 (n = 10)	6 (n = 10)	12 (n = 6)	24 (n = 8)
Total amount, 10^9/l								
6.5 ± 0.1	7.3 ± 0.4	8.5 ± 0.8	7.9 ± 0.8 *	5.9 ± 0.9 *	6.6 ± 0.2 *	7.6 ± 0.3 *	5.5 ± 0.4 *	5.4 ± 0.5 *
Band leukocytes, %								
1.9 ± 0.4	1.2 ± 0.2	2.7 ± 0.8	2.8 ± 0.8	3.7 ± 0.8 *	2.2 ± 0.3	2.5 ± 0.5	2.3 ± 0.7 *	1.5 ± 0.3 *
Segmented leukocytes, %								
27.1 ± 1.5	26.5 ± 1.4	32.8 ± 1.8	37.8 ± 3.4 *	43.2 ± 5.9 *	38.6 ± 1.5 *	39.1 ± 2.9 *	45.5 ± 1.3 *	55.9 ± 3.6 *
Eosinophils, %								
3.5 ± 0.7	1.7 ± 0.3	8.2 ± 2.1	3.0 ± 1.5	2.8 ± 0.6 *	1.4 ± 0.4 *	2.7 ± 0.3	4.7 ± 0.3 *	2.9 ± 0.7 *
Lymphocytes, %								
65.5 ± 1.3	69.5 ± 1.1	54.2 ± 3.0	53.7 ± 3.7 *	48.3 ± 7.2 *	55.7 ± 1.3	53.8 ± 2.7 *	49.2 ± 2.2 *	37.7 ± 4.0 *
Monocytes, %								
1.3 ± 0.2	1.2 ± 0.2	1.8 ± 0.2	2.7 ± 0.5 *	2.0 ± 0.5 *	2.1 ± 0.2	1.9 ± 0.2 *	1.2 ± 0.2	2.0 ± 0.3

Note: * — differences are significant as compared to the control of the corresponding age, $p \leq 0.05$.

increased consumption of lymphocytes, and their destruction. Furthermore, a decrease/increase in lymphocyte pool may result from a decrease in their formation and migration into tissues, since they are capable of recirculation, unlike neutrophils, which do not return to the bloodstream from tissues [10, 16].

An increased percentage of eosinophils (in 12-month-old rats after both STCE regimens) may be a physiological consequence of physical exertion, allergic readiness of the body, or tissue inflammation. Hypersensitivity is always accompanied by enhanced synthesis of eosinophils by the bone marrow. Eosinophils stimulate the accumulation and release of biologically active substances responsible for activation and suppression of inflammatory processes (inflammatory mediators). A reduced percentage of eosinophils (in 24-month-old rats after both STCE regimens) may suggest a decrease in body's resistance to endo- and exogenous factors, as well as physical overexertion [8, 9, 14]. Notably, in the control group, the percentage of eosinophils was almost twice higher in 24-month-old rats as compared to 6-month-old ones. Thus, it was established the fact of restoration of eosinophil percentage in blood of aged rats after STCE, since this index was equal to that in young 6-month-old animals in the control group (Table 1). Eosinopenia can be of hormonal origin and occur as a result of the effect on the nervous system, for example, under stress (cold impact is a powerful exogenous stress factor for the body), when an increase in hormone levels, in particular glucocorticoids, actively affects hematopoiesis and state of peripheral blood [8, 9, 14].

It is known that monocytes participate in regulation of hematopoiesis and formation of specific immunity; provide antitumor effect and interferon production; play a crucial role in destroying dead cells in areas of inflammation, thus enabling tissue regeneration [3, 16]. An increased percentage of monocytes was observed in 6-month-old animals after both STCE regimens, in 12-month-old animals after STCE –12 °C, and no decrease in monocyte percentage was noted. An increase in monocyte number in the blood may be due to the activation of mechanisms that counteract the development of pathological processes and/or changes associated with physiological aging. Since the phenotype and function of monocytes change with age, they contribute to immunosenescence and inflammation and, as a result, play an important role in defective immunity [4].

A decrease in eosinophilic leukocytes, which have detoxification functional activity, as well as in lymphocytes, which are the main cells of the immune system, can be considered as signs of immune suppression (in 24-month-old rats after both STCE regimens). Lymphocytopenia may be associated with the movement of lymphocytes from the blood directly to inflammation sites. The reduction of eosinophil number is also often observed at inflammation onset, but it may also have physiological causes (physical exertion or cold exposure, stress, *etc*.), and then the number of eosinophils in the blood returns to normal for a period [10, 16].

Resistance to certain types of stress reaches its peak at a young age but declines with aging, which is considered to result from genetically programmed inactivation of protective mechanisms and is not associated with the accumulation of damages in the body [6].

Analysis of ILIs changes showed that after STCE –12 °C, they were highest in 6-month-old animals. In 6- and 12-month-old rats, the cellular component of the immune system prevailed in the blood. The inflammation, the effector link of the immunological process, non-specific defense cells, and infectious intoxication were activated, while allergization and immunoreactivity decreased. In addition, 6-month-old rats showed an increased endogenous intoxication and reduced adaptive capabilities of the body, but at the same time, the macrophage system was activated. Macrophages are known to be involved in both active phagocytosis and immunological recognition and presentation of antigens to T-lymphocytes.

In 24-month-old rats, ILIs changes were the lowest, and they also had reduced allergization rate (as in 6- and 12-month-old rats), increased endogenous intoxication (as in 6-month-old rats), and activated immediate-type hypersensitivity processes (Table 2). Notably, after STCE –12 °C, allergization index decreased in rats of all age groups, which was a positive effect of this regimen.

Under STCE 10 °C in animals of all age groups, most of the ILIs changes were unidirectional: the allergization index decreased, an infectious intoxication occurred, the cellular component of immunity prevailed, the immediate hypersensitivity, effector link of the immunological process, and inflammation were activated, and immunoreactivity

decreased (as in 6- and 12-month-old rats after STCE –12 °C), and endogenous intoxication increased, the macrophage system was activated, and the body's adaptive capabilities reduced (as in 6-month-old rats after STCE –12 °C). In addition, the immediate and delayed hypersensitivity reactions were activated in 6- and 12-month-old animals, respectively (Table 2). The age-related changes in ILIs in rats were analyzed in our recent report [12].

As shown previously [13], LTCE also caused unidirectional changes in ILIs in rats of different ages, namely the non-specific defense cells (neutrophils) prevailed, inflammatory processes were activated, the immunoreactivity was disrupted, endogenous intoxication was manifested, allergization and body's adaptation level decreased, the cellular component of the immune system was activated, and the effector link of immunity was enhanced (except for 24-month-old animals). In blood of 12- and 24-month-old animals, the mature neutrophils predominated, and the microphagocytic system was activated; in 12-month-old rats, the delayed-type hypersensitivity processes were activated.

Recently, research has been conducted using the diagnostic and prognostic capabilities of neutro-

Table 2. **Integral leukocyte indices after STCE in rats of different ages**

Control			STCE –12 °C			STCE 10 °C		
Age, month								
6 (n = 15)	12 (n = 6)	24 (n = 6)	6 (n = 6)	12 (n = 6)	24 (n = 10)	6 (n = 6)	12 (n = 6)	24 (n = 8)
Index of neutrophil-to-monocyte ratio								
23.6 ± 2.4	25.7 ± 3.0	20.7 ± 3.1	16.6 ± 1.8 *	30.3 ± 8.0	22.1 ± 2.9	23.7 ± 1.9	42.5 ± 3.7 *	33.6 ± 5.2 *
Index of lymphocyte-to-monocyte ratio								
54.2 ± 2.0	63.4 ± 5.5	32.2 ± 6.0	23.5 ± 3.9 *	35.1 ± 11.2 *	31.1 ± 5.1	32.5 ± 5.3 *	45.6 ± 5.2 *	23.3 ± 5.0
Index of neutrophil-to-lymphocyte ratio								
0.4 ± 0.0	0.4 ± 0.0	0.7 ± 0.1	0.8 ± 0.1 *	1.2 ± 0.3 *	0.7 ± 0.0	0.9 ± 0.1 *	1.0 ± 0.1 *	1.6 ± 0.3 *
Lymphocyte-granulocyte index								
20.9 ± 1.3	24.0 ± 1.4	12.8 ± 1.4	13.0 ± 1.9 *	12.4 ± 3.9 *	13.4 ± 0.8	12.8 ± 1.4 *	9.4 ± 0.7 *	5.2 ± 0.5 *
Allergization index								
3.9 ± 0.4	3.4 ± 0.3	4.0 ± 0.6	2.2 ± 0.3 *	1.9 ± 0.5 *	1.9 ± 0.2 *	2.2 ± 0.2 *	2.2 ± 0.1 *	1.3 ± 0.1 *
Nuclear shift index								
0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Lymphocyte-to-eosinophil ratio								
17.8 ± 2.9	49.9 ± 8.6	12.9 ± 5.1	24.5 ± 10.3	30.2 ± 12.5	28.7 ± 7.6 *	23.6 ± 4.5 *	10.8 ± 1.0 *	12.9 ± 5.6
Leukocyte shift index								
0.5 ± 0.0	0.4 ± 0.0	0.8 ± 0.1	0.8 ± 0.1 *	1.2 ± 0.3 *	0.7 ± 0.0	0.8 ± 0.1 *	1.0 ± 0.1 *	1.7 ±0.3 *
Garkavi adaptation index								
2.6 ± 0.2	2.7 ± 0.2	1.7 ± 0.2	1.5 ± 0.2 *	1.4 ± 0.4	1.5 ± 0.1	1.5 ± 0.1 *	1.1 ± 0.1 *	0.7 ± 0.1 *
Leukocyte index								
2.5 ± 0.2	2.5 ± 0.2	1.6 ± 0.2	1.4 ± 0.2 *	1.3 ± 0.4 *	1.4 ± 0.1	1.4 ± 0.1 *	1.1 ± 0.1 *	0.7 ± 0.1 *
Leukocyte intoxication index by Kalf-Kalif								
0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.1 *	0.3 ± 0.1	0.4 ±0.1 *	0.2 ± 0.0	0.3 ± 0.1	0.5 ± 0.1 *
Index of immune reactivity								
59.5 ± 5.8	65.0 ± 5.7	35.7 ± 5.7	24.6 ± 3.8 *	36.7 ± 11.1 *	34.7 ± 6.6	30.8 ± 2.0 *	47.1 ± 7.0 *	24.7 ± 5.0

Note: * – differences are significant as compared to the control of the corresponding age, $p \leq 0.05$.

phil-to-lymphocyte ratio (N/L) and lymphocyte-to-monocyte ratio (L/M) indices, which reflect the balance between the main components of the immune system [18, 19]. So, the N/L index characterizes the balance between neutrophils, which are key effectors of innate immunity with pro-inflammatory activity, and lymphocytes, which play a central role in adaptive immunity and perform regulatory and anti-inflammatory functions [2]. The L/M index characterizes the balance between lymphocytes and monocytes, which are involved in mechanisms of innate immunity and stipulate the development of chronic inflammation [18]. It has been established that the higher the L/M index, the better the prognosis for treatment [11, 17, 18].

So, since (N/L) index increased after STCE in rats of all age groups at 10 °C, and at –12 °C in 6- and 12-month-old ones, it may be stated that under such conditions, the innate immunity is activated. At the same time, a decrease in L/M index in 6- and 12-month-old rats under both STCE regimens may suggest the activation of inflammation and a decrease in adaptive immunity.

Thus, based on changes in percentage of segmented neutrophils (increase) and lymphocytes (decrease) in the blood, the data coincided for both STCE regimens in rats of all age groups (except for lymphocytes in 24-month-old animals after STCE –12 °C). The ILIs calculation showed allergization and immunoreactivity indices to decrease in rats of all age groups after both STCE regimens, as well as the cellular component of the immune system prevailed in them, except for 24-month-old rats after STCE –12 °C. The Garkavi adaptation index decreased after STCE 10 °C in animals of all age groups, and after STCE –12 °C only in 6-month-old rats.

CONCLUSIONS

Changes in blood leukocyte parameters in rats depend on age and STCE regimen. After both STCE regimens, the TLN increases in 6-month-old rats and decreases in 12- and 24-month-old rats; the percentage of segmented neutrophils augments in all age groups, band neutrophils and eosinophils — only in 12-month-old animals, monocytes — in 6-month-old animals only; the percentage of eosinophils decreases in 24-month-old rats, and lymphocytes — in 6- and 12-month-old animals. The percentage of band neutrophils, eosinophils, and lymphocytes declines in 24-month-old rats after STCE 10 °C.

After both STCE regimens in rats of all age groups, Garkavi adaptation index (except for 12- and 24-month-old rats after STCE –12°C) and those of allergization and immunoreactivity decrease, and the cellular component of the immune system predominates (except for 24-month-old rats after STCE –12°C).

After STCE –12°C, the changes in ILIs were the highest in 6-month-old animals. In addition, 6- and 12-month-old rats showed the activation of inflammation (as evidenced by leukocyte shift index increased), the effector component of the immunological process, non-specific defense cells, and suppression of adaptive immunity; in 6-month-old rats, the macrophage system was activated and endogenous intoxication increased. In 24-month-old rats, changes in ILIs were the lowest, and they also exhibited increased endogenous intoxication (as in 6-month-old rats) and activated immediate-type hypersensitivity.

Moreover, under STCE 10 °C, the animals of all age groups showed activation of immediate hypersensitivity, the effector component of the immunity, inflammation (leukocyte shift index increased), enhanced endogenous intoxication, and activation of innate immunity and macrophage system. In 6- and 12-month-old rats, immediate and delayed hypersensitivity processes were activated, respectively.

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ЕФЕКТИ КОРОТКОЧАСНИХ ХОЛОДОВИХ ВПЛИВІВ
НА ЛЕЙКОЦИТАРНІ ПОКАЗНИКИ КРОВІ У ЩУРІВ РІЗНОГО ВІКУ

Вивчали дію короткочасних переривчастих холодових впливів (КПХВ) при 10 та –12 °С на співвідношення типів лейкоцитів у крові 6-, 12- і 24-місячних щурів та зміни інтегральних лейкоцитарних індексів (ІЛІ). За обох режимів КПХВ загальна кількість лейкоцитів у 6-місячних тварин збільшувалася, у 12- і 24-місячних зменшувалася, відсоток сегментоядерних нейтрофілів у всіх вікових групах збільшувався, лімфоцитів — зменшувався, знижувалися індекси алергізації, імунореактивності, переважав клітинний імунітет (крім 24-місячних щурів при –12 °С). Індекс адаптації Гаркаві знижувався після КПХВ 10 °С у щурів усіх вікових груп, а у 6-місячних тільки при –12 °С. У щурів всіх вікових груп при 10 °С, а у 6- і 12-місячних тільки при –12 °С активувався вроджений імунітет. За обох режимів КПХВ у 6- і 12-місячних щурів знижувався адаптивний імунітет і підсилювалося запалення. Зміни ІЛІ були після КПХВ –12°С найбільші у 6-місячних тварин, найменші — у 24-місячних.

***Ключові слова*:** лейкоцити крові, холодова адаптація, короткочасні холодові впливи, імунна система, вік.