

## Induced Liver Fibrosis Is Accompanied in Young and Old Animals by Age-Dependent Changes in Bone Marrow Cells

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**Abstract**—The purpose of the study is to test the hypothesis stating that the functional characteristics of bone marrow cells (BMCs), such as the proliferation rate and the focus of differentiation, etc., are determined not so much by the age of animals as by some specificities in the microenvironment of the BMCs. To test this hypothesis, two techniques to change the microenvironment are used—in vivo (induction of liver fibrosis in young and old animals) and in vitro (transferal of BMCs isolated from young and old animals of both control groups and groups with fibrosis into the same standard culturing system). It is found that these particular forms of fibrosis induced by administering copper sulfate (CuSO<sub>4</sub>) or carbon tetrachloride (CCl<sub>4</sub>) have different effects on the ratios between BMC types of young and old animals. For example, whereas the relative number of morphologically identifiable cell types decreases for young rats, irrespective of the particular inducer of liver fibrosis, this process is accompanied for old animals by an increase in identifiable cell types in the case of CuSO<sub>4</sub>-induced liver fibrosis and does not change when liver fibrosis is induced by CCl<sub>4</sub>. The proliferative activity of BMCs isolated from old animals and transferred into the in vitro culture exceeds this activity for young rats. This can be explained by the higher (by 167%) content of lymphocytes in the bone marrow of old animals, as well as by the specific composition and characteristics of the microenvironment of their BMCs.

**Keywords:** age, bone marrow cell culture, CuSO<sub>4</sub>-induced fibrosis, CCl<sub>4</sub>-induced fibrosis

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### INTRODUCTION

The problem of aging has always strongly attracted researchers, since this question concerns everybody. But the problem is still far from its solution. There exists no generally accepted definition of this process [8, 17, 18]. The majority of interpretations offered for explaining this general biological phenomenon can be presented as the thesis that it is a degenerative process characterized by the loss of stability in the functioning of biological systems and, as a result, by their increased vulnerability to a diversity of exogenous factors [1, 22].

We can, actually, easily record a growth of “inadequate” adaptive responses to a diversity of exogenous factors in the environment at the later stages of ontogenesis. This is manifested in increased probabilities of malignant diseases, cardiovascular pathologies, neuroendocrine changes, and developing inflammatory responses, i.e., so called age-dependent disorders [21].

There have been some attempts to offer a failure or disorder in the functions of some particular metabolic element or structural change in the genome as possible mechanisms of aging. Examples of this are the telomerase-based and free-radical hypotheses, etc. [7]. The overall diversity of mechanistic monohypotheses

can be grouped into two conceptual approaches, genetic and environmental, or more exactly, into the integrated epigenetic approach. We believe that the latter explains the mechanism of aging as a result of temporal interactions between the metabolic system and continuously changing microenvironment factors. However, studying the multifactor effect of the environment on biological systems would be an exceptionally complicated task.

The existing genetic and molecular biological research methods do not always allow us to explain physiological manifestations of adaptive or psychological responses. This is associated with the multilevel organization of controlling biological processes, the presence of alternative variations in organizing biological responses, and insufficient knowledge of the mechanisms underlying integrative interactions between metabolic processes leading to a consolidated physiological response.

The most effective methodological approach to new knowledge is usually in developing and designing experimental models. These models allow researchers not only to study new phenomena, but also to test the solidity of the already declared hypotheses and predict the behavior of systems in some specified conditions

[2]. Therefore, developing experimental models well-suited to study of the mechanisms of interactions between metabolism and a complex of environmental factors may also be useful for solving the global problem of aging. Cell models may be the most adequate solutions for problems in gerontology at the present research stage, taking into account the multilevel organization of the controlling processes and the presence of alternative metabolic pathways.

We suggest that bone marrow cells (BMCs) can serve as a successful cell model of the age-dependent mechanisms underlying the responses of metabolic systems to microenvironment factors. First, the bone marrow is represented by different cell types, such as stem cells with high proliferative and differentiation potential, bipotent progenitor cells (committed cells), unipotent progenitor cells, and mature cells of the lymphoid and hematopoietic systems [13]. Second, the functional activity (the intensity of proliferation and the focus of differentiation) of BMCs is subject to changes in response to pathological and adaptive shifts in other systems of the body; i.e., they actively respond to the microenvironment arranged inside the body. Third, BMCs can easily and safely be isolated and cultivated in an *in vitro* system. And finally, BMCs from old animals have been shown to acquire genetic and epigenetic alterations leading to reductions in the proliferation rate, telomere shortenings, increased levels of free-radical reaction products, and other changes; i.e., age-dependent disorders at the cell level are observed [6, 16].

We suggest that changes in the functional activity of BMCs are associated in old animals not as much with the reduced stem cell pool or genetic disorders in stem cells as with changes in the BMC microenvironment characteristics in old animals, which are manifested in ontogenesis by alterations in the body's functional characteristics. Therefore, we can regulate both the intensity of proliferation and the direction of BMC differentiation via changing the microenvironment.

To test this hypothesis, we used two (*in vivo* and *in vitro*) approaches in this study to change the microenvironment. The *in vivo* microenvironment was changed in three models: animals of control groups and animals with CuSO<sub>4</sub>- and CCl<sub>4</sub>-induced liver fibroses (CuSO<sub>4</sub>-ilf and CCl<sub>4</sub>-ilf, respectively) in young and old animals.

The development of fibrosis is accompanied by systemic disorders in the body altering the microenvironment characteristics for BMCs [24].

We suggest that different inducers of fibrosis will also create different microenvironment characteristics and, as a result, alter the behavior of cells. How will BMCs isolated from young and old animals respond in these conditions? At the same time, the answer to this question is basic for the understanding of the specific age-dependent mechanisms underlying the organization of adaptive responses at the cell level.

A global change in the microenvironment of BMCs can be attained through transferring BMCs into the *in vitro* system (the second experimental model). When young and old animal cells isolated from control animals and animals with different forms of fibrosis are transferred into the same *in vitro* conditions, we will be able to evaluate the effect of the microenvironment on their proliferative and functional potential.

In this study, we determined the amount of BMC morphotypes in control young and old rats and rats with CuSO<sub>4</sub>-ilf and CCl<sub>4</sub>-ilf, as well as the ability of the cells isolated from these animals to proliferate *in vitro*.

## MATERIALS AND METHODS

The experiments were carried out in sexually mature young (3-month-old) and old (20-month-old) male Wistar rats. All procedures involving animals were carried out with the observance of bioethics rules [12], taking into account circadian rhythms in the organization of biological responses. Therefore, animals always received food at the same time of the day, were kept in standard conditions, and all manipulations (administration of toxic or other preparations) were performed at the same time of the day before their food intake. The animals did not receive any food during 24 hours preceding the isolation of BMCs. The animals were always withdrawn from the experiment from 8:00 to 10:00 a.m. local time.

The animals of each age group were subdivided into the control ( $n = 8$ ) group, the group with CuSO<sub>4</sub>-ilf ( $n = 8$ ), and the group with CCl<sub>4</sub>-ilf ( $n = 8$ ). In total, 48 animals were used in the experiment. CuSO<sub>4</sub>-ilf was induced by six successive intraperitoneal administrations of water solutions of varying copper sulfate concentrations (Fig. 1); the induction scheme was described in [10]. CCl<sub>4</sub>-ilf was induced by six successive carbon tetrachloride administrations (Fig. 1); the induction scheme was described in [15]. Physiological solution was administered to control animals following the same scheme (Fig. 1). The experimental procedures were stopped by immersing rats in ether anesthesia, their BMCs were subsequently isolated, and further the *in vivo* cell morphotype patterns, the proliferative activity of BMCs in the *in vitro* system, and the lifespan of lymphocytes and neutrophils, as well as the pattern of morphotypes, were determined after 48 and 96 hours of cultivation.

BMCs were isolated from two femoral bones of rats, following the procedure described in [4], and cultivated in the Dulbecco's modified Eagle's medium (DMEM), including antibiotics (1% gentamycin and 1% streptomycin) and 20% of inactivated fetal bovine serum. Cultivation was run under the standard conditions at 37°C and atmospheric CO<sub>2</sub> concentration at 5%. The number of cells and their morphotypes were daily determined on the first day through the fourth

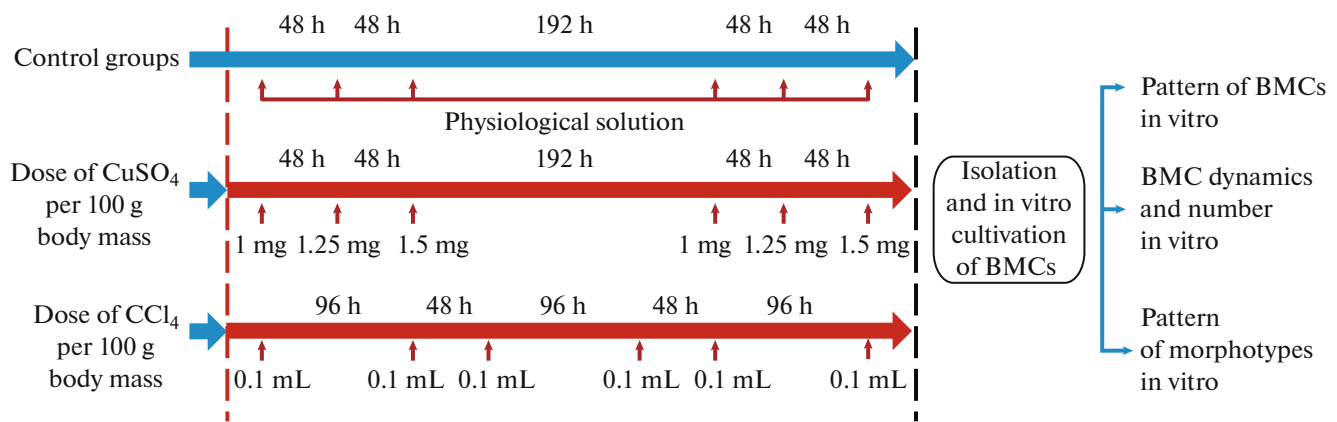


Fig. 1. Scheme of the experiment (bone marrow cells, BMCs).

day of cultivation. The cell culture medium remained unchanged. The initial BMC concentration during the cultivation always reached 2 million cells per mL.

The count and evaluation of viability in animal BMCs were described in [9]. The BMC morphotypes were determined immediately after their suspension was prepared, as well as on the second and fourth day of cultivation, as was described in [20]. The cytological preparations were stained by the Romanovskii–Gimza method, and analyzed with a 100× Zeiss Primo Star iLED microscope (Germany).

Using the light microscopy, we determined the absorption ability of neutrophils after 30-min incubation at 37°C with a microbial test culture (*Saccharomyces cerevisiae*). After 30 min, the percentage of cells absorbing the microbial test culture per 250 cells and the number of neutrophils involved in phagocytosis from total neutrophils was calculated (phagocytic index, PhI). The phagocytic number (PhN) was also calculated after 30 min as the mean number of bacteria inside the cells, as the quotient of dividing the total number of absorbed bacteria by the number of phagocytosing cells.

The activities of neutrophils were determined by the nitroblue tetrazolium test (NBT). The method is based on the ability of neutrophils to absorb nitroblue tetrazolium (under the NBT test). As a result of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidation reaction, diphormazan is formed in cells, creating blue granules. The number of blue-stained granules per 100 cells was determined using light microscopy with 10 × 90 magnification [14]. The spontaneous (without induction) and zymozan-induced levels were determined in the neutrophil-oxidase system, as was described in [3].

To prepare the serum, the blood was collected into dry centrifuge tubes, incubated for 30 min at 20°C, and centrifuged for 15 min at 1500 g. Albumin (g/L), the activity of alanine aminotransferase (ALT, C.U.), aspartate transaminase (AST, C.U.), and  $\gamma$ -glutamyl-

transferase (C.U.) were determined in the blood serum on a Beckman Coulter AU480 biochemistry analyzer (Germany). The content of lipid hydroperoxide was determined in blood serum, following the method described in [5].

The mean standard deviation, mean standard error, and sample size were used as the characteristics of the obtained samples. The statistical significance of differences was evaluated by the nonparametric Mann–Whitney test. The results were statistically treated using the OpenOffice and Origin software packages. The differences between the data of the control and experimental groups were accepted significant at  $p < 0.05$ .

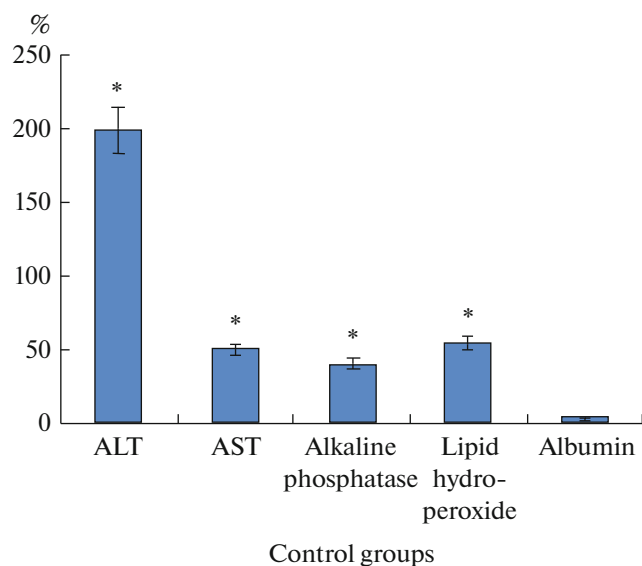
## RESULTS AND DISCUSSION

### *Some Specific Differences between CCl<sub>4</sub>- and CuSO<sub>4</sub>-Induced Liver Fibroses*

Repeated successive CCl<sub>4</sub> administrations to experimental animals are known to induce liver fibrosis [15]. We found that the activity of ALT and AST in blood serum increased 200 and 50%, respectively, 24 hours after the administration of CCl<sub>4</sub> at a dose of 0.1 mL per 100 g of an animal's body mass, compared with the control group (Fig. 2). The albumin content remained unchanged, compared with the control indicator. These alterations in liver occurred against a 56% increase in the content of lipid hydroperoxide in blood serum.

We identified age-dependent alterations in phagocytic activity in the CCl<sub>4</sub>-ilf. For example, PhI in young animals was 25% lower than in the control group, but PhN remained unchanged (Table 1). At the same time, PhI and PhN for old animals decreased 36 and 37%, respectively, compared with the corresponding control group.

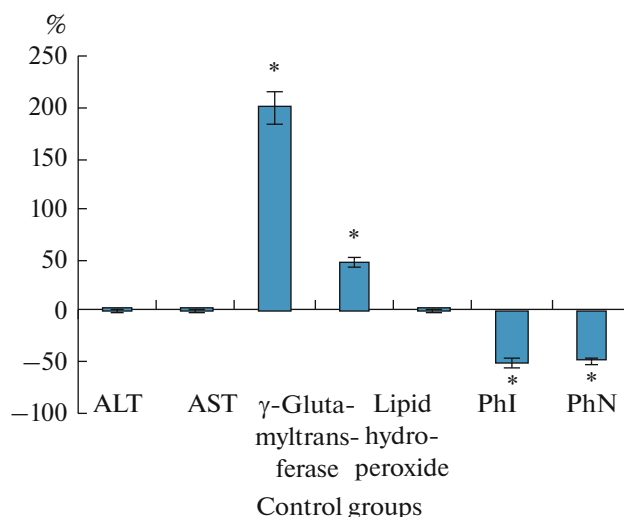
The NBT test has shown that the initial (termed as spontaneous) enzymatic activity level in neutrophils from young animals did not differ from that of the



**Fig. 2.** Blood serum indicators of young and old animals from the  $\text{CCl}_4$ -ilf groups relative to the indicators of the control groups,  $M \pm m$ . Here and in Figs. 3, 6, and 7: \* at  $p \leq 0.05$ , compared with old animals, according to the Mann–Whitney test.

control group, whereas the zymozan-induced level was 24% lower (Fig. 2). At the same time, the spontaneous enzymatic activity was 60% higher for old animals than the control indicators, whereas its stimulated level was 35% lower than the indicators in the control group (Table 1).

The  $\text{CCl}_4$ -ilf development was accompanied by generalized alterations involving hepatic functions, the immune system activity, and, as a result, changes in the composition of blood and the body's redox system. These alterations were also accompanied by expressed jumplike changes in the microenvironment of BMCs. Since it was impossible to characterize the in vitro microenvironment of BMCs completely, we chose another strategy to solve this problem. In the next experimental series, liver fibrosis was induced by administering another hepatotropic toxicant,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ . We can suggest that another (differing from



**Fig. 3.** Blood serum indicators, phagocytic index (PhI), phagocytic number (PhN) in young and old animals of  $\text{CuSO}_4$ -ilf groups relative to the indicators in control groups (accepted as the 0 level),  $M \pm m$ .

$\text{CCl}_4$ -ilf) microenvironment for BMCs will be created in the bone marrow.

Six successive copper sulfate administrations to animals at 48-h intervals between administrations at a dose of 1 mg per 100 g body mass caused in animals both similar and dissimilar (to  $\text{CCl}_4$ ) alterations in the functional and morphological indicators. For example, the ALT and AST activity, as well as the albumin content in animals with  $\text{CuSO}_4$ -ilf did not change, compared with the control group. However, the  $\gamma$ -glutamyltransferase activity, as a marker of toxic effects in the liver, increased 200%, compared with the control level (Fig. 3). The content of lipid hydroperoxide in the serum also demonstrated a 50% increase. The phagocytic activity in terms of PhI and PhN was 50% lower than in the control variant.

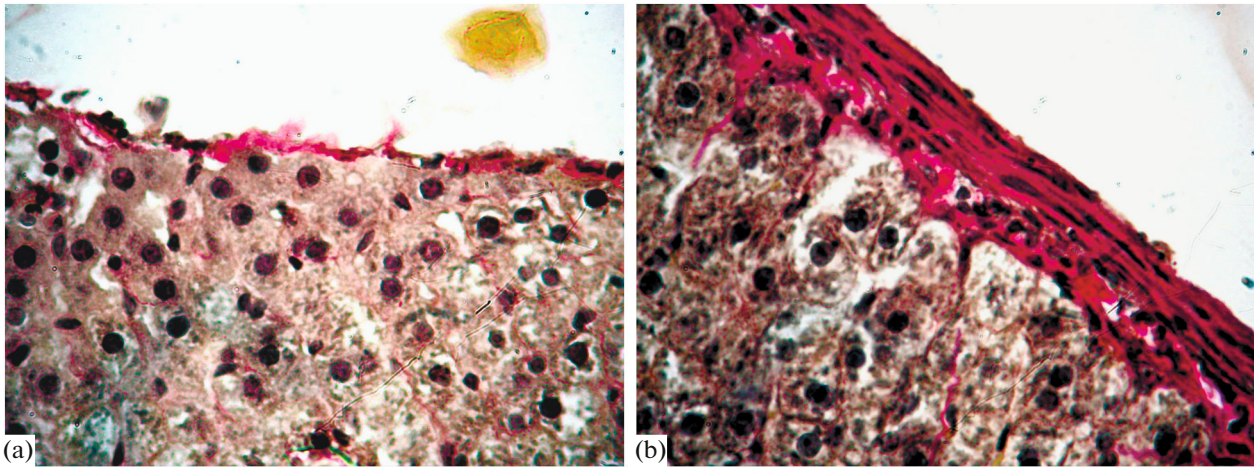
We have previously shown that the collagen content in the parenchyma of the liver was elevated in the animals receiving  $\text{CuSO}_4$  [11]. This was accompanied by the coalescence of liver lobes into a unified structure, which was unobserved in the  $\text{CCl}_4$ -ilf form. The

**Table 1.** Indicators of the phagocytic and neutrophilic activity of young and old animals from control and  $\text{CCl}_4$ -ilf groups,  $M \pm m$

Age of rats	Group	Phagocytic index	Phagocytic number	Activity of neutrophils	
				spontaneous	zymozan-induced
Three-month-old	Control, $n = 8$	$32.1 \pm 0.8$	$2.1 \pm 0.2$	$44.1 \pm 1.9$	$58.0 \pm 2.1$
	$\text{CCl}_4$ -ilf, $n = 8$	$25.8 \pm 1.8^*$	$1.8 \pm 0.1^*$	$48.0 \pm 3.1$	$44.2 \pm 2.4^*$
20-month-old	Control, $n = 8$	$41.1 \pm 1.7$	$2.6 \pm 0.1$	$40.2 \pm 2$	$47.8 \pm 2.4$
	$\text{CCl}_4$ -ilf, $n = 8$	$26.5 \pm 2.0^*$	$1.9 \pm 0.12^*$	$64.6 \pm 4.1^*$	$31.5 \pm 2.4^*$

\* At  $p \leq 0.05$ , compared with old animals by the Mann–Whitney test.





**Fig. 4.** Microphotographs of liver preparations from animals of the control (a) and experimental CuSO<sub>4</sub>-ilmf (b). Stained by Van Gieson, 100× magnified.

histological analysis has shown that the organization of the liver capsule was significantly altered in these animals, compared with the control variant (Fig. 4). Phagocytic cells were observed in the capsule, signaling the presence of an inflammatory process in the liver. Therefore, repeated successive CuSO<sub>4</sub> administrations to experimental animals were accompanied by developing the form of liver fibrosis significantly differing from CCl<sub>4</sub>-ilmf.

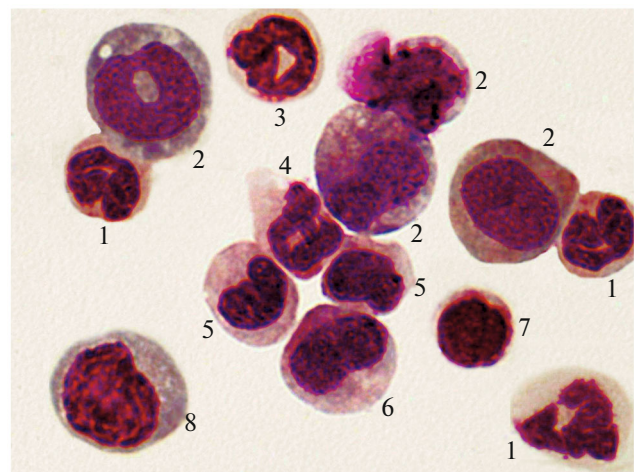
We can suggest that the variant of CuSO<sub>4</sub>-ilmf also leads to alterations in the physicochemical properties of the blood and these alterations differ from those shown in CCl<sub>4</sub>-ilmf.

#### *Pattern of Bone Marrow Cell Morphotypes in the in vivo System*

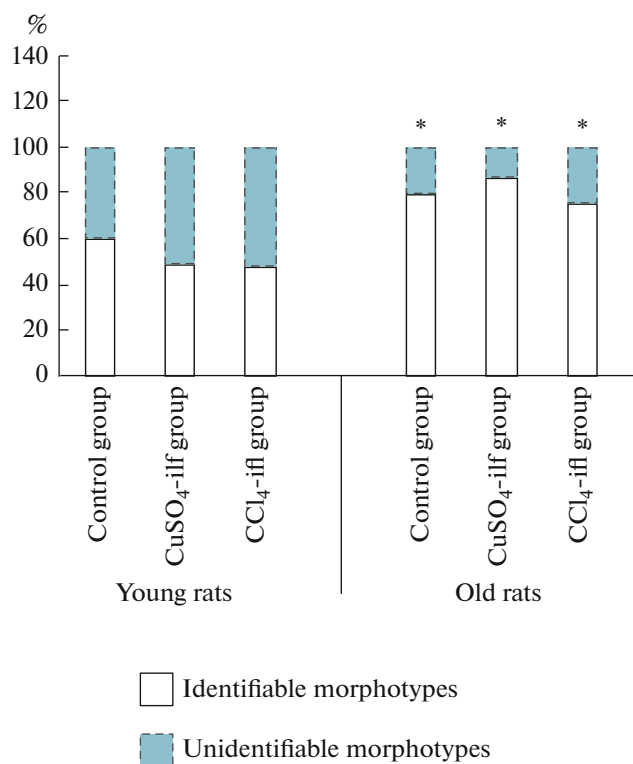
We used a classical visual microscopic analysis of BMC morphotypes in this study (Fig. 5) [19]. This analysis helped to determine the percentage shares of morphologically identifiable cells, including progenitor cells—myelocytes and metamyelocytes—as well as band and segmented neutrophils, lymphocytes, eosinophils, basophils, and monocytes.

We represented all BMCs in two groups: morphologically identifiable (the sum of the indicated eight morphotypes) and morphologically unidentifiable morphotypes (the sum of stem and committed cells). The following has been found: (1) the share of morphologically identifiable BMC morphotypes constituted more than 80% of all BMCs from old animals of the control group, whereas this share was 60% in young animals (Fig. 6); the ratio between identifiable and unidentifiable (i/un) morphotypes was 1.5 in young and 3.8 in old animals (Fig. 7); (2) the diagnosis of liver fibrosis in young animals was accompanied by a decrease in the number of identifiable and an increase in unidentifiable morphotypes of BMCs (Fig. 6), irre-

spective of the particular inducer (CuSO<sub>4</sub> or CCl<sub>4</sub>), which led to a significant reduction in the i/un cell types of young animals to 0.96–0.92 (Figs. 3, 7); BMCs of old animals responded to the induction of fibrosis in a different way, and there was a difference in this response between CuSO<sub>4</sub>-ilmf and CCl<sub>4</sub>-ilmf: the number of identifiable cell morphotypes increased in CuSO<sub>4</sub>-ilmf (Fig. 6), and the ratio of i/un types reached 6.5 (Fig. 7); at the same time, the number of identifiable and unidentifiable cells did not differ in CCl<sub>4</sub>-ilmf from the indicator of old animals of the control group (Fig. 6). Therefore, BMCs of young and old animals responded in a different way to liver fibrosis, i.e., the age-dependent response to a change in the microenvironment has been identified in BMCs.



**Fig. 5.** Some morphological types of BMCs from control rats: 1—segmented neutrophils; 2—undifferentiated cell; 3—band neutrophil; 4—segmented granulocyte; 5—metamyelocyte; 6—myelocyte; 7—lymphocyte; 8—monocyte. Stained by Romanovskii-Giemza, 100× magnified.

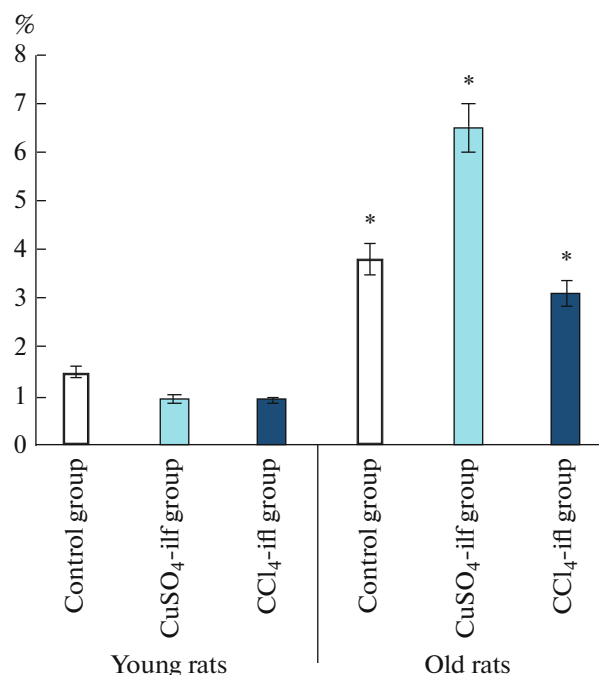


**Fig. 6.** Number of morphologically identifiable and unidentifiable morphotypes in young and old animals of control and experimental groups,  $M \pm m$ .

It has been found that the content of band neutrophils, eosinophils, and monocytes of old animals increased insignificantly, while the number of lymphocytes was 167% higher than that of young animals and the number of segmented neutrophils was 32% lower (Fig. 8a). These results indicate that the rate and direction of differentiation and/or that of the cell transport into the blood flow of old animals differ from young ones.

During the next series of experiments, the researchers determined the number of morphologically identifiable morphotypes in young and old animals with CuSO<sub>4</sub>-ilmf (Fig. 8b) and CCl<sub>4</sub>-ilmf (Fig. 8c). The obtained results allow us to make the following conclusions: (1) different liver fibrosis inducers cause different alterations in the physicochemical blood characteristics and, as a result, the microenvironment of BMCs changed the number of morphological types in the bone marrow in a different way (Figs. 8b, 8c); (2) these alterations, compared with the control animals, were more expressed in CuSO<sub>4</sub>-ilmf than in CCl<sub>4</sub>-ilmf (Figs. 8b, 8c); (3) the BMC response in CuSO<sub>4</sub>-ilmf was bidirectional in young and old animals (Figs. 8b, 8c).

There are data that the stem cell pool becomes reduced in size (depleted) with aging [23]. The obtained results in a relatively reduced share (almost 10%) of undifferentiated BMCs of old animals,



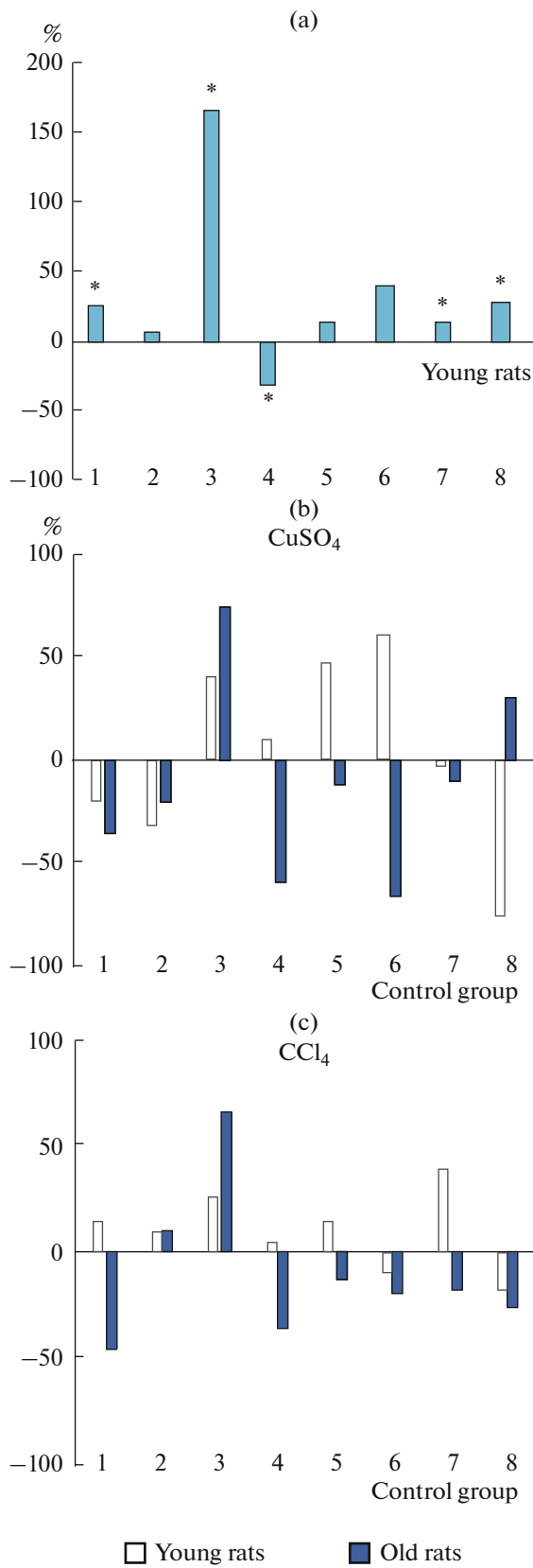
**Fig. 7.** Ratio between identifiable and unidentifiable cell types in young and old animals of control and experimental groups,  $M \pm m$ .

including mesenchymal stem cells, can indirectly confirm these data. Therefore, it is important to evaluate the *in vitro* proliferative activity in the BMCs isolated from young and old animals. In addition, *in vitro* cultivation of cells isolated from animals varying in age allows researchers to exclude the cell microenvironment formed with aging.

#### *The Effect of CuSO<sub>4</sub>-Induced Liver Fibrosis on the Quantitative Dynamics of Bone Marrow Cells Isolated from Young and Old Animals in the *in vitro* System*

To determine the proliferative activity of BMCs isolated from young and old animals, we prepared a suspension to contain 2 million cells in 1 mL of culture medium. The cells were monitored during four cultivation days. It was found that the amount of BMCs isolated from young animals increased 60% for the first 24 hours, while the cells isolated from old animals increased 112% for the same period (Fig. 9a). Subsequently, the cells from young and old animals grew in the *in vitro* system at the same rate, and reached the stationary growth phase on the third day of cultivation. Therefore, the proliferative potential of BMCs in the *in vitro* system was higher for old animals than for young ones.

If BMCs were isolated from young animals with CuSO<sub>4</sub>-ilmf, they were actively proliferating during the first 24 hours of cultivation, and their count even exceeded the control level in that period (Fig. 9b).



Their amount subsequently remained unchanged during four days of cultivation.

When BMCs were isolated from young animals with CCl<sub>4</sub>-iltf, their amount was increasing at a higher rate, compared with the control and animals with CuSO<sub>4</sub>-iltf (Fig. 9c). Therefore, development of CuSO<sub>4</sub>-iltf in young animals was accompanied by epigenetic and metabolic alterations in BMCs, leading to the suppression of their proliferative activity, but in CCl<sub>4</sub>-iltf, on the contrary, their proliferative activity in the in vitro system was increased.

In the case when BMCs were isolated from old animals with CuSO<sub>4</sub>-iltf, the BMCs preserved the ability to proliferate in the in vitro system during four days of cultivation, although this ability was lower than in the control (Fig. 9c). When liver fibrosis was induced by CCl<sub>4</sub> administration to old animals, the proliferative activity hardly differed from the control level. Therefore, CuSO<sub>4</sub>-iltf had a more expressed suppression effect on the proliferative activity of BMCs in the in vitro culture, compared with CCl<sub>4</sub>-iltf, and this activity was expressively age-dependent.

Thus, the proliferative activity of BMCs isolated from control animals and animals with liver fibrosis induced by different agents was different after their transfer into the same cultivation system. The nature of this activity depended on the age of the animals. The presence of liver fibrosis evoked different responses in BMCs, depending on the type of inducer and the age of the animals.

CONCLUSIONS

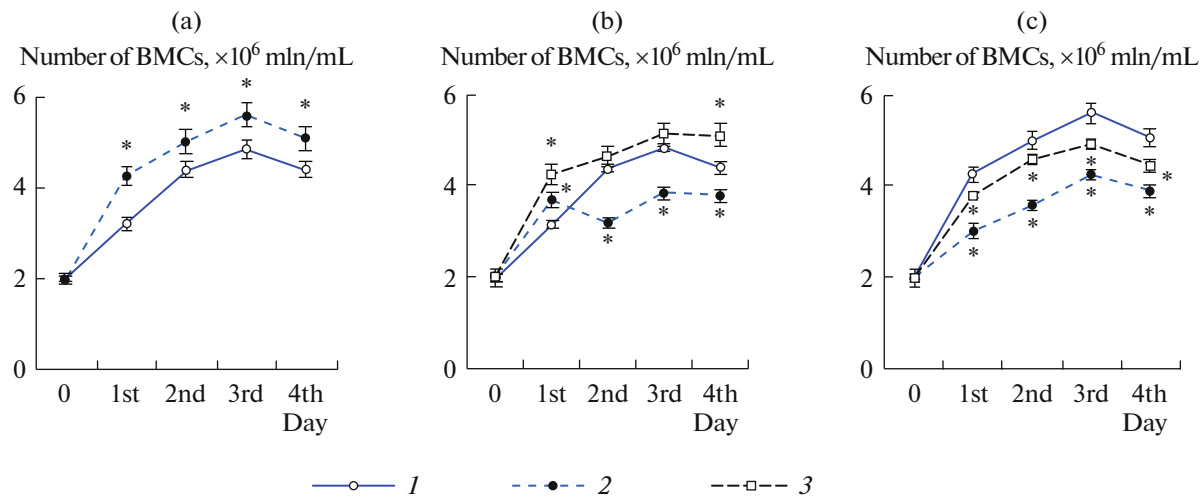
Liver fibrosis induced by the administrations of either CuSO<sub>4</sub> or CCl<sub>4</sub> had age-dependent effects on the ratio between the cell types in the bone marrow and their proliferative activity in the in vitro culture:

—irrespective of the particular fibrosis inducer, the amount of morphologically identifiable cell types from young animals decreased, whereas, on the contrary, their amount from old animals increased in the presence of CuSO<sub>4</sub>-iltf and the identifiable—unidentifiable cell type ratio remained unchanged in the case of CCl<sub>4</sub>-iltf;

—the proliferative activity of BMCs from young animals did not differ from the control in CCl<sub>4</sub>-iltf; BMCs did not proliferate in the culture after 24-h incubation; at the same time, when BMCs were iso-

Fig. 8. Number of band neutrophils (1), metamyelocytes (2), lymphocytes (3), segmented neutrophils (4), myelocytes (5), eosinophils (6), basophils (7), and monocytes (8) from old animals of the control group, compared with the indicators of young animals (accepted as 0)—the part (a); changes in the number of these cell types in young and old animals in the case of CuSO<sub>4</sub>-iltf (b) and CCl<sub>4</sub>-iltf (c), relative to the indicators in the animals of control groups (accepted as the 0 level),  $M \pm m$ ; \* at  $p \leq 0.05$ , compared with young animals by the Mann–Whitney test.





**Fig. 9.** Changes in the number of BMCs on the first through the fourth cultivation day in vitro: (a) isolated from young (1) and old (2) rats of control groups; (b) isolated from young rats of the control group (1), rats with CuSO<sub>4</sub>-ilm (2), and in rats with CCl<sub>4</sub>-ilm (3); (c) isolated from old rats of the control group (1), in rats with CuSO<sub>4</sub>-ilm (2), and in rats with CCl<sub>4</sub>-ilm (3),  $M \pm m$ ; \* at  $p \leq 0.05$ , compared with control animals, by the Mann–Whitney test.

lated from old animals with CCl<sub>4</sub>-ilm, their proliferative activity did not differ from the control level; when they were isolated from old animals with CuSO<sub>4</sub>-ilm, they preserved their proliferative activity, in contrast to young animals, but this activity was lower than the control level.

The proliferative activity of BMCs isolated from old animals of the control group and transferred into the in vitro culture exceeded this activity of young animals. This may be explained by:

—a higher (by 167%) amount of lymphocytes in the composition of BMCs, compared with those of young animals;

—changes in the microenvironment of BMCs isolated from old animals, i.e., the low proliferative in vivo potential of these cells may be explained by their specific microenvironment in old animals.

The induction of liver fibrosis was accompanied by inhibiting the immunity of the cell element (the phagocytic and enzymatic activity of neutrophils), more in old animals and in CuSO<sub>4</sub>-ilm than in CCl<sub>4</sub>-ilm.

#### COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of interests.** The authors declare that they have no conflict of interest.

**Statement on the welfare of animals.** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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