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COLOSTRUM COMPONENTS INVOLVED IN REGULATING THE NUMBER OF IMMUNOCOMPETENT CELLS IN ANIMALS WITH LIVER FIBROSIS

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Abstract

Two different fractions were prepared from whole colostrum: defatted colostrum (DC) and low molecular weight components of colostrum (LMC), and their effect on the growth dynamics of Wistar rats, in which liver fibrosis was induced, and the content of erythrocytes, platelets, and immunocompetent cells (leukocytes, lymphocytes, granulocytes, monocytes). To induce liver fibrosis, rats were injected three times with copper sulfate at a dose of 1 mg /100 g of body weight with an interval of 48 hours between injections. 24 hours after the last administration of copper, the development of the initial stage of fibrosis was observed according to histology data, and this was accompanied by an inhibition of the rate of growth in the body weight of the animals. Hematological parameters were determined on an automatic analyzer Mindray BC - 2008 Vet, (USA). It was found that in animals with fibrosis the number of platelets was increased by 2 times in comparison with the control, other types of cells, in particular immunocompetent cells, remained within the normal range. If animals with Cu-induced fibrosis were injected with OM and NCM at a dose of 0.1 mg /100 g of body weight daily for 6 days, then there was a significant increase in the number of immunocompetent cells in the bloodstream (leukocytes by 80% with OM and 59 % for NCM; lymphocytes by 68% and 30%, respectively; monocytes by 82% for OM and 160% for NCM; granulocytes by 218% and 223%, respectively), with a decrease in the number of platelets compared to animals with fibrosis.

It has been suggested that the components of colostrum have a stimulating effect on the functional activity of bone marrow cells, which can contribute to the elimination of liver fibrosis. DC and LMC had different effects on the content of different types of bone marrow cells.

Keywords: defatted colostrum, low molecular weight components of colostrum, immunocompetent cells, Cu-induced liver fibrosis.

Introduction.

It is known that liver fibrosis, the process of replacing liver cells with connective tissue, is a universal response of the body to damage. [1] Damage in liver tissue can be caused by a variety of factors: toxic chemicals in water, food or air, microorganisms, or physical factors. [2, 3] Fibrosis of the liver is dangerous because it can lead to the development of cirrhosis, which is an irreversible chronic severe condition. [4]

At the same time, in the early stages of development, fibrosis can be eliminated and the impaired liver function restored. Currently, there is an active search for natural compounds that can normalize liver function in fibrosis. [5]

Earlier it was shown that repeated sequential administration of copper sulfate in a dose of 1 mg /100 g of body weight to experimental animals was accompanied by the induction of liver fibrosis. [6, 7]

It is known that colostrum is a natural component, which includes a large number of biologically active compounds, such as immunoglobulins IgA, IgG, IgD, IgE, IgM, cytokines and related interferon, various growth factors - insulin-like factors, platelet, epithelial factors and including the transfer factor. [8, 9, 10] Transfer factor has a wide spectrum of action on biological systems and can normalize liver dysfunction against the background of fibrosis. [11] Isolation of the transfer factor is associated with a fine purification system, which requires expensive equipment on the one hand, and on the other hand, with such a purification system, a number of other biologically active compounds can be removed. It is of interest to separate colostrum into a number of components, in particular, the removal of lipid components and the removal of high molecular weight proteins, to obtain low molecular weight components of colostrum, which also include a transfer factor. With this approach, relatively simple

fractionation methods can be used while maintaining the biologically active properties of the materials.

In this regard, in our work, we investigated the effect of whole skim colostrum and low molecular weight components of colostrum (which also includes a transfer factor) on the growth dynamics and hematological parameters of animals with Cu-induced liver fibrosis.

Materials and methods.

The experiments were carried out on three-month-old male Wistar rats. The animals were kept under standard vivarium conditions. To simulate fibrotic changes in the liver of rats, a solution of copper sulfate was used, which was administered to rats intraperitoneally three times with an interval of 48 hours between injections at a concentration of 1 mg Cu /100 g of body weight, as described earlier. [6, 7, 12]

For fractionation, colostrum was obtained from the Alpha farm (Ukraine), from Ukrainian dairy cows - pockmarked, the second milk yield after calving. Fat was removed by centrifugation of whole colostrum at 3000 g for 20 min at room temperature. After removing the fats, the resulting colostrum was used as defatted colostrum (DC). When obtaining low molecular weight components (LMC), colostrum was passed through membrane filters with a pore diameter of 10 nm, which made it possible to remove proteins with a high molecular weight of more than 10 kd (Fig. 1)

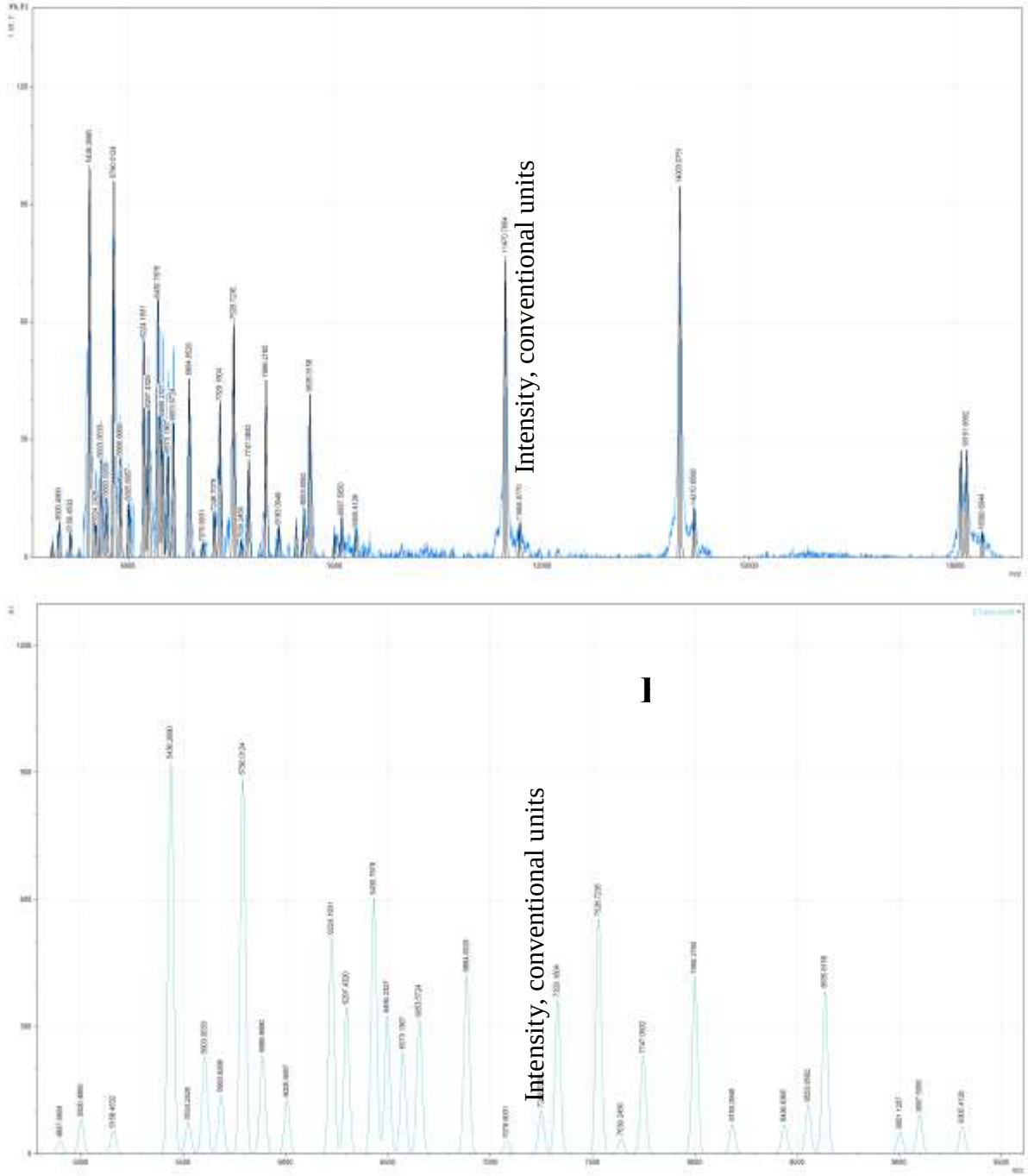

Fig.1. ***A.*** *Protein composition of skim colostrum as determined by mass spectrometry.* ***B.*** *Composition of proteins included in the fraction of low molecular weight components of colostrum.*

The obtained fractions of DC and LMC were dried in a rotary evaporator and stored dry samples at a temperature of minus 15 ºC without access to air.

To study the effect of DC and LMC, animals with induced liver fibrosis with copper sulfate (Cu - ind.fibrosis) were injected *per os* with aqueous solutions of DC and LMC at a dose of 0.1mg/100 g of body weight 6 times every 24 hours (Fig. 3). When working with animals, all recommendations for bioethical standards were observed.

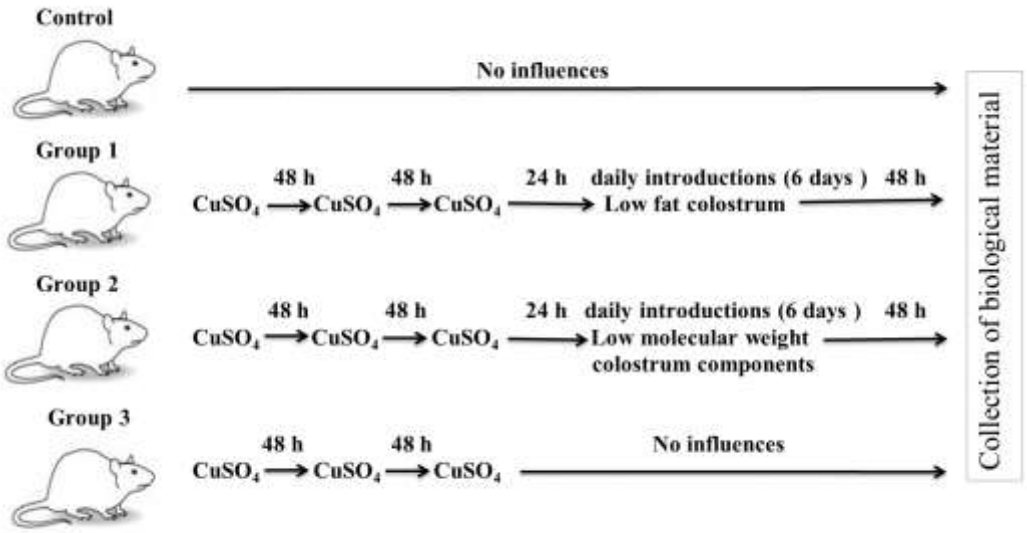

Fig.2. Scheme showing experimental groups of animals, which were sequentially injected with copper sulfate at a dose of 1 mg/100 g for the induction of fibrosis, followed by the administration of DC and LMC. The control variant was not subjected to any additional influences.

The body weight of the animals was determined daily, and after decapitation of the animals, which was carried out under ether anesthesia, blood was collected in tubes with K3 EDTA (3-substituted potassium salt of ethylenediaminetetraacetic acid) for further studies of hematological parameters on an automatic analyzer Mindray BC - 2008 Vet, (USA). The absolute content of leukocytes, lymphocytes, monocytes, granulocytes, erythrocytes, platelets, hemoglobin content, hematocrit, average volume of erythrocytes, erythrocyte anisocytosis, average concentration of hemoglobin in one erythrocyte, relative width of platelet distribution, average platelet volume and platelet volume were determined.

Each experimental group consisted of 6 rats. The results obtained were subjected to statistical analysis using the Exel program. Data in the work are presented as mean (x) and standard error (SE).

Research results.

Influence of exogenous copper ions on the morphology and growth dynamics of rats. Three-time sequential administration of copper sulfate to experimental animals at a dose of 1 mg / 100 g of body weight was accompanied by morphological changes in the liver tissue, which is characteristic of toxic liver damage, in particular: acute coagulation necrosis of hepatocytes in the perilobilar zones, apoptosis of some hepatocytes, dystrophic changes in hepatocytes (protein fatty degeneration). Intoxication with copper ions led to venous hyperemia, the presence of erythrocyte accumulations (aggregates) (Fig. 3 Aa, Ab).

Previously, it was shown that the intoxication of animals with copper sulfate was accompanied by a 30% increase in collagen content and the growth of connective tissue in the liver. [13]

Dysfunction of the liver was accompanied by a loss or delay in the growth of the body weight of the animals. So, if the body weight of the control group of animals increased almost linearly from 1 to 13 days of observation and at the end of the experiment it increased by 25 - 30% of the initial (Fig. 3B, curve 1), then in animals with Cu - induced liver fibrosis by at the end of the experiment, it remained at the level of the initial values (Fig. 3B, curve 2).

In the event that animals with Cu-induced liver fibrosis received DC *per os* at a dose of 0.1 mg / 100 g of body weight, then after growth retardation, from day 5 to 13, they slowly, but gained body weight, and by the end of observations, it increased by 10 - 15% of the initial one (Fig. 3 B, curve 3). If animals with Cu-induced liver fibrosis received LMC, then they increased their body weight with a higher rate, although they lagged behind the control group (Fig. 3B, curve 4).

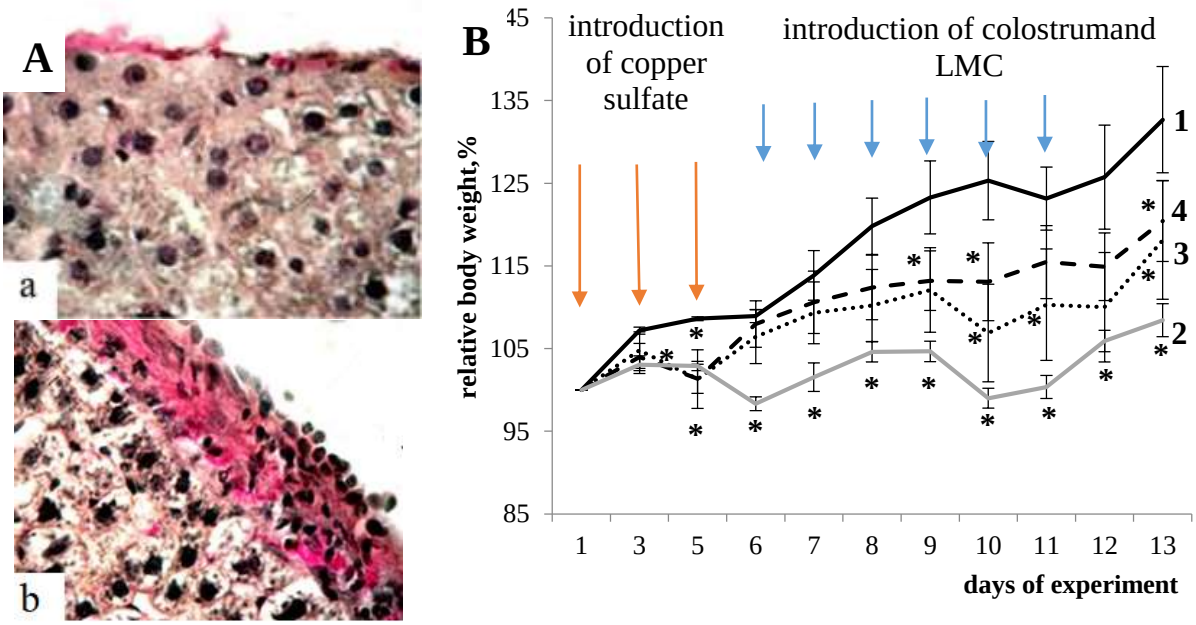

Fig. 3. ***A.*** *Micrographs of histological liver samples from control animals (**a**) and animals that were injected three times with copper sulfate at a dose of 1 mg / 100 g of body weight with an interval of 48 hours between injections (**b**). Magnification x 400 s.* ***B.*** *The dynamics of the body weight of control animals (1), animals with Cu - induced liver fibrosis (2), as well as animals with Cu - induced liver fibrosis, who received defatted colostrum at a dose of 0.1 mg / 100 g of body weight (3) and the group animals with fibrosis who received low molecular weight components of colostrum daily at a dose of 0.1 mg / 100 g of body weight for 6 days with an interval of 24 hours (4). Mean values from 6 animals in each experimental group are shown.*

* - differences were noted between the experimental groups in comparison with the control group for which p <0.05.

Consequently, colostrum intake partially restores body weight growth in experimental animals with Cu-induced liver fibrosis. LMC shows a greater effect in restoring this indicator as compared to DC. However, the full recovery of the growth rate of animals with liver fibrosis did not occur.

The results obtained convincingly confirm the fact that the inflammatory process in the liver, induced by intoxication with copper ions, is characterized by systemic changes at the level of the whole organism. In particular, a change in the functional activity of the digestive system, a shift in the balance of the redox system towards prooxidants [14, 15]. Despite numerous studies of the mechanisms of the development of inflammatory reactions, the questions of the integrative mechanisms of the response to the action of pathogenetic factors remain unresolved. In this case, the excess of copper ions, we believe that the bone marrow, producing blood cells and immunocompetent cells, is a systemic factor in the formation of inflammatory responses.

Hematological parameters in rats with Cu - induced liver fibrosis and after administration of colostrum components. The content of erythrocytes in experimental animals with Cu - ind. liver fibrosis did not differ from intact control. The content of hemoglobin, the average concentration of hemoglobin in the erythrocyte, hematocrit, and the average volume of the erythrocyte and anisocytosis of erythrocytes in animals with Cu - ind. liver fibrosis also remained at the control level (table).

Table

Erythrocyte count, average erythrocyte volume, hemoglobin content, hematocrit and erythrocyte anisocytosis in the intact control group, animals with Cu-induced fibrosis, and animals with Cu-induced fibrosis, who received DC and LMC per os at a dose of 0.1 mg / 100 g body weight. Mean values from three experiments and standard errors of the mean are presented.

Groups of experimental animals	INDICATORS				
	The number of erythrocytes, x10^9 l	Average volume of erythrocytes, fl	Hematocrit, %	Hemoglobin content, g/l	Erythrocyte anisocytosis, %
Intact control	8,52 ±0,37	55,00 ±3,09	46,60 ±0,70	146,00 ±3,79	14,73 ±1,88
Cu – ind. fibrosis	7,6 ±0,24	53,40 ±0,95	41,70 ±0,76	132,00 ±3,45	13,80 ±0,90
Cu – ind. fibrosis and DC injection	7,93 ±0,05	54,20 ±0,69	42,90 ±0,72	134,33 ±1,45	14,17 ±0,58
Cu – ind. fibrosis and the introduction of LMC	7,81 ±0,23	56,05 ±0,90	43,70 ±1,40	138,50 ±3,55	15,08 ±0,78

Administration to animals with Cu - ind. liver fibrosis DC and LMC in doses of 0.1 mg / 100 g of body weight did not affect the parameters of hematopoiesis (table).

Consequently, at the initial stages of the development of Cu-induced fibrosis, hematopoiesis remained within the normal range. Skim colostrum and low molecular weight components of colostrum also had no effect on hematopoiesis, at least when it corresponded to the control level.

The number of leukocytes in animals with Cu - ind. liver fibrosis remained within the control values (Fig. 4A). However, in animals with liver fibrosis, which received DC at a dose of 1 mg / 100 g of body weight for 6 days, the leukocyte content was increased by 80% compared with control (Fig. 4A). In the event that animals with liver fibrosis received LMC, then their leukocyte content was also increased by 59% in relation to the control (Fig. 4A). The manifestation of leukocytosis after the administration of colostrum components to experimental animals can be a positive effect against the background of intoxication, since this is accompanied by the activation of the cellular link of immunity.

Since leukocytes produce interferon and growth factors [16], this can positively affect the restoration of liver function against the background of fibrosis.

The content of lymphocytes in animals with Cu - ind. liver fibrosis was reduced compared to controls by 25% (Fig. 4B). If animals with fibrosis were injected with DC and LMC, then the lymphocyte content was increased in comparison with the control by 68 and 30%, respectively (Fig. 4B).

Consequently, DC caused a more pronounced effect of stimulating the number of leukocytes and lymphocytes compared to LMC.

The class of phagocytic mononuclear cells - monocytes in animals with Cu - ind. liver fibrosis did not differ from the control values (0.33 ± 0.09 and 0.4 ± 0.06). At the same time, administration of DC and LMC to animals with hepatic fibrosis was accompanied by a significant increase in this class of cells by 82 and 160%, respectively, compared with intact controls (Fig. C).

Consequently, LMC was 2 times more effective in increasing the number of monocytes compared to DC. Such a significant increase in phagocytic cells, which are able to remove destroyed cells, denatured proteins and circulating immune complexes from the body, is the most important stage in eliminating pathological processes in the liver.

When assessing the level of nonspecific reactivity of an organism, the index of the ratio of the number of lymphocytes to monocytes is often used. In intact control animals, it was 19.6 arbitrary units, in animals with liver fibrosis, it was significantly reduced to 12.0, after administration of DC to animals with fibrosis, it did not differ from the control level (18.1). However, after administration to animals with fibrosis of LMC, it was almost 2 times lower than the control and amounted to 9.7 arbitrary units.

Consequently, the administration of DC to animals after intoxication with copper sulfate is more preferable compared to LMC, at least for the regulation of the cellular link of immunity.

A relatively leukocytes large proportion is occupied by granulocytic cells, which include basophils, neutrophils and eosinophils. The total number of granulocytes in the control group was $2.0 * 10^9$ cells / l. It did not change in animals with Cu - ind. liver fibrosis ($2.4 * 10^9$ cells / l) (Fig.4D). However, after the administration of DC and LMC to experimental animals, their number increased 2.2 times compared to the control level (Fig. 4D).

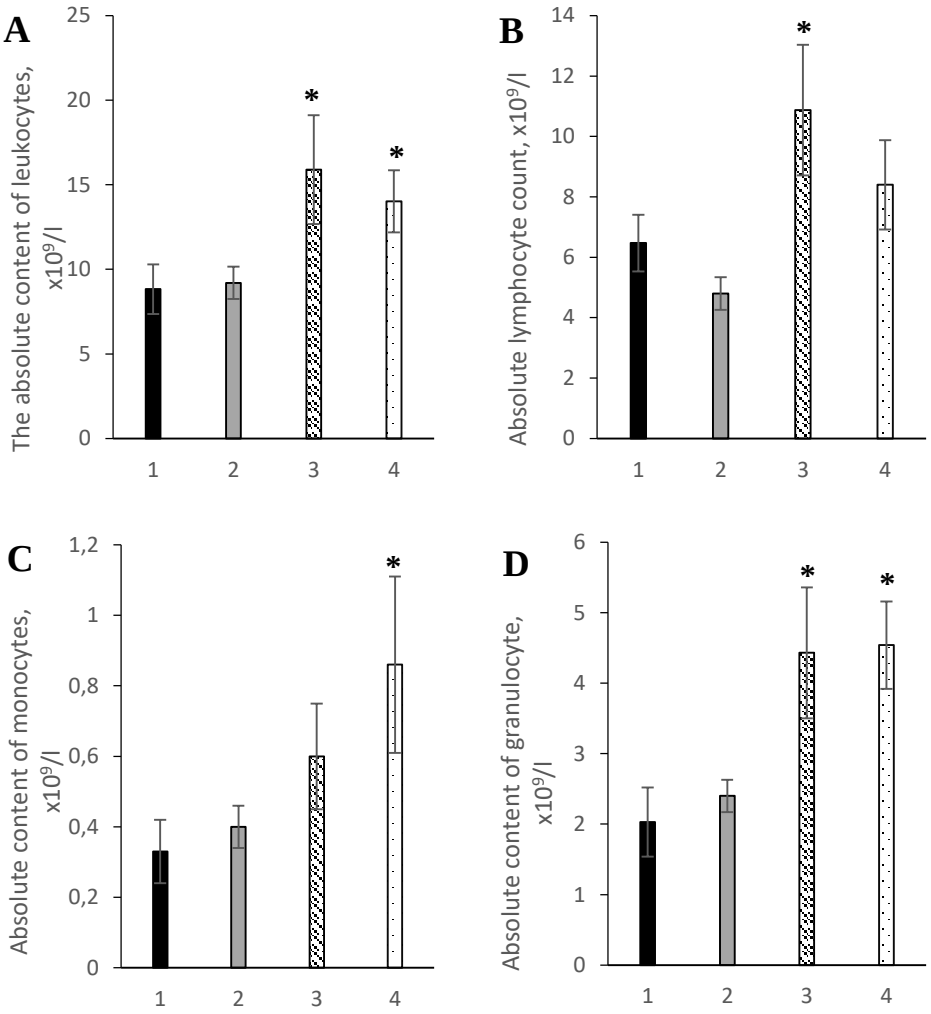

Fig.4. ***A.*** *The absolute content of leukocytes in the intact control group (1), the group of animals with Cu - induced liver fibrosis (2), in the group with Cu - ind. fibrosis, followed by 6-fold administration of DC at a dose of 1 mg / 100 g of body weight (3) and in the group with fibrosis, followed by 6-fold injection of LMC at a dose of 0.1 mg / 100 g of body weight (4);* ***B.*** *Absolute lymphocyte count in the same groups of animals;* ***C.*** *Absolute content of monocytes in the same groups of animals;* ***D.*** *Absolute content of granulocyte in the same groups of animals.*

It is known that granulocyte inducers are interleukins: 1,3,5, granulocyte-stimulating factor, granulocyte-monocyte factor, which are present in colostrum.

Consequently, the administration of colostrum components to animals with liver fibrosis stimulates the formation of immunocompetent cells that can ensure the restoration of the pathologically altered liver.

As it was revealed on histological preparations of the liver with the development of fibrosis, there was a change in the state of the vessels. In this regard, it is of great interest to assess the number and characteristics of platelets, as an indicator of blood coagulation.

It was found that in animals with Cu - ind. with liver fibrosis, the number of platelets was increased 2-fold compared to the control (Fig. 5A), that is, thrombocytosis took place. The causes of thrombocytosis are very diverse, but they are based on an increase in cytokines that stimulate platelet formation.

Thrombocytosis is known to increase the likelihood of blood clots. If animals with fibrosis received DC for 6 days, then the platelet count remained 50% higher than the intact control, but was 26% less than with fibrosis (Fig.5A). LMC had a less pronounced effect of reducing the number of platelets in animals with fibrosis compared with DC (only by 15%) (Fig. 5A).

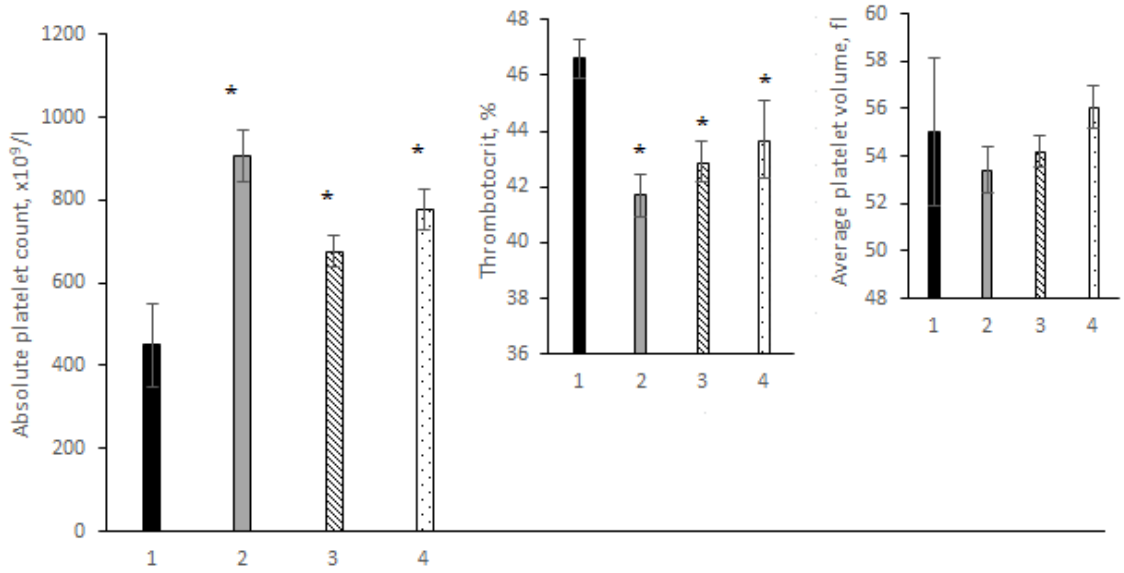

Fig. 5. A. The number of platelets in the intact control group (1), the group of animals with Cu - induced liver fibrosis (2), in the group with Cu - ind. fibrosis, followed by 6-fold administration of DC at a dose of 1 mg / 100 g of body weight (3) and in the group with fibrosis, followed by 6-fold injection of LMC at a dose of 0.1 mg / 100 g of body weight (4); B. Thrombokrit in the same groups of animals; C. the average volume of platelets in the same groups of animals. Mean values and standard errors are given.

* - differences were noted between the control and experimental groups, for which $p < 0.05$.

Consequently, the intake of DC provided a decrease in thrombocytosis, which took place in Cu - ind. liver fibrosis. In favor of the different effect of DC and LMC on platelet formation, data on thrombocrit indicators testify. So, thrombocritis in rats with Cu - ind. liver fibrosis was increased in comparison with the control level by 88%; after the animals with fibrosis received DC, this indicator decreased by 21%, although it remained somewhat higher compared to the control, whereas in the case of receiving LMC by animals, it did not change (Fig. 5B).

The change in thrombocyte was due to an increase in the number of platelets, while maintaining their volume (Fig. 5C).

Discussion

It was previously thought that the induction of fibrogenesis leads to irreversible chronic conditions [17]. Intensive studies in recent years have shown that liver fibrosis can be reversible, however, the features and mechanisms of this process have not been finally established. Understanding the process of loss of the liver's ability to regenerate and remove the formed connective tissue elements in response to tissue damage is a fundamental problem in biomedical research. It is quite obvious that its solution will allow developing methods of treating chronic pathologies and understanding the basic mechanisms of age-related pathologies that have a direct connection with them.

We believe that the problem of "reversible ↔ irreversible" metabolic states depends primarily on the stages of the process development. This can be explained by the fact that at the late (terminal) stages of a metabolic process, metabolic and / or epigenetic memory is formed, which has an adaptive character and, with long-term maintenance, "passes" into a state of "self-support", that is, the process becomes irreversible. At the initial stages of the formation of an adaptive response, which is fibrosis, until the "self-support" system is established, the process can be reversible, that is, a shift of the "degradation ↔ regeneration" equilibrium towards regeneration and restoration of the "previous" homeostatic balance in the body is ensured. Natural biologically active compounds, in particular colostrum components, can act as factors capable of shifting the balance. Colostrum contains a unique natural set of biologically active compounds of a wide spectrum of action, and they can have a systemic effect on the regulation of the process in the body [10, 18].

The effectiveness of the process of restoring the functional activity of the fibrotic-altered liver will depend on the systemic response of the body to the action of the pathogenetic factor. In the formation of the systemic response, the bone marrow plays a central, coordinating (integrative) role as a producer of blood cells and, first of all, immunocompetent cells [19]. Actively phagocytic cells not only remove apoptotic or necrotic cells and circulating immune complexes [20], but are also producers of a wide range of cytokines, low molecular weight regulatory peptides, and other biologically active substances [21]. These biologically active substances take both direct and indirect participation in enhancing the processes of liver regeneration and restoration of its function and other body systems.

The results of this work and previously obtained data on the study of the mechanisms of Cu-induced liver fibrosis indicate and indicate that:

1) 24 hours after the last three times administration of copper sulfate to experimental animals, liver fibrosis develops, which was at the initial stage of development [7]. This is evidenced by moderate morphological changes in the liver, as well as intensive growth of the

liver capsule [22]. These changes in the liver tissue occurred against the background of an increase in the content of lipid hydroperoxides and inhibition of the activity of glutathione peroxidase, aconitase, and other enzymes [14].

2) the reaction of the bone marrow manifests itself insignificantly to these relatively small homeostatic changes at the initial stages of the liver fibrosis development, as evidenced by the preservation of the number of erythrocytes and their structural and functional characteristics within the control values. The results obtained suggest that such a response of the bone marrow to the presence of liver fibrosis will not have an effect on its elimination; and the fibrosis will intensify and progress to the next stage, forming cirrhotic changes in the liver. In other words, relatively small functional changes in the liver that take place in our case may remain "insignificant" for other regulatory systems of the body, in particular for the bone marrow; and the liver can move to the next stage of pathogenesis (Fig. 6).

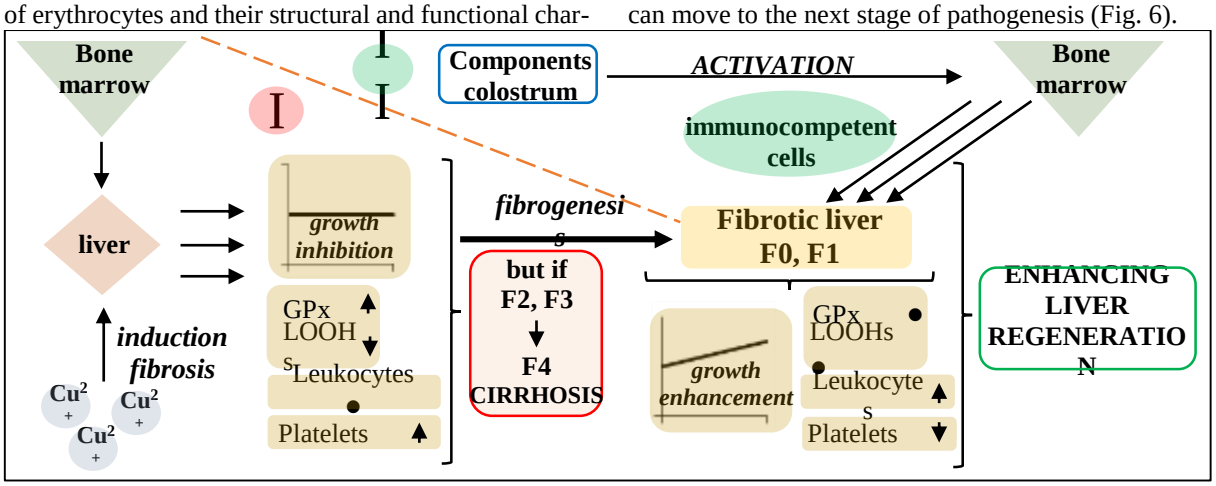

*Fig. 6. Scheme that demonstrates different strategies for the development of liver fibrosis: **I** Introduction of copper ions, which is localized in the liver, induces oxidative stress, an increase in the content of lipyl hydroperoxides (LOOHs), inhibition of antioxidant enzymes (GPx), an increase in the number of platelets, inhibition of animal growth, while bone growth the brain does not "react" to these changes, as evidenced by the preservation of the number of immunocompetent cells at a constant level. **II** In the event that colostrum components are administered to animals with fibrosis, they activate the function of the bone marrow, which is accompanied by a pronounced increase in the content of immunocompetent cells in the bloodstream, elimination of oxidative stress and restoration of body weight growth, which may indicate an increase in the regeneration process of the fibrous liver.*

3) if at the initial stages of the development of Cu-induced fibrosis experimental animals received the components of colostrum, then these components caused a pronounced response at the bone marrow level, which was expressed in an increase in the total number of leukocytes (by 59-80%), lymphocytes (by 30-68%), monocytes (82-160%), granulocytes (62%); at the same time, the number of platelets decreased and approached the control values.

4) these changes caused by the components of colostrum differ quantitatively depending on the composition of these components. This indicates that different regulatory effects can be exerted on bone marrow cells by using different methods for fractionating whole colostrum.

5) changes in induction at the level of hematopoiesis is accompanied by restoration of the activity of antioxidant enzymes, restoration of the immune system [9], restoration of the ability of animals with Cu-induced liver fibrosis to perform work [7] and is accompanied by a slight increase in body weight growth (Fig. 6).

The results obtained suggest that if there are minor structural and functional changes in the liver, which can be relatively easily compensated by hypertrophy of hepatocytes, then such changes will not affect the compensatory functions of the bone marrow, which will not cause a systemic response at the level of the organism. In this case, these structural and functional changes will be “fixed” in the liver, and the balance in the system of “fibrotic changes” will be preserved due to the activation of stellate cells. The rest of the metabolic systems "adjust" (adapt) to the new functional state, since it is not at this stage of development dangerous, and after a certain time (long enough) the next stage of fibrosis will be formed, which will be irreversible due to the fact that most of the body systems “adapted”, that is, formed the mechanisms of“ self-maintenance ”of such a state.

If, at the initial stages of fibrosis development, an “additional” signal is provided by colostrum components to the bone marrow, the activity of immunocompetent cells and the possible activation of other body systems, including the intestinal microbiota, are increased, then the balance can be shifted towards the regeneration of liver cells and ensure the reversibility of fibrotic changes.

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