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Vitamin A Reduces the Mortality of Animals with Induced Liver Fibrosis by Providing a Multi-level Body Defense System

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Background: **Liver diseases remain the most important medical and biological problem. Works devoted to the study of the vitamin A role have shown conflicting results of its effect on the fibrosis development. We tested the hypothesis that an increase of the copper content in the liver, an example of which is Wilson's disease, shifts the balance in the redox system towards pro-oxidants, which leads to the antioxidant systems inhibition, including a decrease in the vitamin A content; this affects the levels of liver function regulation and the development of fibrosis.** ***Methods:*** **In animals with Cu-induced liver fibrosis, neutrophil activity, the immunocompetent cells content, the activity of alanine aminotransferase and γ-glutamylaminotransferase, the content of urea and creatinine in blood serum, as well as the vitamin A content in the liver, copper ions and its regenerative potential were determined.** ***Results:*** **It was found that three consecutive injections of copper sulfate to animals with an interval of 48 h between injections led to the death of 40% of the animals, and 60% showed resistance. The content of vitamin A in "resistant" animals at the beginning of the development of the fibrosis was reduced by 4 times compared to the control, the functional activity of the liver was somewhat reduced, and a connective tissue capsule was formed around the liver lobes in 75% of the animals. If animals with the initial stage of liver fibrosis received daily vitamin A at a dose of 300 IU/100 g of body weight, which was accompanied by its multiple increase in the liver (15 times on day 14), the mortality of animals decreased by almost 7 times, the functional activity of the liver did not differ from control. In the blood of these animals, the number of leukocytes, granulocytes, and monocytes was increased and phagocytic activity was increased. At the same time, the connective tissue capsule was developed more intensively than in animals receiving only copper sulfate, and was detected in 91% of the animals. Fragments of the liver, even more than in the case of fibrosis, lost the ability to regenerate in culture.** ***Conclusion:*** **We came to the conclusion that vitamin A leads to the connective tissue "specialization" formation of the liver and triggers vicious circles of metabolism and includes several levels of regulation systems. Further studies of the vitamin A effect mechanisms on the liver with fibrosis will allow the use of this antioxidant in the treatment.** **(J Clin Exp Hepatol xxxx;xxx:xxx)**

According to statistics, mortality from liver diseases has been ranked 5th in the structure of the world's population death rate.[1] However, pathological changes in the liver are much more common than they are diagnosed. This is due to several reasons: there are no pain symptoms in the early stages of the pathological process development; the process of fibrosis formation and its transition to cirrhosis can be slow and take several years. This feature depends on the efficiency of liver tissue regeneration, or more precisely, on maintaining a balance between the processes of regeneration and destruction of liver tissue. In the event that the balance in the "regeneration ↔ destruction" system is shifted "to the left", then the emerging pathological process will be reversible and the functional activity of the liver will be restored. And vice versa, the inflammatory process "transitions" to fibrosis with a possible subsequent transition to irreversible cirrhosis when the balance shifts "to the right". The essence of the problem solving of liver pathology can be reduced to understanding the processes of regulation in the system "regeneration ↔ destruction" and the development of tools and methods for managing these processes. Currently, several approaches are used to solve this

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Abbreviations: ALT: alanine aminotransferase; CP: ceruloplasmin; GGT: glutamylaminotransferase; Hps: chaperon; HSC: hepatic stellate cells; MT: metallothionein; P: proteins; ROS: reactive oxygen species; TOP: temporal optimality
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problem: elimination of destruction factors; enhancement of regenerative processes in the liver; and improvement of the liver cells' functional activity.[2–4] Despite the great relevance of the problem and the large amount of ongoing studies, the treatment of liver pathologies remains ineffective.[5] Is considered, that this may be due to the fact that it is rather difficult or impossible to eliminate the factors of liver tissue destruction, since they are extremely diverse (different types of radiation, a wide range of chemical compounds, infectious agents, drugs and even components contained in food), and the most important thing is the mechanisms of liver regeneration against the background of the development of pathologies of this organ have not been fully studied.

One of the unresolved aspects of liver regeneration processes will be considered.

Since the liver has a leading role in the metabolism of the whole organism and the maintenance of homeostasis, a multilevel, hierarchical system of liver function regulation has evolved in the course of evolution. The main functional element of the liver is the hepatocyte, which is capable of proliferation,[6] polyploidization,[7] and transformation,[8] and, with slight changes in functional activity, it can regulate homeostasis.

In the case of an increase or chronic effect of toxic factors to the liver, in particular copper ions, a complex of reactions is activated that manifests itself as an increase in the products of free radical reactions, the synthesis of stress proteins, the activation of liver stellate cells, which are residents of mesenchymal stem cells, and an increase in the functional activity of the bone marrow, which ensures the regulation of the immune system activity and other body systems. The elements of this complex hierarchical system of regulation are being actively studied using various models and various methods. However, there is a lack of comprehensive research to understand the mechanism for maintaining equilibrium in the "regeneration ↔ destruction" system.

Due to this fact, the development of models that would solve the problem of regulating the functional activity of the liver is an urgent task, since it is aimed at solving the problem of regulating metabolism and treating liver diseases.

In responding to this problem, two well-known factors were taken into account. First, the basis of the functional rearrangements of the liver, which are caused by pathogenetic factors, is the shift in the balance of "pro-oxidants ↔ antioxidants" towards pro-oxidants. As is known, the redox system is an evolutionarily ancient system of biological systems regulation,[9] and it provides not only the development of pathologies, but also the integration of various body systems into one system. An important link in the formation of the characteristics of a redox system are metal ions with variable valence[10] and, in particular, copper ions. Previously, it was shown that repeated sequential injection of copper sulfate to rats was accompanied by inhibition of the antioxidant enzymes activity and an increase in lipid hydroperoxides, i.e. induced oxidative stress.[11]

Secondly, in recent years, intensive research has been carried out on the role of stellate cells in the processes of liver regeneration and their leading role in the development of fibrosis has been proven, that is, the shift in the "regeneration ↔ destruction" balance, which can be determined by the functional orientation of stellate cells (synthesis of collagen or metalloproteins). However, what factors determine this direction is still not clear. At the same time, however, stellate cells are known to accumulate vitamin A, that is, they are a place for its deposition.[12] Vitamin A may play an important role in the functional activity of stellate cells. It can be assumed that the chronic effect of copper ions, accumulating in the liver, may also affect the metabolism of vitamin A, which in turn may affect the functional characteristics of the liver. The injection of vitamin A into animals against the background of the Cu-induced liver fibrosis development can provide an answer to the question of the role of vitamin A in the injection of fibrosis and contribute to understanding the mechanisms of the functional activity regulation of the liver.

Finally, vitamin A is an active antioxidant and its injection into the body against the background of the prooxidant action of copper ions can shift the balance towards antioxidants and have a positive effect on the functional activity of the liver. In this regard, we tested the hypothesis that one of the options for the development of fibrosis may be associated with an increase in the content of copper ions in the liver, they shift the balance in the redox system towards pro-oxidants, and this leads to inhibition of antioxidant systems, including a decrease in the content of vitamin A, the vitamin A content in stellate cells decreases, which is accompanied by the launch of fibrogenesis. The bone marrow and cellular immune system respond actively to this background, ensuring that the body's overall homeostasis is maintained.

In order to verify this hypothesis, liver fibrosis was induced in a group of experimental rats by repeated injection of copper sulfate, after which vitamin A was injected *per os* daily at a dose of 300 IU/100 g of body weight and a number of physiological parameters (change in body weight, anatomical and histological changes in the liver, the ability of the liver to regenerate in an organotypic culture, the time of death of animals) and biochemical indicators of liver activity (the content of copper ions and vitamin A in the liver, the activity of alanine aminotransferase and glutaminotransferase, the content of urea and creatinine in blood serum) were determined, as well as the number of immunocompetent cells and the activity of the neutrophils phagocytosis.

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MATERIAL AND METHODS

Experimental Facilities

Experiments on laboratory animals, including assessing the effect of copper sulfate, were carried out in agreement with the bioethical committee of V.N. Karazin Kharkiv National University, which is guided by the provisions of the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Strasbourg, March 18, 1986).[13]

The experiments were carried out on young mature (3-month-old) male *Wistar* rats. The animals were kept under standard vivarium conditions throughout the experiment, with free access to food and water. The sample of experimental animals was randomized.

In order to induce liver fibrosis, we have to carry out the experimental group of animals (n = 68) intraperitoneal injections of copper sulfate three times with an interval between injections of 48 h (Figure 1II), as described in Ref. 14. The control (intact) group (n = 27) was not subjected to any influences, and instead of copper sulfate they received saline (Figure 1, group I).

ptLater, to simulate the state of "hypervitaminosis against the background of fibrotic changes" in the liver of animals after intoxication with copper sulfate, they were injected with *per os* oil solution of vitamin A at a concentration of 300 IU per 100 g of body weight (90 μg/100 g of body weight) daily for 21 days (III). The dose was established by us on a model of liver fibrosis, which provided a therapeutic effect and did not lead to toxic manifestations in preliminary studies. An additional group of animals (n = 34) that received only vitamin A (300 IU/100 g body weight) was used as a control for the fibrosis group treated with vitamin A (Figure 1). After 4, 7, 14, and 21 days of vitamin A injection, 3–5 rats were removed from each group, depending on the series of the experiment for material sampling.

Material Sampling

Animal Decapitation

The animals were decapitated under ether anesthesia. After decapitation, the presence and degree of development of

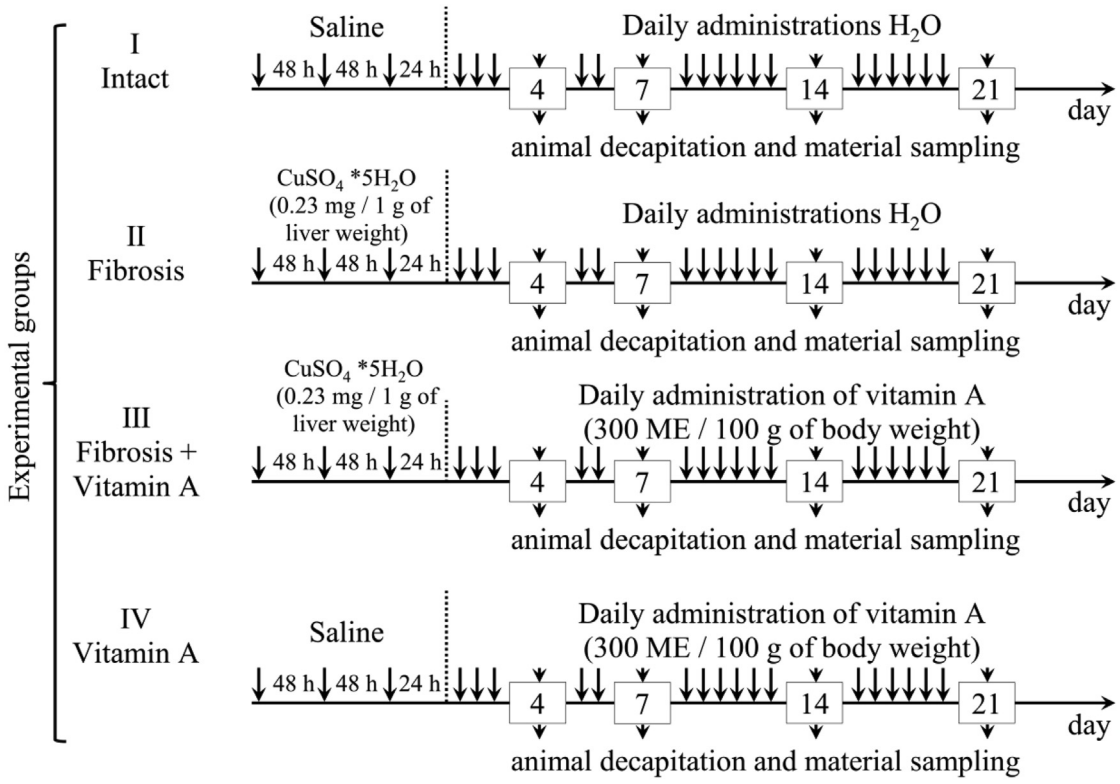

Figure 1 The scheme demonstrates manipulations with various experimental groups of animals: I – a group of intact animals (n = 27) – instead of intraperitoneal injections of copper sulfate (fibrosis inducer) and subsequent injection of vitamin A *per os*, they received saline and water, respectively; II – a group of animals (n = 34) – they received copper sulfate three times (1 mg/100 g of body weight or 1 mg/100 g of body weight) (fibrosis induction), and after 24 h – water for 21 days; III – a group of animals (n = 34) – they received copper sulfate three times as a group II (1 mg/100 g of body weight or 1 mg/100 g of body weight), and after 24 h they received injections of vitamin A (300 IU/100 g body weight); IV – a group of animals (n = 34), which received a saline solution instead of the injection of copper sulfate, followed by the injection of vitamin A (300 IU/100 g of body weight) for 21 days; every 4, 7, 14, and 21 days, 3–5 rats were removed from each group, depending on the series of the experiment, to determine the studied parameters.

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the connective tissue between the liver lobes and adipose tissue were assessed, this was expressed in arbitrary units. The first drops of blood were collected in tubes with EDTA (3-substituted potassium salt of ethylenediaminetetraacetic acid) to study hematological parameters, which were evaluated on an automatic analyzer Mindray BC-2800 Vet. (USA),[15] and in test tubes with heparin to assess the phagocytic activity of neutrophils[16]; and also received blood serum. For this, the samples were centrifuged after clot formation at 1500 g for 20 min.

Collection and Use of Liver Samples

To take histological samples, liver fragments were taken and fixed in a 10% formalin solution. Samples were routed as described in Ref. 17.

The rest of the liver was divided into three parts. The first part was used to assess liver regeneration *in vitro* culture according to the method of V.S. Khavinson improved by us and Chalisova N.I.[18] In the second part, the content of copper ions was determined. In the third part, the content of vitamin A was determined.[19]

ANALYTICAL METHODS

Neutrophil Activity

The activity of neutrophils was determined according to the method of total redox activity in the nitroblue tetrazolium reduction test (NBT test),[20] which is based on the ability of neutrophils to absorb nitroblue tetrazolium dye (NBT) and reduce its granules to insoluble diformazan in the form of blue granules under the influence of superoxide anion formed in NADPH oxidase reaction, inducing the process of phagocytosis stimulation. The NBT test characterizes the oxygen-dependent anti-infective systems of the phagocyte.

Alanine Aminotransferase Activity in Serum

The activity of alanine aminotransferase (ALT) (EC 2.6.1.2) in blood serum was determined as described in Ref. 21. ALT catalyzes the transition of the amino group from L-alanine to α-ketoglutarate, which leads to the formation of pyruvate and L-glutamate. The resulting rate in absorption decrease is proportional to ALT activity. The measurements were carried out on a STAT-FAX 1908 spectrophotometer (USA).

γ-Glutamylaminotransferase Activity in Serum

The method for determining γ-glutamylaminotransferase (GGT) activity[22] is based on the fact that γ-glutamylaminotransferase (EC 2.3.2.2) catalyzes the transition of the glutamyl group L-γ-glutamylcarboxy-4-nitroanilide to glycylglycine with the formation of 5-amino-2-nitrobenzoate, the amount of which is directly proportional to GGT activity and is measured kinetically at a wavelength 405 nm (STAT-FAX 1908, USA).

Urea Content in Serum

The concentration of urea in the blood serum of the experimental groups of animals was determined as described in Ref. 23. Urea is hydrolyzed by urease to form ammonia and carbon dioxide. The resulting ammonia reacts with α-ketoglutarate in the NADH presence, resulting in the formation of glutamate. Oxidation of NADH in the reaction leads to a decrease in absorption at 340 nm, which is proportional to the urea content. The test samples were incubated at 37 °C and 30 s, at 60 s determination time (STAT-FAX 1908, USA).

Serum Creatinine Content

The creatinine content was determined according to the described method.[24] Creatinine in this case reacts with picric acid in an alkaline environment and forms a colored compound with absorption at a wavelength of 510 nm (STAT FAX 1908, USA). The level of formation of a color compound is proportional to the level of creatinine in the sample.

Assessment of Liver Regeneration *In Vitro* Culture

The organotypic culture of the liver was obtained according to the method of V.S. Khavinson modified by us and Chalisova N.I.[18] Liver fragments 1 × 1 cm in size were placed in a solution of 0.9% sodium chloride (Yuriya Pharm, Ukraine). The fragments were cooled to a temperature of +26 °C. Cylindrical explants were isolated and placed in sterile disposable plastic Petri dishes (Biosigma, Italy) on a nutrient medium (90% DMEM/F12 medium, 10% fetal bovine serum (Biowest, France), 50 μg/ml of gentamicin (Zdorovye, Ukraine) and amphotericin (BioloT, Russia)), incubated for 48 h at 37.0 ± 0.5 °C under conditions of hypoxia and elevated carbon dioxide concentration. The degree of adhesion (DA) was calculated – the ratio of the number of attached explants to the total number of explants. For this purpose, the area of the central zone (explant zone) and the peripheral zone (exclusion and growth zone) were determined on photographs of the obtained cultures according to the formula:

$$AI\ (\%) = \left(\frac{CZ + GZ}{CZ} \times 100\ \%\right) - 100\ \%$$

where: *CZ* is the area of the central zone (explant), and *GZ* is the growth zone; in mm^2 or pixels.

Determination of the Content of Copper Ions in Biological Samples

The content of copper ions in intracellular fractions of the liver was determined by atomic absorption spectrometry

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A

Groups	N gen. pcs	Number survivors, pcs	Number deaths, %	Connective tissue formations#, % of the total number of animals
I Intact	27	27	0	norm 100; formations 0
II $CuSO_4$ *$5H_2O$	34	20	41	norm 25; formations 75
III CuSO4 *$5H_2O$ &Vit A	34	32	6	norm 9; formations 91
IV Vit A	34	34	0	norm 100; formations 0

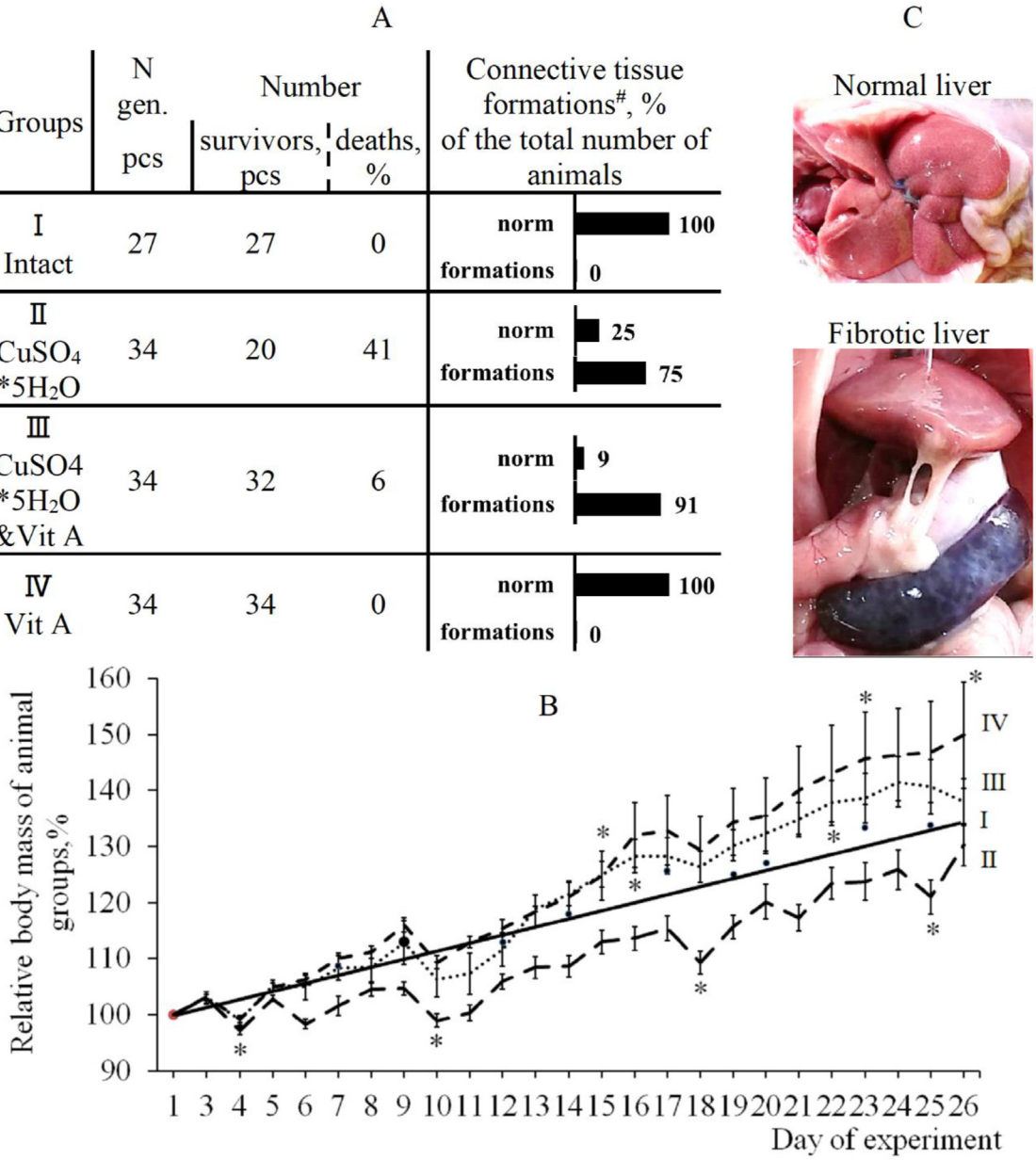

Figure 2 Anatomical changes in the group of control animals (I, n = 27); in the group of animals treated three times with copper sulfate at a dose of 1 mg/100 g of body weight with an interval of 48 h (II, n = 34); in the group of animals treated with copper sulfate and vitamin A, similarly to groups II and IV (III, n = 34); and in the group of animals receiving daily vitamin A at a dose of 300 IU/100 g body weight (IV, n = 34): A – the number of animals that survived after copper sulfate intoxication, as well as the presence of connective tissue formations around the liver as a percentage of the total number of animals in the group; B – change in body weight as a percentage of the original; C – the appearance of the liver in the group of intact animals without connective tissue formations (normal) and the appearance of the liver in the presence of connective tissue formations and the formation of a common capsule around the liver lobes (fibrotic); * – significant values were noted ($P < 0.05$) compared with the intact level (Mann–Whitney U test); # – connective tissue formations of animals that survived to decapitation; animals that died during the experimental part were not taken into account.

(AAS) with electrothermal atomization on a KAS 120 type C 115-MI spectrophotometer (Ukraine). The copper content was determined by pre-drying the liver and dissolving 50 μg of the sample in 2 ml of a mixture of sulfuric and nitric acids (1:1), as described. The principle of the AAS method is based on measuring the absorption level of light rays passing through the atomic vapor of the sample (324.7 nm for Cu^{2+}). The ohmic resistance of the graphite cell (atomizer) during the passage of current ensures its heating up to 2500 °C; this temperature is necessary for the transition of Cu^{2+} to the gas state.

Definition of Vitamin A Content

The content of vitamin A in the liver was determined according to the well-known method[19] which is based on the complex formation of the vitamin with boron

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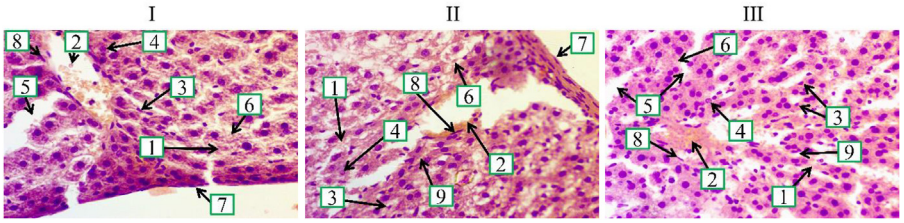

Figure 3 Micrographs of liver preparations of intact control animals (I); experimental animals that were injected three times with copper sulfate at a dose of 1 mg/100 g of body weight with an interval of 48 h between injections (II); animals that received copper sulfate (1 mg/100 g body weight) three times and then vitamin A (300 IU/100 g body weight) 24 h later (III); microscope GRANIUM R6003, camera SIGETA M3CVOS 14000, magnification 400×; the following structures were noted: 1 – discomplexation of the hepatic beams, 2 – blood vessel, 3 – Ito cells, 4 – lymphocytes, 5 – multiple tissue ruptures, 6 – autolysis of hepatocytes, 7 – liver capsule, 8 – endotheliocytes, 9 – Disse space.

trifluoride etherate and determination of the this complex decomposition rate.

Statistical Analyses

The data are presented as group means and standard error ($x \pm SE$). Data analysis was performed using Excel 2013 (Microsoft Corporation., USA) and STATISTICA 8 (Statsoft, USA). Visualization was performed using the Microsoft Excel software package 2013. Significant differences between groups according to the data: body weight dynamics were determined using the non-parametric Mann–Whitney U test; changes in biochemical parameters and vitamin content – r/ANOVA; indicators for assessing the regenerative potential of the liver – the Mann Whitney U test for the area index and the Fisher test (selective shares) for the degree of adhesion. Differences between the control and experimental groups were considered significant at $P < 0.05$.

RESULTS

Toxic Effect of Copper Ions and the Effect of Vitamin A on Some Physiological and Anatomical Parameters

Multiple successive injections of copper sulfate to rats (Figure 1, II) led to the death of a relatively large number of experimental animals treated with copper sulfate: in a group of 34 animals, 41% died during the observation period (21 days) (Figure 2A, group II). The body weight in this group of animals in the first few days after the start of the copper sulfate injection decreased, and after 5–6 days it began to recover (Figure 2B, curve II); but the animals of this group lagged behind in growth compared to the control animals throughout all days of observation (Figure 2B, curve I&II).

As noted earlier, multiple consecutive injections of copper sulfate led to the growth of connective tissue around the liver, i.e. manifestation of "adhesive" disease.[25] Thus, in 75% of the surviving animals in the group treated with copper sulfate (group II), an intensive synthesis of connective tissue was observed, which was accompanied by the formation of adhesions between the lobes of the liver and the formation of a common capsule from the connective tissue around the liver, and in some cases, the spleen (Figure 2C, fibrotic liver). Along with this, there were no adhesions around the liver in 25% of the animals treated with copper sulfate; anatomically, the liver did not differ from the control (Figure 2C, normal liver).

It is known that Ito cells are actively involved in the induction of fibrogenesis.[26] Vitamin A is involved in the activation of these liver cells.[27] In the next series of experiments, 34 rats were daily injected vitamin A for 21 days at a dose of 300 IU/100 g of body weight. It was found that after 14 days from the start of the experiment, the animals began to intensively increase their body weight and exceeded the control by 20–30% (Figure 2A, group IV), and all 34 animals in this group survived (Figure 2B, curve IV). No anatomical changes, including the induction of connective tissue growth, were found in the liver region of animals treated with vitamin A.

In the event that, after three injections of copper sulfate, the animals were given vitamin A daily, the number of dead animals was no more than 6% (Figure 2B, curve III), i.e. 6.8 times less compared to the group of animals that received only copper sulfate (Figure 2B, curve II). At the same time, the growth rate of animals that received copper and vitamin A (III) exceeded the intact control (I) and significantly exceeded the group of animals that received only copper sulfate (II) and did not differ from animals that received only vitamin A (IV) (Figure 2A).

At the same time, in the group of animals with a triple injection of copper sulfate and subsequent daily injection of vitamin A (III), 91% of surviving animals showed an intensive growth of connective tissue, which was superior to that in animals that received only copper sulfate (Figure 2B,C).

Consequently, repeated sequential injection of vitamin A to animals after intoxication with copper sulfate significantly reduced the mortality of animals, and the amount of connective tissue around the liver was increased in surviving animals, and these animals outperformed the control in terms of body weight growth. It can be assumed that the growth of connective tissue or "adhesive" disease is a manifestation of the adaptive reaction of the body.

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Table 1 The Content of Copper Ions in the Liver Tissue, Fractions of Mitochondria and Endoplasmic Reticulum.

Test sample	Group	
	I Intact	II $CuSO_4$ $*5H_2O$
	X ± SE	
Liver tissue, mcg/1 g tissue dry matter	0.048 ± 0.002	*8.040 ± 0.900
Fraction of mitochondria, mcg/1 g protein	0.037 ± 0.008	*1.800 ± 0.340
Fraction of endoplasmic reticulum, mcg/1 g protein	0.040 ± 0.0037	*0.170 ± 0.050

*Significant values ($P < 0.05$) compared with the intact level (ANOVA) are noted.

Morphology of the Liver and Signs of Fibrosis

Intoxication with copper ions was accompanied by morphological changes in the liver tissues, which are characteristic of toxic liver damage (Figure 3, group I&II). In particular, partial autolysis of hepatocytes is observed (6); often there is a discomplexation of the hepatic beams (1); the vessels are filled with blood (2); the Disse space is reduced (9) and Ito cells are quite common in it (3); endothelium dystrophic, discontinuous (8); especially pronounced thickening of the capsule compared to intact animals (7) (Figure 3, group I&II).

In the case when animals with induced fibrosis received vitamin A (doses of 300 IU/100 g), more pronounced changes were observed compared with liver fibrosis (Figure 3, group III). Along with this, there was a decrease in the turgor of the liver tissues, which is a direct sign of the manifestation of organ ischemia, which is an indirect sign of the accumulation in the tissues and cells of the liver of an increased content of toxins (metabolites, metal ions, etc.). These changes indicate the activation of the immune system after the injection of vitamin A against the background of Cu-induced fibrosis; it can be assumed that the injection of vitamin A enhances the induction of the body's adaptive response to the action of toxic compounds.

Relationship between Copper Ion Content and Vitamin A Content

The liver of intact control animals contained a small amount of copper ions, which was evenly distributed between the mitochondria and the membranes of the endoplasmic reticulum (Table 1, group I). After a triple injection of copper sulfate with an interval of 48 h between injections, the content of copper ions in the liver tissue increased more than 150 times (Table 1, group II). At the same time, the nature of the distribution of copper in the cell compartments changed. So, in the fraction of mitochondria, significantly more copper ions accumulated than in the fraction of the endoplasmic reticulum (Table 1, group II).

Against the background of an increase in copper ions in the liver, the content of vitamin A decreased by 4 times in the group with Cu-induced fibrosis (Figure 4A, curve II) compared with the intact level four days after the last injection of copper sulfate. Later, after 7 days, the content of vitamin A increased and remained below the control values, and on the 14th day, its content did not differ from the control (Figure 4A, curve II). However, on the 21st day after the injection of copper sulfate, the content of vitamin A was again below the control values by 37%, while the differences between the control and the experiment were not significant (Figure 4A, curve II).

Daily injection of vitamin A to intact animals was accompanied by a linear increase in its content in the liver up to 7 days, after which the rate of vitamin deposition slowed down from days 7 to 14, and further its content in the liver decreased against the background of daily injections of vitamin A (Figure 4C, curve Ⅳ). After that, despite the continued intake of vitamin A in the body, its content in the liver decreased (Figure 4C, curve Ⅳ).

In the event that vitamin A was injected in the same dose and according to the same scheme to animals with Cu-induced liver fibrosis, then its content on the 4th day from the start of injection was 2 times lower compared to animals that received vitamin A (Figure 4C, curve III). However, if in the group of control animals the content of vitamin A on the 7th day of daily injections increased 10 times from the initial level, then in animals with fibrosis it increased by 15 times over this period from the initial level, while the absolute values of the control and experimental groups did not differ from each other (Figure 4C). Differences between the studied groups after 14 and 21 days of the experiment were not detected.

Consequently, an increase in the content of copper ions in the liver was accompanied by a decrease in the content of vitamin A. And the daily injection of this vitamin to animals with Cu-induced fibrosis was accompanied by a lag in the rate of vitamin A accumulation in the liver from control animals in the first 4 days, and later due to a higher relative rate accumulation of vitamin A in the liver with fibrosis, its absolute content did not differ from the group of intact control animals treated with vitamin A.

The Ability of Liver Tissue to Regenerate in Model Experiments

In the first series of experiments, the dynamics of liver fragments' adhesion to the culture vessel (plastic) was

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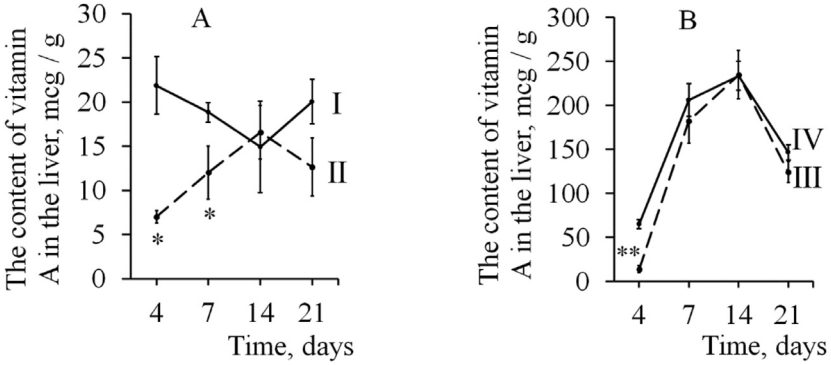

Figure 4 The content of vitamin A in the liver tissue (A and B) in intact control animals (I); in experimental animals, which were injected three times with copper sulfate at a dose of 1 mg/100 g of body weight with an interval of 48 h between injections (II); in animals that received copper sulfate (1 mg/100 g body weight) three times, and vitamin A (300 IU/100 g body weight) in 24 h later (III); intact animals injected with vitamin A (300 IU/100 g body weight) (IV); vitamin A was determined after 4, 7, 14, and 21 days after the last injection of copper; x ± SE are shown * Significant values ($P < 0.05$) compared with the intact level (ANOVA) are noted; ** Significant values ($P < 0.05$) compared with intact level with vitamin A (ANOVA) are noted.

determined, which is an indicator of the functional activity of hepatocytes. It was found that the liver explants began to attach to the surface after 24 h of cultivation and this process was "completed" after 30 h (Figure 5A). Therefore, liver fragments are able to attach to the substrate, and this requires 30–40 h of incubation in the culture medium. In a further study, this exposure was used.

The ability of the liver to regenerate *in vitro* culture was judged by the formation of an "eviction and/or growth" zone around the explant (Figure 5B), which was assessed by the area index. It was found that after 30 h, the area index for liver fragments obtained from intact animals was 30%, and by 50 h it reached 80% (Figure 5C). In a

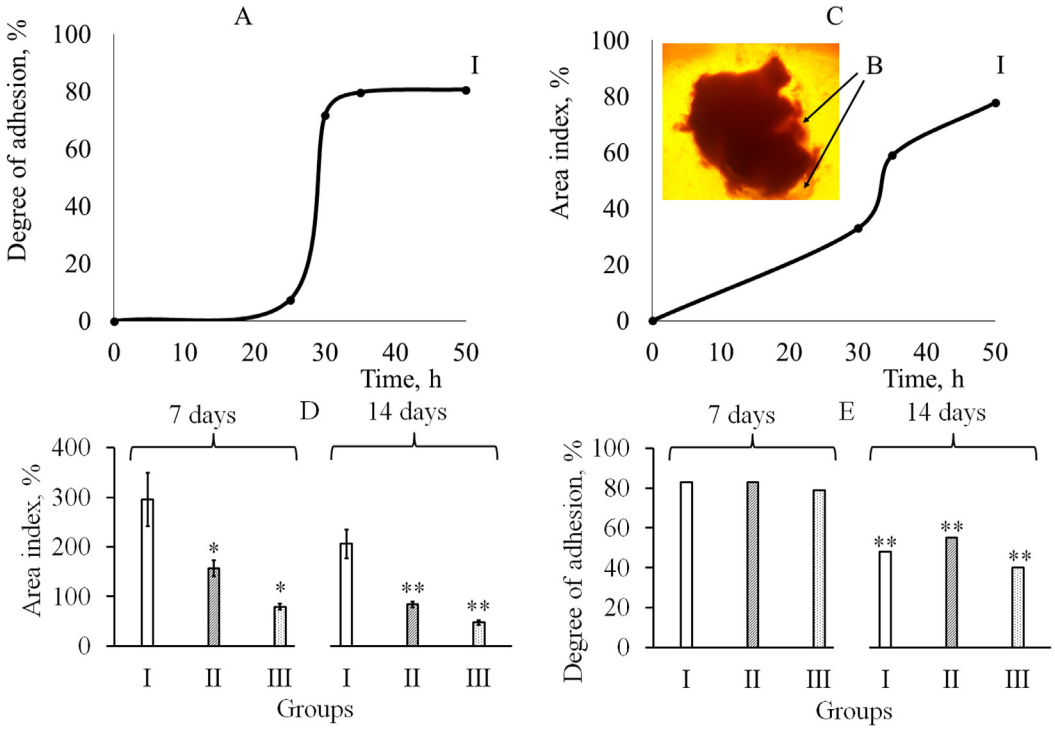

Figure 5 Percentage of liver fragments attached to the surface of the culture vessel from the 1st to the 50th hour of cultivation on the medium (45% DMEM, 45% F12, and 10% calf serum) (A); zone of "eviction and/or growth", which is formed around the explant by the 50th hour of cultivation *in vitro* (B), and an increase in the area index of "population" by liver cells from the 1st to the 50th hour of cultivation (C); area index (D); and the degree of adhesion of liver fragments to the surface of the culture vessel (E) in intact control animals (I), in experimental 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 h between injections (II) and animals, injected with copper sulfate three times (as in group II), and vitamin A (300 IU/100 g body weight) 24 h later (III) on days 7 and 14 after vitamin A injection; * – $P < 0.05$ compared with intact control; ** – 14-day exposure compared to 7-day exposure for which $P < 0.05$; (Mann–Whitney U test, Fisher's test for sample fractions).

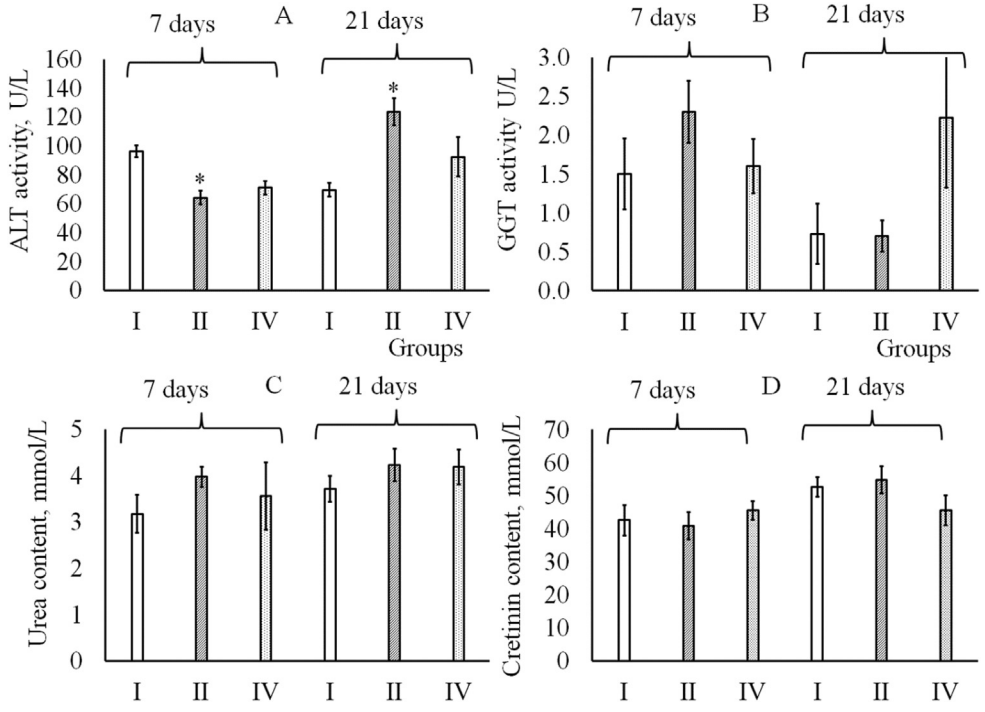

Figure 6 Activity of alanine aminotransferase (A), γ-glutamylaminotransferase (B), urea (C), and creatinine (D) in blood serum on days 7th and 21st in intact control animals (I); in experimental animals, which were injected three times with copper sulfate at a dose of 1 mg/100 g of body weight with an interval of 48 h between injections (II); and in animals treated with copper sulfate three times as in group II (1 mg/100 g body weight), and vitamin A (300 IU/100 g body weight) 24 h later (III); * – significant values ($P < 0.05$) compared with the intact level (r/ANOVA) are noted. Distribution of the experimental groups (n = 5 animals per group).

comparative study, an exposure of 50 h was used to estimate the area index.

It was found that the degree of adhesion for liver fragments after intoxication with copper sulfate 7 and 14 days after the copper sulfate injection to animals did not differ from the liver fragments adhesion of control animals.

The area index for liver fragments with liver fibrosis did not differ significantly from the control in 7 days after the start of the experiment, and after 14 days it was significantly reduced compared to the control (Figure 5D).

When determining the effect of fibrosis on the ability of liver fragments to attach to the substrate, it was found that after 7 days about 80% of the fragments were attached to the substrate of the culture vessel, and after 14 days – about 50% (Figure 5E, group II). Consequently, during the development of fibrosis, liver fragments somewhat lost their adhesive properties.

When animals with Cu-induced liver fibrosis received daily vitamin A for days 7th and 14th, the ability of liver fragments to attach also decreased from days 7th to 14th (Figure 5E). However, vitamin A had no significant effect on adhesion compared to fibrosis for fragments obtained on both days 7 and 14 (Figure 5E).

When animals with liver fibrosis received daily vitamin A, the area index was lower compared to fibrosis for both 7-day and 14-day liver samples (Figure 5D).

Therefore, increasing the content of vitamin A in the liver with Cu-induced fibrosis reduced the ability of liver fragments to adhesion and to proliferation in culture, however, this did not reduce the functional activity of the liver. This may indicate that vitamin A may enhance the compensatory activity of the organ.

Functional Activity of the Liver

The functional activity of the liver was judged by the activity of alanine aminotransferase (ALT), γ-glutamylaminotransferase (GGT), serum urea, and creatinine levels. It was found that 7 days after the increase in the content of copper ions in the liver, ALT activity decreased, which is typical for the initial stages of fibrosis development, and on day 21st of observations, it increased compared to the control (Figure 6A, group II). GGT activity also increased slightly on day 7 and remained at the control level on day 21st (Figure 6B, group II). Urea and creatinine levels remained at control levels (Figure 6C&D, group II).

Therefore, despite the presence of some structural disorders of the liver and the induction of the inflammatory process, the functional activity of the liver was close to the control values, which indicates the initial stages of fibrosis.

When animals with liver fibrosis received vitamin A daily for 7 days, ALT activity did not differ from the control

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Table 2 The Content of Some Types of Cells in the Blood of Experimental Groups of Animals in 7 days After the Injection of Vitamin A.

Index	Group		
	I Intact	II $CuSO_4$ $*5H_2O$	III $CuSO_4*5H_2O$ &Vit A
		X ± SE	
Absolute leukocytes count, 10^9*L	9.20 ± 0.10	*6.65 ± 1.25	*· **11.85 ± 1.85
Absolute granulocytes count, 10^9*L	1.83 ± 0.29	*2.50 ± 0.70	*2.90 ± 0.85
Absolute monocytes count, 10^9*L	0.17 ± 0.17	*0.25 ± 0.05	*0.33 ± 0.15
Absolute erythrocytes count, 10^{12}*L	8.54 ± 0.67	8.07 ± 0.51	8.42 ± 0.29
Hemoglobin content, g/L	145.33 ± 10.20	142.50 ± 2.5	146.33 ± 4.06
Hematocrit, %	44.43 ± 3.12	43.25 ± 0.95	44.97 ± 1.04

* – significant values were noted ($P < 0.05$) compared with the intact level (Mann–Whitney U test). Distribution of the experimental groups (n = 5 animals per group), ** – significant values were noted ($P < 0.05$) compared with the copper intoxication group (Mann–Whitney U-test). Distribution of experimental groups (n = 5 animals per group).

level, and on day 21st it was 26% lower compared to fibrosis and reached control (Figure 6A, group III).

GGT activity after injection of vitamin A to animals with fibrosis corresponded to the intact control on the 7th day after the start of the experiment. Thus, its activity after the injection of vitamin A decreased by 31% compared with fibrosis and did not differ from the intact control (Figure 6B, group III).

The injection of vitamin A to experimental animals with Cu-induced fibrosis did not have any effect on the excretory system activity, as judged by the content of urea in the blood serum (Figure 6C, group III) and creatinine (Figure 6D, group III).

It can be concluded that an increase in the content of vitamin A in the liver with Cu-induced fibrosis led to the normalization of the activity of ALT and GGT; the functional activity of the liver did not differ from the control.

Hematological Parameters in Experimental Animals

An increase in the content of copper ions in the liver did not significantly affect the quantitative characteristics of blood cells 7 days after the last injection of copper ions. The number of erythrocytes, hemoglobin content in erythrocytes, hematocrit did not change compared to the control (Table 2, groups I&II). Injection of vitamin A to animals with fibrosis had no effect on erythrocyte counts (Table 2, group III).

As is known, in intact animals, the number of leukocytes ranges from 8 to $9 \cdot 10^9$ cells/L. In our experiment, the intact control group contained about $9 \cdot 10^9$ leukocytes/L, and in animals with Cu-induced fibrosis, their number was reduced by 28–30% compared to the control (Table 2, groups I&II). It should be noted that the administration of vitamin A to animals after intoxication with copper ions was accompanied by an increase in the number of leukocytes by 78% and slightly exceeded the control level by 28% (Table 2).

In the event that animals with Cu-induced fibrosis received vitamin A for 7 days, the number of leukocytes was increased by 78% compared with Cu-induced fibrosis and even slightly exceeded the control level (by 28%) (Table 2, group III).

The injection of vitamin A led to an increase in the number of granulocytes and monocytes compared with control animals by 58 and 94% (Table 2, group III).

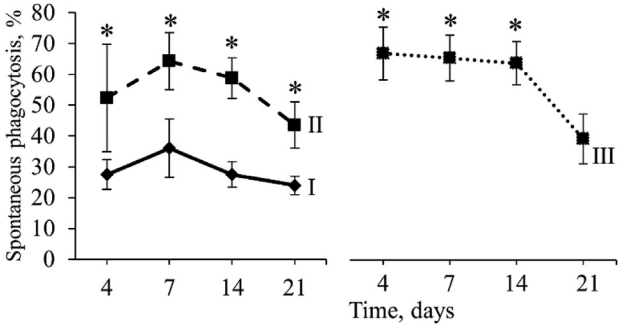

Figure 7 The ability of blood neutrophils to absorb model microorganisms in culture, which is expressed as a percentage of the total number of neutrophils, after 4, 7, 14, and 21 days of the experiment in intact control animals (I); in experimental animals, which were injected three times with copper sulfate at a dose of 1 mg/100 g of body weight with an interval of 48 h between injections (II); in animals treated with copper sulfate three times as in group II (1 mg/100 g body weight), and 24 h later they were injected with vitamin A (300 IU/100 g body weight) (III); * – significant values ($P < 0.05$) compared with the intact level (Mann–Whitney U test) were noted; distribution of the experimental groups (n = 3 animals per group).

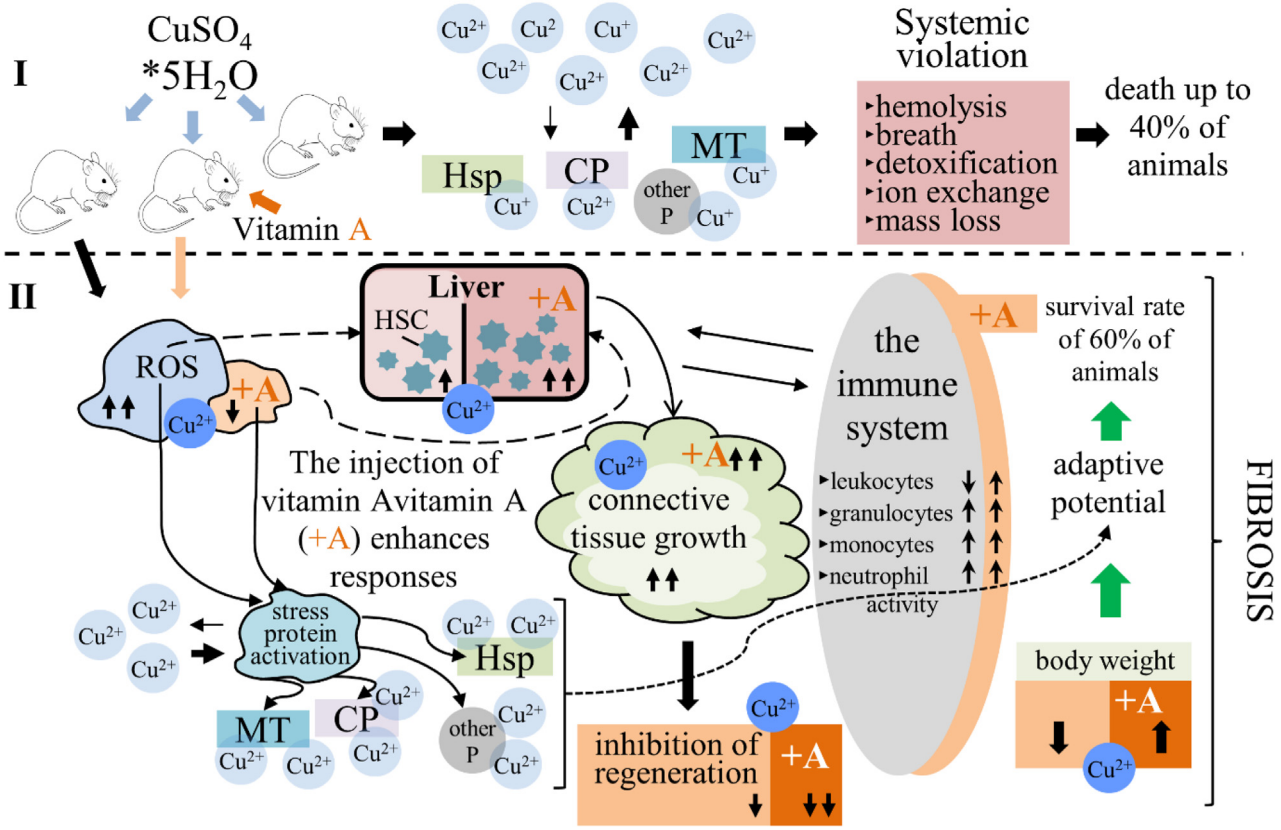

Figure 8 The scheme demonstrates the division of the studied cohort of animals into two different groups: with the manifestation of toxicity, i.e. sensitive (I) and the formation of resistance – resistant (II); in the sensitive group, the balance between free and protein-bound copper ions is shifted in favor of free ions, which induce systemic disorders in the vital systems of the body, which is accompanied by high mortality; at the same time, in the resistant group, which is about 60%, most of the copper ions bind to various proteins, which eliminates the manifestation of acute toxicity and triggers free radical processes that activated Ito cells with the formation of connective tissue, manifestation of the immune system reaction, and inhibition of processes liver regeneration. If such animals were injected with vitamin A, then an additional activation of Ito cells occurred, which was accompanied by an even greater increase in the growth of connective tissue, normalization of liver function, activation of the cellular link of the immune system and an increase in body weight, while the ability of the liver to regenerate was inhibited to an even greater extent, i. e. specific metabolic patterns were formed that can be defined as vicious circles of metabolism; an increase (↑) or decrease (↓) in the corresponding indicators was noted (more than one arrow indicates a quantitative increase in the indicator); Hps — chaperon, CP — ceruloplasmin, MT — metallothionein, P — proteins, HSC — hepatic stellate cells, ROS — reactive oxygen species.

Therefore, the injection of vitamin A to animals with Cu-induced liver fibrosis increased the content of granulocytes and monocytes in comparison with the control, which indicates the activation of the cellular link of the immune system.

Phagocytic Activity of Neutrophils in Experimental Animals

As is known, the cellular link of the immune system plays an important role in eliminating degradation products of cells and cellular structures and can lead to a shift in the balance in the "pro-oxidants ↔ antioxidants" systems.

It was found that the activity of phagocytosis of neutrophils obtained from animals with Cu-induced fibrosis was increased by 90, 77, 117, 81% compared with the control level on days 4, 7, 14, and 21st after the injection of copper sulfate (Figure 7, curve I&II).

The injection of vitamin A to animals with Cu-induced liver fibrosis for 4 days caused an increase in phagocytosis activity by 27% compared with Cu-induced liver fibrosis and by 141% compared with control animals (Figure 7, curve III). However, there was no effect on the phagocytic activity of neutrophils compared to that in animals with liver fibrosis after 7, 14, 21 days of daily injection of vitamin A to animals with fibrosis. And by 21 days, when a decrease in vitamin A content was observed, this indicator approached the control values (Figure 7, curve III).

Therefore, the injection of vitamin A to animals with Cu-induced liver fibrosis increased the number of leukocytes in the blood compared to animals with fibrosis, stimulated the phagocytic activity of neutrophils in the blood only on the 4th day of daily injection, and later this

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indicator remained at a constant level and only on 21st day of the experiment, it decreased approaching the control.

DISCUSSION

There is evidence that liver fibrosis is a reversible condition.[28–31] However, treatment and prevention of the transition to the irreversible last stage of the cirrhosis, is a rather complicated and yet unsolved problem of hepatology. According to some experts,[32–37] one of the most promising approaches in the treatment of fibrosis is the use of various natural compounds. In this regard, such natural compounds as carotenoids, vitamins, in particular vitamin A, and other compounds are of great interest. Previous studies of vitamin A effect on the liver fibrosis development indicate rather contradictory results: some authors point to positive effect of vitamin A,[38–40] while others point to negative effect, i.e. the ability to accelerate the liver cirrhosis formation.[41,42]

We believe that such inconsistency in the available data of the vitamin A effect on the development of liver fibrosis reflects a number of fundamental properties of biological systems and, above all, the principle of "temporal optimality" – the principle of "TOP". By "optimality" is meant that the body as a self-regulating system chooses such metabolic options in response to environmental changes and forms such new patterns that can ensure the preservation of homeostasis in a state close to normal at this particular moment in time, against the background of constantly acting negative factors. In other words, the organism always seeks to survive "here and now" with minimal cost, without regard to possible future consequences. The implementation of the TOP principle is possible due to the multifunctionality of most elements of biological systems in solving adaptive problems, i.e. the body can use the same elements (enzymes and other molecules) to solve different problems.[43] In addition, a high degree of stability of biological systems is provided by a multilevel system of regulation.

We suppose that the first level of an adaptive response to the actions of negative factors is the use of existing elements of the body (synthesized before extreme exposure): molecules are able to "switch" to perform additional or other functions due to their polyfunctionality (i.e. principle of "initial state").

The second level of the adaptive response is the induction and synthesis of additional elements similar to existing molecules, however, if their increase does not ensure the preservation of homeostasis, then at the next, third level, the induction of new elements starts.

At the fourth level, the cell-tissue pool is restructured, which leads to cooperative changes in the entire biological system, i.e. there is a formation of specific adaptive metabolic patterns, which will ensure the preservation of homeostasis in the changed conditions of existence.

If we proceed from the principle of temporal optimality (TOP), then the processes of pathologies development and, above all, pathologies of the liver should be considered in the context of adaptatiogenesis, i.e. the formation of fibrosis, one of the cellular and tissue levels of adaptation, is aimed at maintaining the function of the liver, which ensures the overall homeostasis of the body. The effectiveness of an adaptive process depends on the speed (temporality) of its implementation. An emergency response can be implemented by switching polyfunctional elements to new functions, which will "allow" you to move to subsequent levels of protection. It can be assumed that if for some reason the first level of protection cannot be realized, then the process of destruction of cellular components will lead to a critical deviation from homeostasis and the death of systems will occur, which was realized in 40% of animals in our experiment (Figure 8, I).

In the event that the body effectively used the first level of protection and "gained" time to move to subsequent levels of adaptive protection, then in this case, adaptive changes can be saved (injected) and go into a chronic pathological state, in our case, into a formed liver fibrosis. This was manifested in 60% of the experimental animals (Figure 8 II).

The results obtained indicate the decisive role of the initial metabolic state in the formation of a multilevel adaptive response to the action of copper ions. This is convincingly indicated by the results of our previous work on the induction of metallothionein synthesis in animals of different ages, i.e. animals with initially different functional state.[11]

As is known, copper ions in the body bind to a variety of proteins, primarily ceruloplasmin and albumin. In addition, copper ions are able to induce the synthesis of metallothioneins and other stress proteins in the liver, which are also able to specifically bind copper ions and to some extent eliminate of toxic effect ones[44,45] (Figure 8). In the event that copper ions remain "free", i.e. do not bind and/or are not retained for a long time by these proteins, they bind to functionally active enzymes and inhibit their activity, which manifests itself as toxicity. It can be assumed that in 40% of animals, the initial number of elements (ceruloplasmin, albumin, etc.) that can specifically bind copper ions is not enough to eliminate their acute toxicity, i.e. provide the first level of protection. For example, it is known that copper ions not associated with specific copper-binding proteins exhibit a hemolytic effect,[46] disrupt the functions of the nervous system,[47] the gastrointestinal tract (diarrhea),[48] inhibit the function of respiration and the excretory system,[49] i.e. death occurs as a result of systemic changes in the functions of a number vital organs (Figure 8). It should be noted that in this group of animals there were practically no connective tissue adhesions around the liver, which indicates that the cellular tissue level of adaptive protection was not fully realized.

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At the same time, in the group of animals (60%) that showed resistance to the toxic effect of copper ions, the following was recorded: inhibition of growth rate; changes in the morphology of the liver; slight inhibition of the functional activity of the liver; a slight increase in the number of granulocytes and monocytes, and at the histological level there was a violation of the structure of hepatocytes, which was expressed in a thickening of the liver capsule and in which leukocytes and other types of immunocompetent cells were incorporated. It is also possible to note the violation of the epithelium integrity (Figure 3).

Partial disruption of the epithelial tissue and, probably, capillary vessels is accompanied by the start of fibrogenesis. Fibrogenesis is based on the activation of hyaluronidase, which is accompanied by an increase in the permeability of the basement membrane and vessel walls, which in turn leads to the exudation of lymph, leukocytes, macrophages and fibrinogen. Macrophages localized in the resulting fibrin matrix differentiate into fibroblasts, which actively produce collagen.[50] As a result, 75% of the surviving animals (showed adaptation) had intense growths of the connective tissue around the liver – adhesive disease (Figure 2), i.e. the fourth, cellular-tissue level of protection was fixed. The results obtained indicate the initial stages of the liver fibrosis development (F0/F1) in these animals (Figures 2 and 3). Along with these changes, these animals showed inhibition of the liver regeneration process and a 4-fold decrease in the amount of vitamin A in the liver compared to control animals (Figures 4, 5, and 8).

An increase in the content of vitamin A in the liver with fibrosis, at the initial stages of its development, was accompanied by normalization of the functional activity of the liver (Figure 7), restoration and, moreover, activation of the growth rate of animals (Figure 2). It was accompanied by an increase in the number of immunocompetent cells compared to the control (leukocytes, monocytes, granulocytes), including their incorporation into the liver capsule (Figure 3). It was accompanied by an increase in the ability to phagocytosis and a decrease in the mortality of experimental animals by almost 7 times compared with animals that did not receive vitamin A. However, along with this increase in vitamin A content, there was an increase in the growth of connective tissue around the liver (detected in 91% of this group animals), as well as a decrease in the ability of liver fragments to regenerate (Figures 2 and 5). It should be noted that a decrease in the ability of liver fragments to regenerate occurred against the background of the immune system activation and normalization of liver function. It can be assumed that the compensatory effects of the functional activity of the liver in this case were provided not at the level of proliferation, but at the level of hypertrophy.

It is known that the stroma of the liver, which significantly increased after the injection of vitamin A into animals, is represented by 4 types of tissue structures: capsule, perivascular connective tissue, tracts and extracellular matrix, which perform an important structural and metabolic function[51]; this connective tissue is actively involved in maintaining homeostasis of the body under the conditions of the copper toxic effect, i.e. it performs an important compensatory-regulatory function.

As you know, the main producer of the connective tissue of the liver are: Ito cells (excrete all the components of the matrix, all types of collagens, proteoglycans, glycoproteins); fibroblasts and partially hepatocytes (participate in the synthesis of laminin and type XVIII collagen), endotheliocytes (synthesize type IV collagen).[52,53] In the normal functional state of the liver, Ito cells are inactive. When the liver is damaged, partial activation of Ito cells occurs and they begin to produce components of the connective tissue matrix.[54]

It is known that the products of free-radical reactions, cytokines, transforming growth factor, proteolysis products, and other biologically active products that are formed against the background of inflammatory reactions take part in the activation of Ito cells.[55,56] However, retinoids play a leading role in the activation of Ito cells.[57,58] The main depot in the liver of vitamin A are Ito cells.[59] The loss of vitamin A by Ito cells during their activation leads to the transformation of these cells into myofibroblasts and promotes their migration to areas of inflammation and necrosis.[12,60] The results of this work indicate that vitamin A contributes to additional activation of fibrogenesis, and possibly not only through additional activation of Ito cells, but also through the activation of other cell types that are involved in fibrogenesis.

Along with this, vitamin A contributed to the activation of the cellular link of the immune system, which could also contribute to the restoration of the functional activity of the liver without the participation of regeneration processes. Previously, we have shown that compensatory processes in the liver can be provided both by an increase in proliferation and an increase in the ploidy of hepatocytes and their hypertrophy.[61] If we consider the obtained data from the standpoint of the "TOP" principle, then it can be argued that the injection of vitamin A to animals with liver fibrosis at the initial stages of its formation ensured the implementation of all possible levels of the body adaptive defense, while there was a transition of fibrosis to a potentially chronic state.

Further studies of the role of vitamin A in the regulation of fibrogenesis and its relationship with the functional activity of the liver and the overall metabolism of the body are extremely promising in hepatology.

Thus, we can conclude that multiple successive injections of copper sulfate at a dose of 1 mg/100 g of weight to experimental animals led to the death of up to 40% of the animals within 27 days. In the rest of the animals that survived, there was a lag in body weight growth, while

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in 75% of these survivors there was an proliferation of connective tissue around the liver and a change in the morphology of the liver (the shape of the hepatic lobes changed, a connective tissue capsule was formed), and inhibition of the functional activity of the liver was also noted, i.e. there was an induction of liver fibrosis in the initial stages of development (F0/F1). The formation of fibrosis was accompanied by a decrease in the content of vitamin A in the liver by 4 times compared with the initial level.

An injection of vitamin A led to its increase in the liver by more than 15 times from the initial one, while the mortality of animals decreased by almost 7 times. More than 90% of the animals treated with vitamin A had an even more pronounced growth of connective tissue, their liver morphology was changed, and the number of immunocompetent cells was increased. In such animals, incorporation of immunocompetent cells into an enlarged liver capsule was observed, which indicates an increase in the immune response to Cu-induced liver damage. After the injection of vitamin A into animals with fibrosis, the activity of ALT and GGT was restored to the control level. An increase in connective tissue growth correlated with survival and body weight, but at the same time reduced the ability of the liver to regenerate. Partial loss of regenerative potential by liver fragments during the restoration of liver activity indicates that compensatory processes in the liver are provided by polyploidation and hypertrophy of hepatocytes.

1 Three times the injection of copper sulfate to rats at a dose of 1 mg/100 g of body weight is accompanied by both the manifestation of acute toxicity (in 40%) and the formation of resistance (in 60%) to copper ions.
2 Against the background of a multiple increase in copper ions in the liver (150 times), the content of vitamin A in the liver decreased by 4 times, and such animals developed liver fibrosis.
3 The injection of vitamin A to animals at the initial stages of the liver fibrosis development reduced the toxic effect of copper by almost 7 times, normalized liver function, while increasing the formation of connective tissue, and the liver lost its ability to regenerate.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Anatoly I. Bozhkov: Term, Conceptualization, Writing - Review & Editing. Anna V. Novikova: Investigation, Formal analysis, Writing - Reviewing. Elena M. Klimova: Investigation, Writing- Reviewing and Editing. Igor A. Ionov: Investigation, Writing- Reviewing. Rustam A. Akzhyhitov: Investigation, Formal analysis, Visualization. Nataliia I. Kurhuzova: Project administration, Investigation, Resources. Svitlana G. Bilovetska: Investigation, Writing - Reviewing. Vitalii B. Moskalov: Investigation, Formal analysis. Stanislav S. Haiovyi: Investigation.

CONFLICTS OF INTEREST

There is no conflict of interest, all co-authors agree with the publication of the work.

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SUPPLEMENTARY DATA

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