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VITAMIN A ACCELERATES THE PROCESS OF LIVER REGENERATION IN THE INITIAL STAGES OF CU - INDUCED FIBROSIS

Svitlana Bilovetska, Anatoly Bozhkov

***Aim**: To test the hypothesis about the possible role of vitamin A in normalizing the functional activity of the liver with Cu-induced fibrosis by increasing the regeneration process.*

***Materials and methods:** Experiments were conducted on 20 sexually mature male Wistar rats, which were divided into 4 groups: a control group that was not exposed to copper sulfate and vitamin A, a group that was at the initial stage of liver fibrosis, which was provided by three consecutive administrations of copper sulfate at a dose of 1 mg/100 g of weight (one series of injections), a group that was at the stage of intensive development of fibrosis (F2), which was carried out by two consecutive series of copper sulfate injections with an interval of 3 days between injections, and a group that received vitamin A three times daily in a dose of 300 IU/100 g of weight between two series of intoxication. Body weight dynamics, relative liver weight, histological changes in liver tissues and the number of binuclear hepatocytes were determined.*

***Results:** It has been found that animals with Cu-induced liver fibrosis did not gain or lose body weight, and the introduction of vitamin A ensured the restoration of body weight growth, and they slightly lagged behind the control group. In animals with liver fibrosis that received vitamin A, the relative weight of the liver was slightly increased and there were 2 times more binuclear hepatocytes. The structural organization of the liver tissue changed to a minor extent, and to the greatest extent there was an increase in the thickness of the Glisson's capsule, in which immunocompetent cells were incorporated.*

***Conclusions:** Vitamin A contributed to the normalization of liver function against the background of the development of fibrosis. The mechanism of normalization can be ensured due to an increase in the number of binuclear hepatocytes, a slight increase in the relative weight of the liver, and was accompanied by an increase in the thickness of the Glisson's capsule, in which immunocompetent cells were incorporated*

***Keywords:** Cu-induced liver fibrosis, vitamin A, binuclear hepatocytes, morphological characteristics of the liver*

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1. Introduction

As it is known, the process of formation of liver pathologies goes through a number of stages: hepatitis, fibrosis, cirrhosis, which is the terminal stage and is a threatening and irreversible condition [1–3]. According to the available data, liver diseases are a serious medical and biological problem that is far from being solved. The complexity of its solution is explained by a number of reasons. However, the most important of them is the fundamental role of the liver in the body system.

First, functional changes in the liver occur under the influence of an extremely wide range of physical, chemical and biological factors [4–6]. In this context, various exogenous signals are reflected and transformed in the liver as a "mirror", which can be accompanied by the development of inflammatory reactions and the formation of pre-pathological conditions [7–8]. Secondly, since the liver is a "coordinating" and "executive" element of the body's metabolic system, that is, it provides vital functions, an effective system of regeneration and repair of cellular elements of the liver was formed in the process of evolution. The processes of maintaining and restoring the functional activity of the liver are carried out by polyploidization of hepatocytes with their hypertrophy and regeneration of cellular elements of the liver [9, 10], that is, it has a wide arsenal of adaptive mechanisms. On the one hand, these features provide a wide variability of the functional activity of the liver, and on the other hand, they "mask" the manifestation of pathological processes, and they appear already in late and irreversible chronic conditions.

Based on this, the development of liver fibrosis can be imagined as a shift in the balance between the necessary regeneration, after continuous deviations from homeostatic parameters, and the development of fibrogenesis, which can be considered as an appropriate adaptive response to damaging factors. It can be assumed, that the increase in liver regeneration processes in the initial stages of liver fibrosis can provide a shift in the equilibrium or balance towards regeneration processes and, as a result, inhibit or eliminate unwanted fibrogenesis.

Currently, methods of treating liver fibrosis are being actively developed, since according to the available data, this stage of the evolution of fibrosis can be reversible. [11, 12]. For this, a wide arsenal of medical measures is used, which includes: elimination of causative factors, use of drugs with anticytotoxic activity, hepatoprotective ef-

fect, antifibrotic activity, antioxidant properties, inhibitors of the renin-angiotensin system, etc.

Vitamin A (retinol) can be considered a promising natural component, potentially able to shift the balance in the liver towards regeneration. There are several reasons for this: it can change the rate of proliferation of epithelial cells [13], about 85 % of vitamin A is localized in stellate cells of the liver, which play a central role in fibrogenesis, and with the development of fibrosis, the content of vitamin A in the liver is reduced [14]. There is evidence that vitamin A inhibits the formation of fibrotic liver [15]. Along with this, there are indications that vitamin A, on the contrary, can accelerate the development of fibrosis [16]. We believe that the study of the effect of vitamin A on the processes of regeneration and preservation of the structural and morphological organization of liver cells against the background of the development of fibrosis is important in understanding the mechanisms of fibrogenesis and its practical application.

The aim of the study is to test the hypothesis about the possible role of vitamin A in normalizing the functional activity of the liver with Cu-induced fibrosis by increasing the regeneration process

2. Materials and methods

The research was conducted in 2021–2023 on the basis of the Research Institute of Biology of Kharkiv National University named after V.N. Karazina, Kharkiv, Ukraine.

The experiments were conducted on sexually mature male Wistar rats with a body weight of 80–130 g. Throughout the experiment, the animals were kept in standard vivarium conditions and had free access to food and water. All manipulations were carried out in agreement with the bioethical committee of Kharkiv National University named after V. N. Karazin (Protocol No. 1 dated 03.03.2023), governed by the provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, March 18, 1986) [17]. The solution of the injected substances was calculated per body weight (a single dose did not exceed the volume of 1 ml per animal).

To test the working hypothesis, 4 groups of animals were formed from 20 ones: 1 – a control group that was not exposed to copper sulfate (inducer of liver fibrosis) or vitamin A, 2 – animals with liver fibrosis in the initial stages of development (F0/F1), for this purpose, 5 animals were injected three times with copper sulfate at a dose of 1 mg/100 g of weight with an interval of 48 hours, as described in the article [18], 3 animals with liver fibrosis at the stage (F1/F2), for this purpose, 5 animals underwent two cycles of introduction of copper sulfate at a dose of 1 mg/100 g of body weight three times with an interval between cycles of 3 days; 4–5 animals that were in the initial stages of development of fibrosis received three consecutive injections of vitamin A at a dose of 300 IU/100 g of body weight, followed by a second cycle of copper sulfate administration. 6 days after the end of the last manipulation with the animals, they were taken out of the experiment by means of ether anesthesia.

Every 3 days, in the morning before feeding, the body weight of all groups of animals was determined. Anesthetized animals were dissected, the liver was removed, its relative weight was determined, and an expert assessment of connective tissue formations of the intra-abdominal cavity was carried out. Anatomical studies consisted in evaluating the degree of formation of connective tissue adhesions around the lobes of the liver: a low level of adhesion disease of 1–3 points and a high level of 4–5 points in the case of complete fusion of the lobes with connective tissue.

Liver fragments were taken for histological examination and fixed in 10 % formalin solution. The samples were processed as described in [19], after one or two days of fixation of liver pieces in a fixation liquid (10 % formalin). The samples were dehydrated using ethyl alcohol. To do this, the pieces were placed in ethyl alcohol one by one with an increasing concentration from 70 % to 96 %, in wide-mouthed jars for 12 hours in each portion. After that, the samples were placed for 1–3 hours in xylene, then for 2–3 hours in melted paraffin. Pieces, carefully cut from the fixed material with a sharp blade, in the form of thin plates, 2–4 mm thick and 1–2 cm in area, washed and slightly dried on filter paper, were placed in 4 portions of isopropyl alcohol for 1 hour in each portion; after that, the pieces were transferred to a solvent for 1 hour and transferred to melted paraffin, heated in a thermostat to 57 °C. After that, the samples were stained with hemotoxylin-eosin and microscoped. The number of binuclear hepatocytes per 100 cells was determined. Microscopy was carried out using a Carl Zeiss microscope, magnification ×200, photos were recorded with a SIGETA M3CVOS 14000 camera.

Data are presented as group means and standard error (x±SE). The data analysis and visualization were performed using Excel 2013 (Microsoft Corporation, USA). ANOVA (Kruskal–Wallis H test) with a posteriori comparisons and Mann–Whitney U test with Bonfironi correction were used to determine significant differences between groups. Differences between the control and experimental groups were considered reliable under the condition of $p<0.05$ (the Bonferroni correction was 0.008).

3. Research results

As you know, the characteristics of changes in body weight of animals are an integral indicator of the functional state of the organism. The development of chronic pathologies, as a rule, is accompanied by inhibition of body weight growth in young, growing animals.

Body weight in the intact group of animals during the experiment (20 days) increased by 20–25 % from the original (Fig. 1, curve 1), which corresponds to the accepted standard for rats. In that case, if liver fibrosis was induced in the experimental animals by multiple consecutive injections of copper sulfate, the growth of their body weight stopped (Fig. 1, curve 2), i.e. their body weight was 15–20 % less compared to the control. Such growth retar-

dation indicates suppression of the general metabolism of animals with liver fibrosis.

If the effect of copper sulfate continued for 13 days, then such animals lost more than 5 % of their initial body weight, and at this time they lagged behind the control group of animals by at least 20–25 % in body weight (Fig. 1, curve 3).

If the animals received vitamin A after the first cycle of intoxication, then the second cycle of intoxication did not inhibit the growth rate of such animals, on the contrary, they exceeded the growth rate of animals with liver fibrosis and slightly lagged behind the control animals in terms of growth rate (Fig. 1, curve 4).

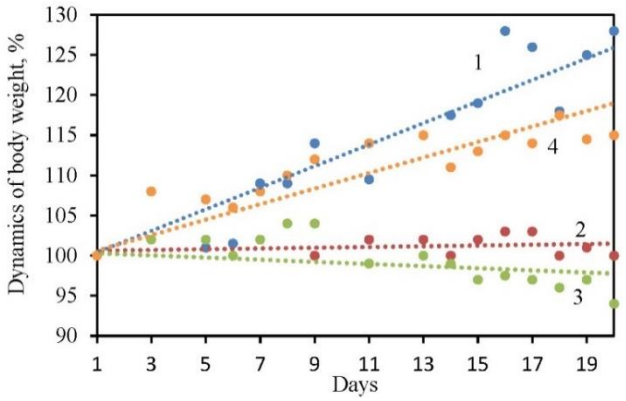

Fig. 1. Dynamics of body weight: 1 – control group of animals, 2 – animals with liver fibrosis in the initial stages of its development, 3 – animals with pronounced development of fibrosis, which was assessed by the growth of connective tissue formations around the liver, 4 – animals that received vitamin A at the initial stage of fibrosis development with subsequent exposure to the fibrosis inducer. Linearized curves based on the average value of body mass are presented

Therefore, the administration of vitamin A to animals during the development of liver fibrosis, that is, between two cycles of copper sulfate intoxication, normalized the growth intensity of such animals, and they did not differ in growth rate from animals of the control group and significantly exceeded animals with Cu-induced liver fibrosis.

The obtained results allow us to assume that the administration of vitamin A to animals with fibrosis in the initial stages of its development ensured the normalization, at least partially, of liver functions, which ultimately ensured the restoration of the growth of the animals' body weight.

Such normalization of liver functions may be based on various mechanisms, including cell regeneration or hypertrophy, or perhaps both. A slight increase in the relative weight of the liver in the group of animals that received vitamin A against the background of the development of fibrosis (Fig. 2, *a*) and an increase in the number of binuclear hepatocytes (Fig. 2, *b*), can indirectly testify to this.

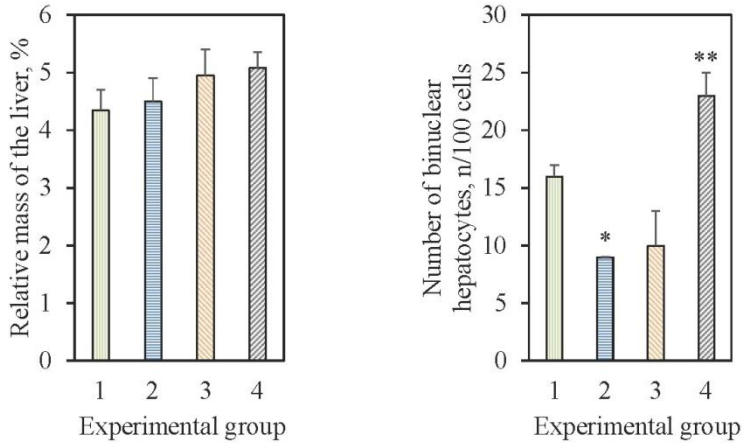

Fig. 2. Dynamics of integral indicators of animals of experimental groups: *a* – relative weight of the liver; *b* – number of binuclear liver hepatocytes; 1 – control group; 2 – animals with fibrosis in the initial stages of development; 3 – animals with pronounced fibrosis; 4 – animals that received vitamin A in a dose of 300 IU/100 g of weight three times in the initial stages of fibrosis development against the background of long-term exposure to copper sulfate. Average data and standard errors are given. Variants, for which $p<0.05$ compared to the control, are marked with an asterisk

Thus, the number of binuclear hepatocytes in the liver with fibrosis, regardless of the stages of its development, was reduced compared to the control (Fig. 2, *b*). At the same time, if vitamin A was administered to animals with fibrosis in the initial stages of its development, the number of binuclear hepatocytes increased more than 2 times compared to animals that did not receive vitamin A and ones with fibrosis in the initial stages of its development. It is important to note, that in the group of animals with fibrosis that received vitamin A, the number of binuclear hepatocytes was significantly higher even compared to the control group (Fig. 2, *b*).

It was previously shown that in Cu-induced fibrosis, there is a significant decrease in the content of vitamin A in the liver, and the triple administration of vitamin A at a dose of 300 Me/100 g of weight was accompanied by the restoration of its content in animals with fibrosis to the control level [20]. It can be assumed, that maintaining the balance of vitamin A with other biologically active compounds also restores the balance between regenerative and destructive processes, at least in the initial stages of liver fibrosis. The fact that the formation of connective tissue around the lobes of the liver during Cu-induced fibrosis (Fig. 3, *a*), which was described in detail [18], may not lead to irreversible structural and functional changes in hepatocytes and, as a result, ensure the strengthening of the regeneration process of the liver, as indicated by the data of histological studies.

It has been found that in the intact liver, there is a partial, weakly expressed autolysis of hepatocytes while preserving the structural organization of the liver lobules, blood filling is normal (Fig. 3B, *a*).

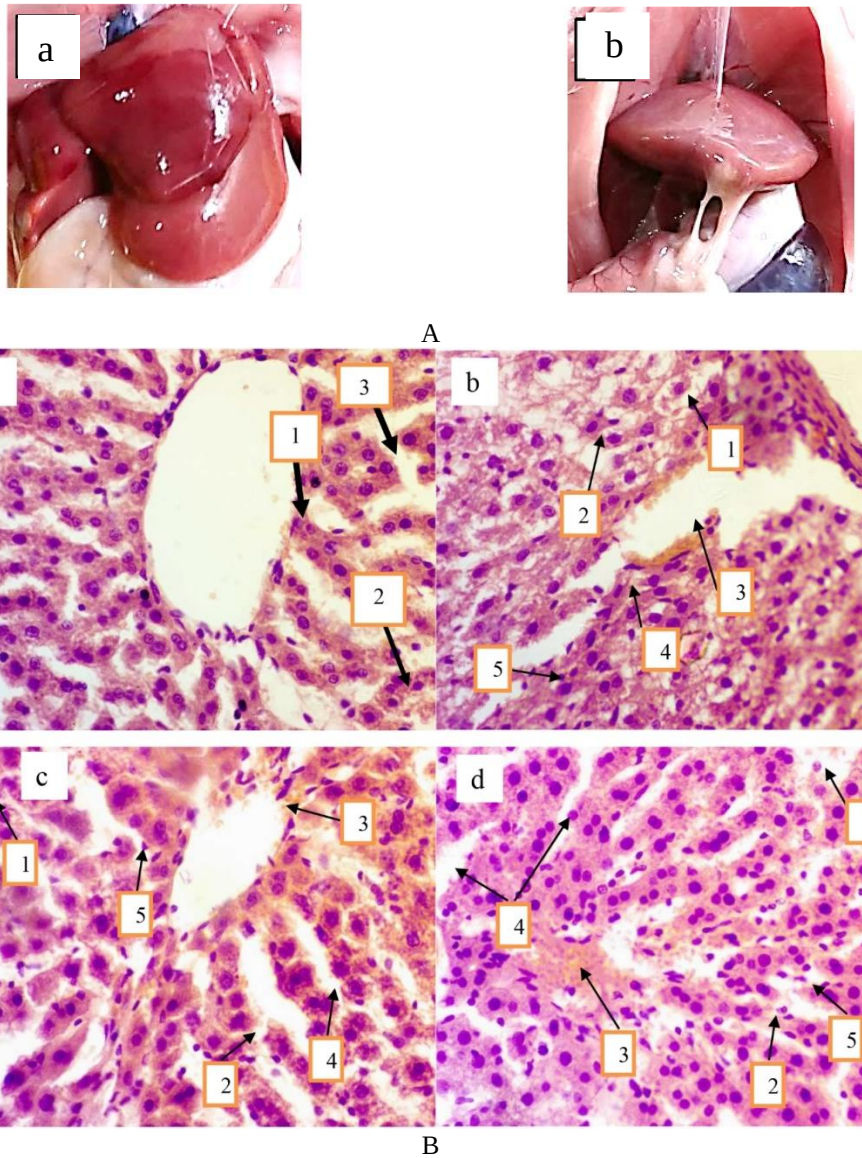

Fig. 3. Formation of connective tissue formations around the liver at the early stages of Cu-induced liver fibrosis (A) and histological preparations (B); *a* – intact control, *b* – copper control, *c* – two-time introduction of copper without vitamin A, *d* – two-time introduction of copper with vitamin A

After the first cycle of intoxication with copper sulfate, which corresponds to the initial stage of fibrosis, partial autolysis of hepatocytes occurs (1), discomplexation of hepatic tubules is common (2). The vessels are filled with blood (3), but the Disse space is reduced (4), in which Ito cells are often found (5) Fibroblasts are few, endotheliocytes are found in moderate quantities most often near blood vessels. The endothelium is dystrophic, discontinuous (Fig. 3B, *b*). These small structural changes in the liver compared to intact controls were accompanied by small changes in the activity of specific liver enzymes.

After the second cycle of intoxication of the body with copper sulfate, characterized by a more pronounced development of liver fibrosis, dystrophic changes took place, the intercellular space, hepatic tubules (2) were partially destroyed (Fig. 3B, *c*). In such animals, there is no blood filling in the veins and arteries (3), in some cases, hemorrhage in the central vein was observed. Violated integrity of the endothelium. Binuclear hepatocytes are present, a moderate number of lymphocytes is observed in the liver.

4. Research results and discussion

Therefore, after two cycles of intoxication with copper sulfate, more pronounced structural changes occurred in the liver compared to one cycle of intoxication.

In that case, if vitamin A was administered to the animals three times between two cycles of intoxication, the thickening of the Glisson's capsule was observed, the number of stellate cells increased, and the hepatic tubules retained their structure (Fig. 3B, *d*). The number of binuclear hepatocytes increased both compared to control and compared to fibrosis. It should be noted, that the most pronounced morphological changes in the group of animals that received vitamin A in the initial stages of the development of fibrosis with the subsequent effect of a hepatotoxic factor on these animals were manifested in the increase in the thickness of the Glisson's capsule, in which immunocompetent cells were incorporated, which may indicate the manifestation of an immune response to damage in the liver, and an increase in binuclear hepatocytes, which indicates hypertrophy or regeneration of the liver.

Some researchers believe that vitamin A can contribute to the restoration of liver function in fibrosis [21]. However, there are data that the additional administration of vitamin A can lead to an accelerated transition of fibrosis to cirrhosis [16], the work shows the stimulation of lipogenesis in the liver by vitamin A. The authors believe that vitamin A-induced lipogenesis increases the likelihood of developing steatosis and exacerbates fibrosis. In our study, we took into account the temporal characteristics of the mechanism of the effect of vitamin A on the development of liver fibrosis and investigated the stages, at which the introduction of vitamin A contributed to the restoration of liver function, which may explain the contradictions in the data available in the literature.

In conclusion, we note that the introduction of vitamin A to animals with liver fibrosis in the initial stages of development ensures an increase in regeneration processes and shifts the balance towards the restoration of the functional activity of the organ, which ensures the body's resistance to the further negative effects of copper sulfate.

Limitations of the study. Studies of the possible mechanisms of action of vitamin A are associated with a number of methodological difficulties and, first of all, the study of the dynamics of both vitamin A metabolism and metabolic processes in the liver in the in vivo system. In the existing studies of this problem, indicators are determined in different periods of the development of fibrosis, after which a temporal reconstruction of possible dynamic changes is carried out. However, such an approach will always be associated with a number of assumptions that are difficult to test experimentally.

Prospects for further research. An explanation of the inconsistency of the available data can make it possible to justify and implement in practice the treatment of a number of liver pathologies.

5. Conclusions

1. Administration of vitamin A to experimental animals in the initial stages of development of Cu-induced liver fibrosis eliminated inhibition of animal weight growth.

2. In animals with liver fibrosis that received vitamin A, the number of binuclear hepatocytes increased by 2 times and the relative weight of the liver was slightly increased.

3. Administration of vitamin A to experimental animals with liver fibrosis was accompanied by an increase in the thickness of the Glisson's capsule, in which immunocompetent cells were incorporated.

Conflict of interests

The authors declare that they have no conflict of interest in relation to this study, including financial, personal, authorship, or any other, that could affect the study and its results presented in this article.

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Data availability

Data will be given upon reasonable request.

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Svitlana Bilovetska, Postgraduate Student, Department of Molecular Biology and Biotechnology, V. N. Karazin Kharkiv National University, Svobody sq., 4, Kharkiv, Ukraine, 61022

Anatoly Bozhkov, Doctor of Biological Sciences, Professor, Department of Molecular Biology and Biotechnology, V. N. Karazin Kharkiv National University, Svobody sq., 4, Kharkiv, Ukraine, 61022

***Corresponding author:** Anatoly Bozhkov, e-mail: niibio@karazin.ua*