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Original Article

Vitamin A intake forms resistance to hypervitaminosis A and affects the functional activity of the liver

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SUMMARY

Disruption of the exchange of copper ions in the body is accompanied by the development of a number of pathologies and, most often, liver fibrosis. Ito cells, which deposit vitamin A, play a key role in fibrogenesis. For the purpose of determining the impactof vitamin A on the functional characteristics of the liver with fibrosis, we studied the dynamics of vitamin A accumulation in the liver during its daily administration (up to 21 days) in intact animals and animals with Cu-induced liver fibrosis, as well as physiological (body weight and relative weight organs) and biochemical parameters (activity of alanine aminotransferase, alkaline phosphatase, concentration of cholesterol and urea). It was shown that daily administration of vitamin A to experimental animals at a dose of 300 IU/100 g of body weight was accompanied by its accumulation in the liver, and, after reaching a concentration of 250–300 μg/g, its content decreased even against the background of further administrations. The development of Cu-induced liver fibrosis was accompanied by a decrease in vitamin E in the liver by 40% compared with the baseline level. The administration of vitamin A to animals with liver fibrosis was also accompanied by its accumulation in the liver, but its increase was observed later, and the rate of decrease was faster. There is an inverse relationship between the vitamin A content and the vitamin E content in the

Abbreviations: ALT, Alanine aminotransferase; ALP, Alkaline phosphatase; MDA, Malondialdehyde; PC, Protein carbonylation; GP, Glutathione peroxidase; RBP4, Retinolbinding protein 4; HSCs, Hepatic stellate cells.

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liver. Administration of vitamin A to animals with liver fibrosis was accompanied by normalization of ALT activity, cholesterol content, and restoration of the growth rate of animals.

1. Introduction

Unfortunately, liver disease is responsible for approximately 2 million deaths per year worldwide, of which 1 million are due to complications of cirrhosis. Cirrhosis is currently the 11th most common death in the world. [1] In this regard the investigation of the impaired functional activity's mechanisms of the liver leading to diseases remains an urgent task of biomedical research.

Currently, five main etiological factors of liver pathology are distinguished: viral lesions, autoimmune pathologies, tumors, alcohol and metabolic disorders. [2].

Metabolic diseases are of great clinical interest. In particular, metabolic disorders in the liver can be caused by an extremely wide range of genetic and environmental factors and their combination. However, only some of them are satisfactorily described at present. The violation of iron metabolism in the body leads to the development of hemochromatosis, and a disruption of copper metabolism leads to Wilson's disease. [3] The last one is a serious disease of the central nervous system and liver. [4] Wilson's disease is associated with a mutation in the gene ATP7B and lack of protein copper-transporting adenosinetriphosphatase involved in the excretion of copper ions from the body, [3,4] which leads to excessive accumulation of copper in the brain and liver. It was previously shown that the accumulation of copper ions in the liver tissues could be modeled by repeated administration of copper sulfate, which also leads to the development of liver fibrosis, similar to that described in Wilson's disease [5].

Medical scientists and doctors are especially concerned about metabolic disorders in the liver appeared due to drug therapy, the so-called drug liver damage. The drug liver damage markedly increased as a result uncontrolled consumption of vitamin preparations (according to the DILIN – Drug induced liver injury network). Therefore, the uncontrolled use of vitamins, in particular vitamin A, is of great danger, though there is an opinion that they cannot lead to any negative consequences.

In fact, we are faced with several important and controversial opinions related to various aspects of the action of vitamins, and in particular vitamin A. First, irrespective of the fibrosis inducers, the general body response is related to excess free radical production and oxidative stress. [6–8] Indeed, the elimination of oxidative stress in Cu-induced liver fibrosis was accompanied by the normalization in the functional characteristics of the liver. [7–9] Since vitamins A and E poses antioxidant properties, they could be used in the treatment of liver fibrosis, i.e. elimination of oxidative stress, at least in the initial stages of the development of this pathology, leading to normalization of the liver functional activity.

Secondly, there is evidence that long-term intake of vitamin A, even at therapeutic doses, may be accompanied by the development of drug liver damage. [10–14] Moreover, it was demonstrated that taking vitamin A in the presence of liver fibrosis can, on the contrary, accelerate the development of cirrhosis, [13] [-15] i.e. showing a negative effect. In addition, it is known that the main depot of vitamin A in the body are hepatic stellate cells (HSCs), which play an important role in the formation of liver fibrosis. [16–18] It has been shown that with liver fibrosis there is a loss (rapid utilization) of vitamin A by stellate cells. [17–19] It remains unclear how the response of the liver with fibrosis will change in the case of constant “replenishment” of the depot (hepatic stellate cells) with vitamin A, i.e. its long injections. Therefore, the available data related to the relationship of vitamin A nutrition and status with the development of fibrosis and the possibility of its clinical use in the treatment of this pathology deserve more attention and additional research.

We believe that such a complex nature of the body's response to the action of vitamin A depends on several factors. Primarily, it depends on the administered dose of vitamin A, and more precisely on the

balance between the dietary intakes of vitamin A, its accumulation in the liver, metabolic rate and on the features of the liver's functional state while taking vitamin A.

The aim of this work was to study effects of high dietary vitamin A provision to healthy rats and rats with Cu-induced fibrosis with specific emphasis to the effects of prolonged intake of vitamin A on the metabolic status of the liver (some biochemical parameters of animals' liver functional activity) (1); the relationship of the content of vitamins A with E in the liver of intact rats and rats with Cu-induced fibrosis (2); as well as the effect of vitamin A on some somatometric indicators in young rats (change in body weight, relative weight of liver, spleen and kidneys), as an indicator of toxicity (3).

2. Material and methods

2.1. Experimental facilities

The experiments were carried out on the mature 3-month-old male *Wistar* rats. The animals were kept in the standard conditions of the vivarium and they had food and water *ad libitum*. Twelve hours before the end of experiment animals were deprived of feed. The experimental animals were divided into 4 groups. The first (control) includes intact animals that fed a standard diet and kept in standard conditions. The second group consisted of intact animals which were daily administered *per os* with vitamin A at a dose of 300 IU/100 g body weight (90.00 μg/100 g body weight) in the morning before feeding. The third group included rats with Cu-induced liver fibrosis (the experimental fibrosis in animals was induced by injection of copper sulphate three times administration with interval between injections of 48 hours at a dose of 1 mg/100 g body weight as described in [6]) and they were also injected daily with vitamin A, as in the second group. And the fourth group of animals was based on animals with Cu-induced fibrosis without additional vitamin A supplementation. For sampling animals from each group were exposed to anesthesia on days 4, 7, 14, 21 after the start of the experiment (Fig. 1).

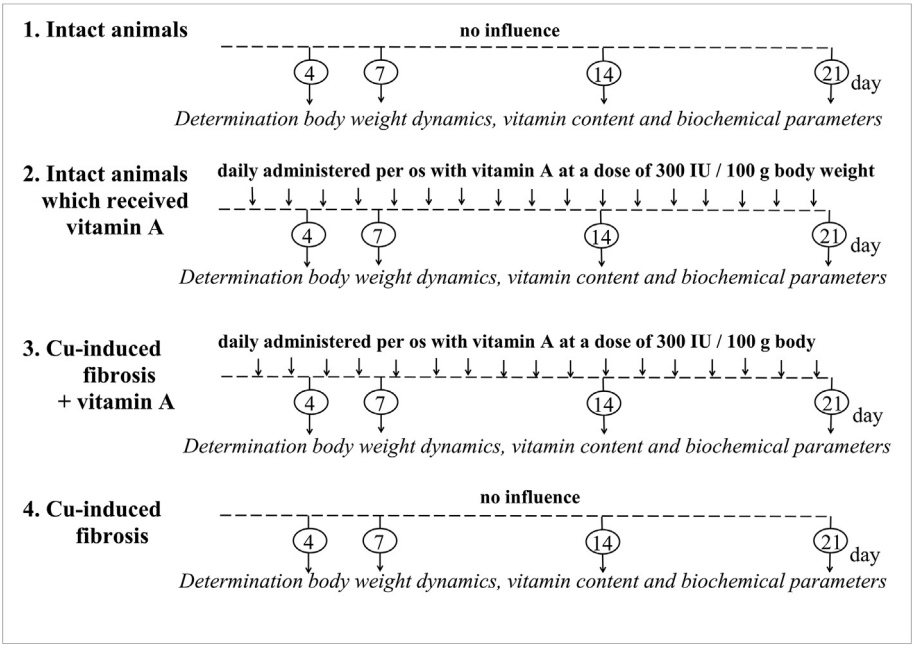

Fig. 1. The scheme demonstrates the sequence of administrations of vitamin A to animals and the procedure of removing animals from the experiment on days 4, 7, 14, 21.

All recommendations for bioethical standards were observed when working with animals [20] and the experimental protocol was approved by ethical committee of the university.

In the process of experimental preparation, the body weight was determined by weighing animals every day before feeding from 8 to 9 a.m. local time.

2.2. Isolation of serum and organs

After decapitation, blood was collected under anesthesia. To obtain serum, the blood was kept at temperature of 26 ° C for 30 minutes, and then it was centrifuged at 1500 g for 10 min at room temperature. The blood serum was transferred into sterile test tubes. The liver, spleen, kidneys were removed, and the relative organs mass in relation to the body weight of the animal of all experimental groups was determined.

3. Analytical methods

3.1. Alanine aminotransferase and alkaline phosphatase activity in serum

The activity of alanine aminotransferase (ALT) (EC 2.6.1.2) in blood serum was determined as described in. [21] The determination of ALT activity based on the following that 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 activity of alkaline phosphatase (ALP) (EC 3.1.3.1) in blood serum were determined as described in. [22] In the reaction of the determination of this enzyme, p-nitrophenyl phosphate is hydrolyzed to p-nitrophenol and inorganic phosphate. The level of p-NPP hydrolysis is directly proportional to the alkaline phosphatase activity.

Absorption was defined at a wavelength of 340 nm and temperature 37°C, than incubated for 60 seconds, and 60-seconds determination time (STAT-FAX 1908, USA). Activity of ALT expressed as arbitrary units (AU).

3.2. Cholesterol and urea content

The content of cholesterol in the blood serum was determined according to the method [23], and the concentration of urea in the blood serum of the experimental groups of animals was determined as described in [24]. 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 seconds, at 60-seconds determination time (STAT-FAX 1908, USA).

3.3. Vitamin A content

The content of vitamin A in the liver was determined according to the well-known method, [25] which is based on the complex formation of the vitamin with boron trifluoride etherate and determination of the this complex decomposition rate.

3.4. Vitamin E content

The content of vitamin E was determined by the method, [26] which based on the Emmery-Engel's reaction after its preliminary purification by thin layer chromatography.

3.5. Statistical analyses

All experiments were repeated at least 3 times. Data analysis was performed using Excel 2013 (Microsoft Corporation., USA) and STATISTICA 8 (Statsoft, USA) (for analysis of variance with repeated

measures (rANOVA). Visualization was performed using the Microsoft Excel software package 2013. The data are presented as group means and standard error (x ± SE), which were subjected to statistical processing using a nonparametric Mann – Whitney U-test. Differences were considered significant at $P < 0.05$.

4. Bioethical standards

Experiments on laboratory animals using copper sulfate were carried out in agreement with the V.N. Karazin Kharkov 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).

5. Results

5.1. Vitamins content in the liver after daily injections of vitamin A

5.1.1. Content of vitamin A

The vitamin A content in the liver of 3-month-old control animals was 16–18 μg/g of tissue and it remained at this level for 21 days of observation (Fig. 2A, curve 1).

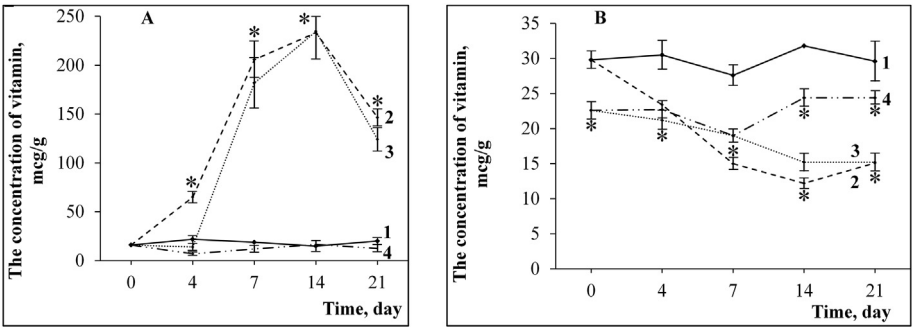

Fig. 2. The content of vitamin A in liver tissues (x ± SE): in intact control animals (1, n = 3), in animals daily administered with vitamins A at a dose of 300 IU/100 g body weight (or 90.00 μg/100 g body weight) for 21 days (2, n = 3), in animals with Cu-induced liver fibrosis, and also with administration of vitamin A daily (3, n = 3) and in animals with Cu-induced fibrosis (4, n = 3); the content of vitamin A is determined before the start of the experiment (0 days), and 7, 14, 21 days after the start of the application of vitamin A **(A);** in these same groups of animals, the content of vitamin E was determined **(B)**; * – significant values are noted ($P < 0.05$) compared to the intact level (nonparametric Mann – Whitney U test).

When intact animals were administered with vitamin A *per os* at a dose of 300 IU/100 g body weight (or 90.00 μg/100 g body weight) daily for 4 days the following changes were observed. Content of vitamin A in the liver by days 7 from the start of the experiment increased 11 times compared with the control level (Fig. 2A, curve 2). If vitamin A was administered for 14 days, then its amount in the liver increased only slightly compared to 7 days of vitamin A administration (Fig. 2.A. curve 2). If intact animals received vitamin A daily for 21 days, then its content in the liver 21th day decreased by 60% compared to 7th day of the experiment, however, it remained 8 times higher in comparison to the control animals (Fig. 2.A. curve 2).

Consequently, there was a U-shaped character of changes in the content of vitamin A in the liver from 1 to 21 days of the experiment against the background of daily administration of vitamin A to intact animals. That is, its content increased at the start of the experiment, and after reaching a certain concentration (about 250–300 μg/g) in the liver, its content decreased, despite the constant intake of vitamin A.

To determine the ability of liver tissue with fibrosis (i.e., the one that is in a different functional state) in comparison with the control to accumulate exogenous vitamin A, we evaluated its content in the liver in animals with liver fibrosis. It turned out that the content of vitamin A in the liver with Cu-induced liver fibrosis was 38–40% lower than in intact animals (Fig. 2A, curve 4). It should be noted that its content in this group of animals was quite variable. So, in a group of 10 animals, its content was 7.2 μg/g, and in another group, also of 10 animals, it was 17.4 μg/g.

If animals with liver fibrosis were administered with vitamin A *per os* at a dose of 300 IU/100 g body weight, then after 4 days the content of this vitamin in the liver did not change compared to the average initial level (Fig. 2A, curve 3). After 7 days of daily administration, the content of vitamin A in the liver increased 15 times compared to the initial level (versus 11 times in intact animals) (Fig. 2A, curve 3). At the same time, the content of vitamin A in the liver with fibrosis after 14-day administration of vitamin A slightly increased compared to 7 days of administration and did not differ from its content in intact animals against the background of administration of vitamin A (Fig. 2A, curve 3). After 21 daily administration of vitamin A to animals with liver fibrosis, its content decreased by 89% compared to 14-day administration; i.e. to a greater extent compared to intact animals (Fig. 2A, curve 3). Consequently, the content of vitamin A in the liver with fibrosis was lower than in the liver of intact animals. Daily oral administration of vitamin A (at a dose of 300 IU/100 g body weight) was accompanied by a relatively high rate of vitamin A accumulation in the liver and, after reaching the maximum concentration (on the 14th day); it began to decrease despite the daily administration of new doses of vitamin A for the next 7 days. That is, there was a U-shaped change in the content of vitamin A in the liver with fibrosis, as in the case of an intact liver.

5.1.2. Content of vitamin E

The vitamin E content in the liver of intact rats was 27–31 μg/g liver and remained unchanged from the first to the 21st day of the experiment (Fig. 2B, curve 1). If intact animals received daily vitamin A, then after 4 days the content of vitamin E was 24% less compared to the control level, and after 7 days of administration of vitamin A – by 46% and even 62% less after 14 days compared to the control. (Fig. 2B, curve 2). Therefore, there is a negative relationship between the content of vitamin E in the liver and an increase in the content of vitamin A in the liver of healthy animals on the background of the daily administration of exogenous vitamin A.

If the animal with liver fibrosis was administered daily vitamin A, then the content of vitamin E in the liver decreased by 28% to 14 days, and in the future, 21 days remained unchanged compared to 14 days (Fig. 2B, curve 3).

Therefore, there is a negative inverse relationship between vitamin A and vitamin E content in the liver.

5.2. Some biochemical parameters of animals' liver functional activity with induced hypervitaminosis A

5.2.1. Alanine aminotransferase (ALT) activity

The ALT activity was reduced by 34% compared to the intact control at 7 days after the start of the induction of fibrosis, and 21 days later, on the contrary, was increased by 79% (Fig. 3). Previously, it was shown during histological and biochemical studies that copper-induced fibrosis at such exposures is at the initial stages of development (F0, F1) [8,27]. It is known that at the initial stages of the liver fibrosis development, a decrease in activity ALT with its subsequent increase can be observed, and this characterizes the stages of pathology development [28].

If animals with liver fibrosis were repeatedly injected with vitamin A, then the ALT activity was reduced only by 26% to 7th day (Fig. 3), and after 21 days, the enzyme activity did not differ from the control values (Fig. 3). Therefore, administration of vitamin A to animals with fibrosis had a positive effect on ALT activity compared to fibrosis.

It was of interest to study the effect of vitamin A on ALT activity in healthy animals without liver fibrosis. We found that ALT on 7th day did not have significant differences compared to the control. On 21th day after the induction of fibrosis, ALT activity did not differ significantly from the control (Fig. 3, curve 4). These results indicate that long-term administration of vitamin A (21 days) at a dose of 300

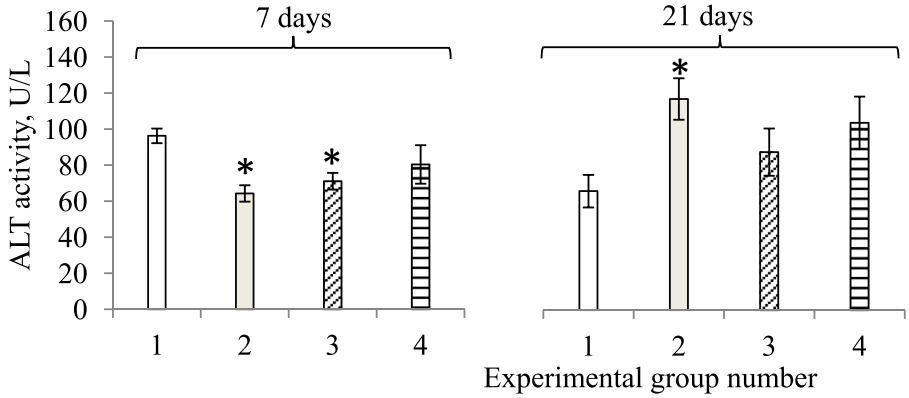

Fig. 3. *ALT* activity [U/L] in blood serum at 7th and 21st days (x ± SE) in intact control animals (1, n = 3), in animals with induced liver fibrosis (2, n = 3), in animals with liver fibrosis and daily administration of vitamin A *per os* at a dose of 300 IU/100 g body weight (or 90.00 μg/100 g body weight) (3, n = 3), and in intact animals with daily administration of vitamin A (4, n = 3); * – significant values are noted (P <0.05) compared to the intact level RANOVA before (7 days) after (21 days).

IU/100 g of body weight does not affect the ALT activity, and in animals with liver fibrosis it bring the ALT activity to the control values on the 21st day of the experiment.

5.2.2. Alkaline phosphatase (ALP) activity

As it is known, ALP activity is a marker of cholestasis [29].

The activity of ALP was reduced in comparison with intact control by 47%, and after 21 days, like ALT activity, it was increased by 42% in relation to control after 7 days from the start of the induction of liver fibrosis (Fig. 4).

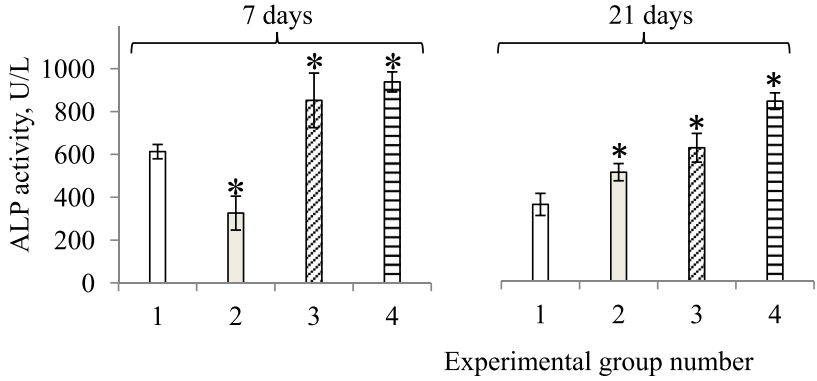

Fig. 4. The activity of alkaline phosphatase [U/L] in blood serum at 7th and 21st days (x ± SE) in intact control animals (1, n = 3), in animals with induced liver fibrosis (2, n = 3), in animals with liver fibrosis and daily administration of vitamin A *per os* at a dose of 300 IU/100 g body weight (or 90.00 μg/100 g body weight) (3, n = 3), and in intact animals with daily administration of vitamin A (4, n = 3); * – significant values are noted (P <0.05) compared to the intact level RANOVA before (7 days) after (21 days).

These data indicate that an increase in the content of copper ions in the liver leads to the development of cholestasis, which is at the initial stages of fibrosis development.

If the experimental animals with liver fibrosis were injected with vitamin A daily for 7 days, then the activity of alkaline phosphatase was increased by 39% compared with the intact control and by 162% compared with the fibrosis of animals that did not receive vitamin A (Fig. 4). On the 21st day of

the experiment, the alkaline phosphatase activity in the blood serum was increased in comparison with the intact control and did not differ from that one with liver fibrosis, which did not receive vitamin A (Fig. 4).

It is necessary to pay attention to the fact that the administration of vitamin A to healthy (intact) animals was accompanied by a more pronounced increase in the activity of alkaline phosphatase in the blood serum compared to the effect of vitamin A in animals with liver fibrosis, and this was manifested both after 7 and 21 days of the experiment (Fig. 4).

Consequently, the administration of vitamin A to animals with liver fibrosis is accompanied by an increase in ALP activity, but this was less pronounced than after administration of vitamin A to intact animals.

Consequently, the effect of vitamin A on the activity of the studied enzymes was different in healthy animals and animals with liver fibrosis.

5.2.3. The serum cholesterol concentration

The liver plays an important role in lipid metabolism and the manifestation of hypercholesterolemia with a simultaneous increase in ALP and gamma-glutaminetransferase (GGT) indicates the presence of cholestasis. It turned out that on the 7th day after the induction of fibrosis, the content of cholesterol did not significantly differ from the control, and on the 21st day it was increased by 44% (Fig. 5), which confirms the initial stages of the development of cholestasis in animals with an increased content of copper ions in the liver.

If animals with liver fibrosis received vitamin A, its content remained at the control level after 7 and 21 days (Fig. 5). This suggests that vitamin A is able to normalize liver function.

In intact animals, the cholesterol content did not change in comparison with the control after 7 days of vitamin A administration, and on the 21st day it increased by 77% (Fig. 5). In animals with fibrosis and the administration vitamin A, the cholesterol content remained at the control level after 7 and 21 days (Fig. 5).

Consequently, the administration of vitamin A to experimental animals with liver fibrosis was accompanied by the normalization of serum cholesterol by 21th day of the experiment. Whereas in intact animals' vitamin A increased cholesterol content by 77% compared to control.

5.2.4. The serum urea concentration

The urea content after 7 days of the experiment start remained at the control level, both in animals with liver fibrosis and in animals with liver fibrosis and administration of vitamin A (Fig. 6).

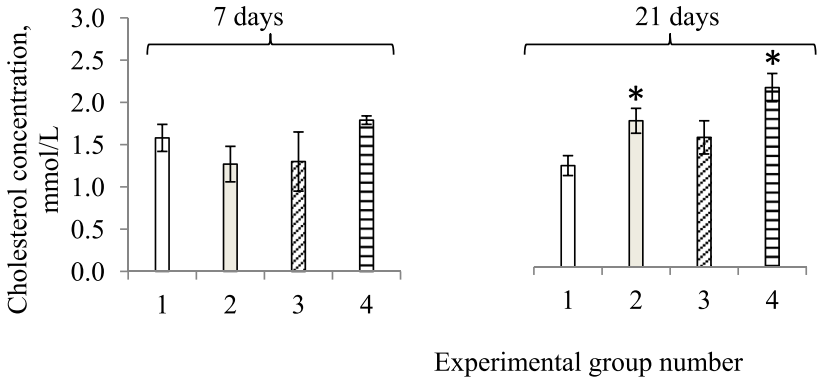

Fig. 5. Cholesterol content (mmol/L) in blood serum at 7th and 21st days (x ± SE) in intact control animals (1, n = 3), in animals with induced liver fibrosis (2, n = 3), in animals with liver fibrosis and daily administration of vitamin A *per os* at a dose of 300 IU/100 g body weight (or 90.00 μg/100 g body weight) (3, n = 3), and in intact animals with daily administration of vitamin A (4, n = 3); * – significant values are noted (P <0.05) compared to the intact level RANOVA before (7 days) after (21 days).

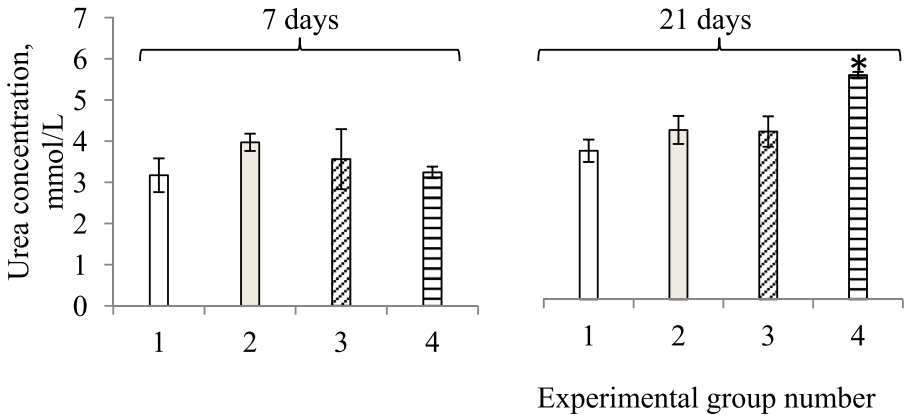

Fig. 6. Serum urea concentration (mmol/L): in blood serum at 7th and 21st days (x ± SE) in intact control animals (1, n = 3), in animals with induced liver fibrosis (2, n = 3), in animals with liver fibrosis and daily administration of vitamin A *per os* at a dose of 300 IU/100 g body weight (or 90.00 μg/100 g body weight) (3, n = 3), and in intact animals with daily administration of vitamin A (4, n = 3); * – significant values are noted (P <0.05) compared to the intact level RANOVA before (7 days) after (21 days).

However, the urea content increased by 50% in comparison with the control on the 21st day of administration of vitamin A to intact animals (Fig. 6). At the same time, the serum urea content in animals with fibrosis after 21 days of vitamin A intake remained at the level of intact control (Fig. 6). Consequently, different effects of vitamin A in intact animals and animals with liver fibrosis were also manifested in terms of cholesterol and urea content.

The effect of vitamin A on the studied biochemical parameters in animals with fibrosis had a positive effect on the 21st day of admission to normalize the ALT activity, cholesterol content, did not affect the urea content and did not affect the ALP activity in the blood serum. At the same time, vitamin A in the studied doses had a hepatotoxic effect in healthy animals, which was manifested in an increase in the activity of ALT and ALP, an increase in the content of cholesterol and urea in the blood serum on the 21st day of taking vitamin A.

5.3. Some somatometric parameters in animals with hypervitaminosis A

The body weight of the intact control animals increased from 1st to 21st days of observation by 34–35 % of the initial body weight (Fig. 7A, curve 1). It should be noted that the dynamics of body weight growth was close to linear.

In the event that liver fibrosis was induced in animals by three times administration of copper sulfate, then they lost body weight in the first 3–5 days, and then it slowly recovered and on days 20–23 slightly differed from the control (Fig. 7A, curve 2).

If, against the background of Cu-induced liver fibrosis, the animals were injected with vitamin A, they slightly lost body weight from 1 to 5 days from the beginning of the experiment (Fig. 7A, curve 3). After that, their body weight increased over the next 5 days, and further superiority was observed in comparison with the control in the increase in body weight (Fig. 7A, curve 3).

In the group of animals that received vitamin A daily, the following was observed: a slight loss of body weight starting from 3 to 5 days by 5–6% of the initial weight; in the period from the 6th day of the experiment, their body weight increased by 8–10% and remained until the 9th day. As a result, on day 21, an increase in body weight of 20–25% was observed in healthy animals in comparison with the control (Fig. 7A, curve 4).

Consequently, the administration of vitamin A to animals with liver fibrosis was accompanied by an acceleration of body weight growth, and they exceeded the control in this indicator.

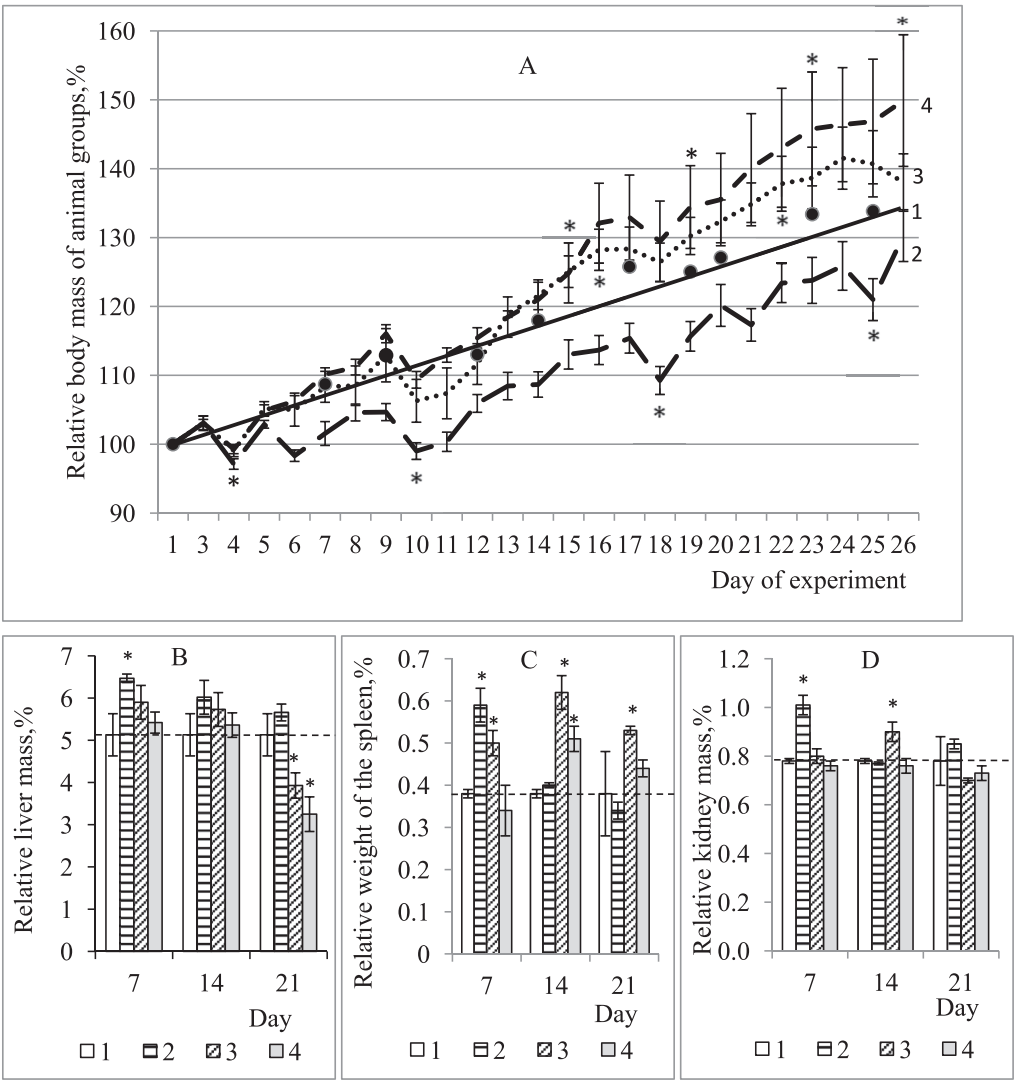

Fig. 7. Dynamics of body weight in intact control rats (1, n = 3), animals with Cu-induced liver fibrosis (2, n = 3), rats with Cu-induced liver fibrosis, which were also injected daily with vitamin A (3, n = 3), intact animals, which were injected daily with vitamin A *per os* at a dose of 300 IU/100 g of body weight (or 90.00 μg/100 g of body weight) for 21 days (4, n = 3) (A); changes in the relative mass of the liver (B), the relative mass of the spleen (C) and the relative mass of the kidneys (D) in the intact group (1, n = 3), in the group with Cu-induced liver fibrosis (2, n = 3), in the group with Cu-induced liver fibrosis and daily injections of vitamin A (3, n = 3), as well as in intact animals, which were injected daily with vitamin A *per os* at a dose of 300 IU/100 g of body weight on days 7, 14 and 21 of observation (4, n = 3); * - significant values are noted ($P < 0.05$) compared to the intact level (nonparametric Mann-Whitney U-test); the dotted line marks the control level from 7 to 21 days of the experiment.

If healthy animals received vitamin A, then their growth exceeded the control group of animals on days 15–26 of vitamin A intake, and animals with fibrosis did not differ in growth intensity. This may indicate a positive role for vitamin A in restoring body weight.

Changes in body weight, as a rule, correlate with changes in organ weight. [30] The relative liver mass of the control group of animals remained unchanged from the first to the 21st day of the experiment (Fig. 7B). The relative weight of the liver with fibrosis was increased in comparison with the control on the 7th day and returned to normal on the 14th and 21st days (Fig. 7B). If vitamin A was

administered to animals with Cu-induced liver fibrosis, the relative liver weight on days 7 and 14 did not differ from the control, and on day 21 it decreased compared to the control (Fig. 7B). The administration of vitamin A to intact animals for 7 and 14 days had no effect on the relative weight of the liver, and on day 21 it decreased almost 2 times in relation to the control.

Consequently, vitamin A normalized the relative liver mass in animals with Cu-induced fibrosis at the initial stages of its administration, and subsequently led to a decrease, which may be associated with the effect of vitamin A (Fig. 7B).

In animals with fibrosis, the relative weight of the spleen was increased by 7 days in the development of this pathology. However, later on, on days 14 and 21, the relative weight of the spleen did not differ from the control. Animals with fibrosis who received vitamin A also had an increased spleen weight compared to controls on days 7 and 14. The administration of vitamin A to intact animals had no effect on the relative weight of the spleen on day 7, increased this indicator on day 14, and on day 21 it did not differ from the control. These data suggest that vitamin A in animals with liver fibrosis had an ambiguous effect on the function of this immunocompetent organ.

The relative weight of the kidneys changed insignificantly in the studied groups of animals.

6. Discussion

The results of the investigation allow us to draw several conclusions that need to be discussed.

1. Previously, it was shown that repeated sequential administration of copper sulfate (30% of the lethal dose) to experimental animals was accompanied by the accumulation of copper ions in the liver, where they bind to mitochondria (the appearance of oxidative stress), to the endoplasmic reticulum, and leads to dysfunction of hepatocytes and the start of fibrogenesis [31]. These changes were similar to those caused by carbon tetrachloride [32]. Consequently, copper sulfate induces the development of toxicogenic liver fibrosis (Cu-induced fibrosis) and can be used as a model of fibrosis at the initial stages of its development.
2. In this work, we have shown that animals with Cu-induced liver fibrosis lagged behind intact controls, had a complex dynamics of changes in the indicators of liver functional activity (ALT, ALP, cholesterol content decreased to 7th day with a subsequent increase to 21st day of development of pathological process (Fig. 3,4,5). These changes occurred against the background of a decrease in the

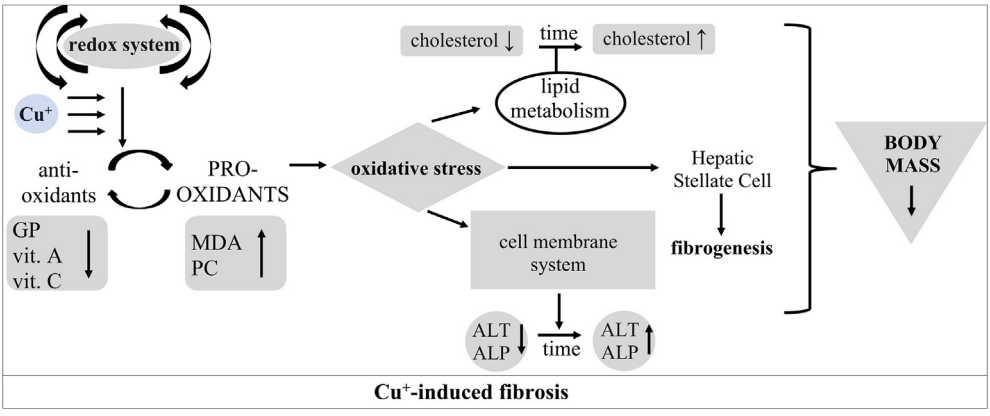

Fig. 8. The scheme demonstrates a possible sequence of metabolic changes induced by multiple successive copper sulfate injections: the primary reaction of changes in the ionic composition in the body and antioxidant activity inhibition, including a decrease in the content of vitamin A, E (↓); oxidative stress leads to hepatocyte membranes damage, activates hepatic stellate cells, this triggers fibrogenesis and affects energy and lipid metabolism at the body level, and leads to body weight loss.

content of vitamins A and E in the liver (Fig. 8). A decrease in the vitamins content (with antioxidant properties) is associated with the manifestation of oxidative stress, which was shown earlier [33].

3. If experimental animals with Cu-induced fibrosis were injected daily with vitamin A at a dose of 300 IU/100 g body weight, then the accumulation of vitamin A in the liver was observed, and after reaching a concentration of 250–300 μg/g, a decrease in its content was observed (Fig. 2), restoration or approach of the liver functional activity to the control animals (assessed by the indicators of ALT, ALP, cholesterol, the relative mass of the liver, spleen, kidneys and growth restoration of animals). This allows us to consider the possibility of using vitamin A in the elimination of liver fibrosis.

If experimental animals with Cu-induced fibrosis are injected daily with vitamin A at a dose of 300 IU/100 g of body weight, then the accumulation of vitamin A in the liver was observed.

4. If vitamin A was administered to intact control (healthy) animals, then its accumulation in the liver occurred faster and it was metabolized/excreted faster than in animals with fibrosis; the functional activity of the liver, based on the studied parameters, was inhibited, while this did not affect the growth rate of the animals, and then they exceeded it in comparison with the control (Fig. 2,3,4,5). Consequently, the biological activity of vitamin A depends not only on the dose, but also on the functional state of the liver, that is, the effect of vitamin A in intact animals and animals with liver fibrosis was different.

The presence of a complex characteristic of the time concentration dependence of the vitamin A content in the liver (the main depot of its accumulation in the body) indicates the formation of resistance to excessive accumulation of this vitamin in the body. The «need» for the formation of resistance to excess vitamin A is due to the manifestation of toxicity at high doses of this vitamin, at least for healthy (intact) animals, as evidenced by numerous data [15,34] and the results of this work.

The mechanism of toxicity of vitamin A upon reaching 450–500 mcg/g of liver can be explained by the membranotropic effect of this vitamin.

As it known, vitamin A in the body exists in three forms: retinol, retinal and retinoic acid. They depend on the degree of carbon atoms oxidation. [18,35] By nature, a lipid molecule has a lipophilic ring on one, and an alcohol group on the other end of the molecule. [18] As a lipid, vitamin A exhibits a membranotropic effect with respect to all types of membranes manifested in antioxidant and hepatotropic effects. The results of this work may also indicate this. The accumulation of vitamin A in the target organ depends on the functional state of the liver; this can be explained by the peculiarities (level and direction) of metabolism. It was shown that for intact animals with hypervitaminosis A, the membranes of lysosomes are disrupted, which leads to the release of hydrolytic enzymes, cell destruction and the development of inflammatory reactions, [14] disruption of mitochondrial membranes can lead to apoptosis, and erythrocyte membranes to hemolysis. [36] It should be noted that the “concentration boundary” of the manifestation of antioxidant/prooxidant properties of vitamin A has not been established, but, perhaps, depends on the state of the organism metabolic characteristics, and, in particular, the liver.

The mechanism of resistance to super-large doses of vitamin A is of great interest, since this one can be attributed to general biological effects. Similar resistance is shown to toxic doses of radiation (hormesis effect), [37] heavy metal ions [38] and other toxicants.

The mechanism of induced resistance to large doses of vitamin A can be implemented at several levels of regulation; at the level of transport from the gastrointestinal tract (GIT) to the liver, at the level of its deposition in the liver and metabolic rate, as well as the rate of excretion from the liver (Fig. 9).

It is known that the «absorption» of vitamin A is carried out by enterocytes of the epithelium of the small intestine. Vitamin A from micelles diffusion penetrates through enterocyte membranes, then forms chylomicrons and is transported to lymph [39].

Available data indicate that cholestasis leads to the formation of fibrosis, [40] [[,41] and on the other hand, liver fibrosis, induced by metabolic disorders in the liver, affects the formation of bile. [41,42] This suggests that in animals with liver fibrosis, the mechanism of absorption and transport of vitamin A to

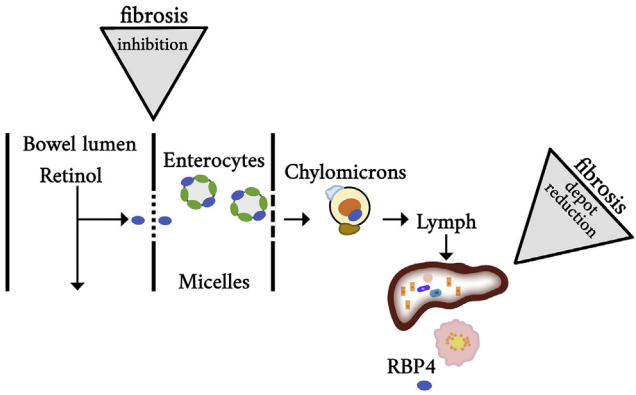

Fig. 9. The scheme demonstrating the main stages of the transport of vitamin A from the digestive tract to the liver, their deposition in stellate cells of the liver. In animals with fibrosis, the transport of vitamin A through enterocytes and their deposition in hepatic stellate cells (HSCs) can be inhibited is manifested in apoptosis of connective tissue elements.

the lymph is impaired. This is indirectly evidenced by the data on the presence of diarrhea in animals with Cu-induced fibrosis [43,44].

The next step in regulating and maintaining the concentration of vitamin A at a certain level in the liver is intracellular transport and the formation of a depot, especially in stellate cells of the liver. [16–18] Vitamin A binds to a retinol binding protein in the cells. Currently, there are 4 known families of these proteins that are part of the subfamily lipid carriers. The main is retinolbinding protein 4 (RBP4) provides transport, protection against oxidation and the action of hydrolases [45] and is able to deposit vitamin A. [46,47] There is evidence that in the case of large doses of vitamin A, RBP4 binds this one, protecting the cell membranes from its toxic effects. [48] It is known that in hepatitis there is lipid accumulation [49], which indicates the participation of RBP4 in this process and, as a result, may lead to a decrease in intracellular transport and accumulation of vitamin A in hepatic stellate cells (HSCs). Moreover, these proteins are multifunctional; it is shown that RBP4 is a hormone that is activated by insulin resistance. [46,50,51] RBP4 is involved in the regulation of cardiomyocyte activity and is a risk factor for cardiopathologies; it is clear that RBP4 performs different functions in fibrosis than in intact animals. [52] Moreover, it was found that the content of RBP4 correlates with body mass index. [53] This can be attributed to an increase in the growth dynamics of animals receiving vitamin A with some depression of liver function (Fig. 7.A).

Therefore, we can assume that the excess intake of vitamin A into the body, binding to RBP4 (which is aimed at eliminating the toxic effect of vitamin) leads to the manifestation of a whole complex of metabolic consequences, which was also manifested in a change in body weight, as can be seen from our experiment, and this happens differently in intact animals and animals with liver fibrosis.

It is known that 90% of liver vitamin A is localized in HSCs. [54,55] In the case of «excess» vitamin A, large lipid vesicles form in HSCs, [56,57] which leads to the transformation of HSCs into myofibroblasts, [6] which produce collagen and aggravate the course of liver fibrosis, which can turn into cirrhosis. Since HSCs are already activated by growth factors in case of Cu-induced fibrosis, large doses of vitamin on the one hand eliminate the manifestation of oxidative stress and, on the other, trigger another chain of metabolic events in the development of liver pathology, affecting HSCs. Against the background of such changes, a nonlinear process of loss and partial restoration of body mass took place. A significantly lower accumulation of vitamin A in the liver with fibrosis compared with the intact liver may indicate a lesser depot ability to activate HSCs during fibrosis, which is explained by a lower content of vitamin A in the liver with fibrosis.

In conclusion, we note that the absorption, transport, metabolism, and deposition of vitamin A in the liver can be altered with liver fibrosis.

The most important issue in understanding the mechanisms of action of vitamin A is the effect of excess vitamin A on vitamin status in the liver. The decrease in the content of vitamins E and C against the background of hypervitaminosis A can be explained by both direct and indirect interaction of vitamins. Direct interactions include the following: vitamin C restores the oxidized form of vitamin E, in turn, the activity of vitamin E depends on the action of vitamin A, the presence of selenium and sulfur-containing amino acids. [58] The indirect effect of vitamin A on the content of other vitamins (in particular E and C) can be realized in different ways. It may be changing the composition and content of proteins involved in the metabolism of vitamins, the compensatory effect in the case when different vitamins perform similar functions (antioxidant), competition for «substrate», etc. Practitioners prescribe vitamins E and C for hypervitaminosis A. [59] This confirms the results of a decrease in these vitamins with hypervitaminosis A. It is important to note that the decrease in the content of vitamin E against the background of hypervitaminosis A is less pronounced than in the liver of intact animals, which indicates the relationship between the general metabolism in the liver and the metabolism of vitamins.

Consent to publication

All co-authors of this article (Anatoly Bozhkov, Igor Ionov, Nataliia Kurhuzova, Anna Novikova, Oleg Katerynych, Akzhyhitov R.) agree to publish.

Author contributions

Anatoly Bozhkov: idea of work, processing of results, writing a manuscript; Igor Ionov: took part in the experiment; participation in data analysis, design and revision of the article; Nataliia Kurhuzova: determination of enzyme activity; participation in data analysis, design and revision of the article; Anna Novikova: biochemical research; participation in data analysis, design and revision of the article; Oleg Katerynych: determination of vitamin A content; participation in data analysis, design and revision of the article; Rustam Akzhyhitov: work with experimental animals; participation in data analysis, design and revision of the article.

Bioethics

Experiments for laboratory animals were carried out in agreement with the bioethical committee of V.N. Karazin, 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).

Conflict of interest

This work was carried out with the financial support of V.N. Karazin Kharkiv National University.

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

The authors are interested in publishing the manuscript and there is no conflict of interest.

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