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Ontogenetic Approach to the Study of Mechanisms of Copper-Induced Liver Fibrosis

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Abstract

On the model of Cu-induced liver fibrosis, the relationship between the activity of prooxidant-antioxidant system, immune system parameters, liver morphology and several physiological parameters (body temperature and performance ability of the animals, taking into account their ages) was investigated. Classical biochemical, immunological, histological and physiological methods of investigation were used. The subjects of the study were male Wistar rats of 3-month (young) and 20-month (old) age. For the induction of liver fibrosis, experimental animals were successively injected with copper sulfate (three times at intervals of 24 hours at a dose corresponding to 33% of the lethal one). It was shown that after five days of sulfate copper administration inflammatory reactions in the liver, damage of the vessel epithelium, an increase in collagen content, and other morphological changes were detected. At this time, the content of lipid hydroperoxides in the liver and blood serum was increased, the activity of a number of antioxidant enzymes was reduced, and the activity of aconitase was two times less compared to values in the control group. These changes correlated with a decrease in the amount and activity of phagocytic cells in the blood of experimental animals. Inhibition of the general metabolism was accompanied by a decrease in body temperature, loss of body weight and performance ability. The relationship between a specific metabolic pattern in animals with Cu-induced fibrosis was age-dependent. The formed specific adaptive metabolic pattern is unstable, and in the future it can be realized in one of three possible adaptive strategies, the choice of which is influenced by age.

Keywords

Lipid Hydroperoxides, Glutathione Peroxidase, Aconitase, Liver Fibrosis,

Ontogenesis

1. Introduction

According to the World Health Organization for the past 20 years, there has been a steady increase in chronic liver diseases of various etiologies, and mortality from liver diseases has reached the 4th place in the world [1] [2].

Thus, the study of the mechanisms of induction of liver pathologies development is an urgent objective. As the liver is the central organ of homeostasis regulation and provides the functions of detoxification of various xenobiotics, a disturbance of the structure of the liver cells will induce systemic changes in the body. It is known that in response to hepatotoxic compounds, regardless of their nature, the development of a complex of inflammatory reactions is induced [3] [4].

Inflammatory reactions in the liver can be accompanied by: 1) the formation of foci of regeneration with the subsequent restoration of the homeostatic functions of the liver; 2) the formation of fibrosis; 3) further transition of fibrosis to cirrhosis.

The essence of the problem of liver pathologies can be reduced to an understanding of the mechanisms for the formation of a metabolic strategy for the development of induced fibrosis. The solution of this problem is of great practical importance, since this knowledge can be the basis for developing new ways of liver pathologies treatment and understanding the fundamental mechanisms of reprogramming the activity of liver cells.

Toxicological fibrosis is of particular interest. Among the toxic compounds of hepatotropic action, heavy metal ions and, especially, copper ions are of great concern.

Disorders of copper metabolism in the organism can be accompanied by the accumulation of copper ions in the liver—Wilson-Konovalov's disease [5].

Age is known to be one of the most important factors both in the formation and in the treatment of various pathologies. It is shown that the outcome of fibrosis and other liver diseases treatment depends on the age of the patients [6]. However, the principles and the mechanisms of the age effect on these processes remain unclear. At the same time, studies of the influence of age on the mechanisms of the development of pathologies are the most important task of gerontology.

The ontogenetic approach to the study of liver pathology mechanisms has several practical and fundamental aspects. We have shown earlier that repeated successive administration of copper sulfate to the experimental animal was accompanied by the accumulation of copper ions by the liver, these ions were bound to copper-binding proteins of the liver cell cytosol, to mitochondria and the microsomal fraction [7]. Such an experimental approach allows modeling a non-genetic analog of the development of Wilson-Konovalov's disease. Using

this model, it was shown that a multiple increase in the content of copper ions in the liver cells was accompanied by an increase in the collagen content in this organ and by the manifestation of oxidative stress [8], which is characteristic for the development of fibrosis.

In the present work, a system study of the morphology, biochemistry and functional activity of the animals with experimental Cu-induced fibrosis was carried out, namely, histological changes in liver cells, the content of lipid hydroperoxides and the activity of enzymes (aconitase, glutathione peroxidase, glutathione reductase and glutaredoxin) in mitochondria and blood serum, some parameters of the cellular immunity (phagocytic number, phagocytic index, completeness of phagocytosis) and of humoral component of immune system (peptides of intermediate molecular weight), acute phase proteins (ceruloplasmin, haptoglobin) at 24 hours after the last administration of copper sulfate and at five days after its first administration in young (3 months) and old (20 months) rats, as well as changes in their body weight, rectal temperature and performance in a swimming test with a load.

2. Materials and Methods

The research was conducted on males of Wistar rats maintained at standard vivarium conditions and carried out in accordance with the guidelines of the European Convention for the Protection of the Vertebrata using for the experimental and scientific aims [9] [10]. The last was confirmed by Bioethics Committee of V.N. Karazin Kharkov National University.

Decapitation of animals and all experimentations started always at the same time (8 a.m.). Control and experimental animals were weighed daily before feeding. The rectal temperature was measured at the same time by Micro Therma 2T Hand Held (Braintree scientific, INC., USA). The performance ability of the animals was estimated by swimming test with load at the water temperature 12˚C - 14˚C [11]. At decapitation the blood was collected for serum obtaining.

The liver was weighed and ratio of liver weight to body weight was determined.

After animal decapitation, the liver fragments were taken from the same lobe of liver and fixed in 10% formalin for 48 hours for histology analysis. Liver samples were analyzed histologically by standard methods according to Van-Gison [12]. Histological samples were analyzed microscopically at magnification ×100 and ×400. The mitochondria were obtained by differential centrifugation [13]. The liver was perfused by physiological solution, taken out and weighed, in the liver aliquot the collagen amount was determined [14]. Lipid hydroperoxide content was determined by the method of Ohkawa *et al.* [15] in hepatocytes and subcellular fractions of liver, and by the method of Asakawa *et al.* [16] in the blood. The content of lipid hydroperoxides was expressed in equivalent quantities of (MDA) per 1 mg of protein or 1 ml of serum.

Glutathione peroxidase activity (GP, EC 1.11.1.9) was determined in cytosolic fractions, liver mitochondria serum spectrophotometrically at 340 nm [17].

The activity of glutathione reductase (GR, EC 1.6.4.2) in liver homogenates and mitochondria was measured spectrophotometrically by decrease of NADPH content [18].

Aconitate hydratase activity (aconitase, AG, EC 4.2.1.3) was determined as described [19]. Determination of glutaredoxinactivity in rat liver mitochondria was performed by spectrophotometric method of Raghavachari [20] with minor modifications Gallogly [21].

Phagocytic activity of neutrophils was assessed by the absorption and elimination of microbial cells *Saccharomyces cerevisiae* neutrophilic granulocytes (NG) using light microscopy method [22]. The concentration of haptoglobin was determined by the method of Ravin [23], and ceruloplasmin—by the method of Gabrieljan *et al*. [24]. The peptides of intermediate molecular weight amount were determined by the method described in Bozhkov and Nikitchenko [25].

3. Results

The dynamics of body mass and performance ability after three successive injections of copper sulfate

The body mass of 3-month-old (young) control rats increased during the experiment by 8%, *i.e.* it was the same as in the animals under standards conditions. For the same period body mass of 20-month-old control animals did not change accordingly with standard growth rate in this age (**Figure 1**).

As a result of intraperitoneal administration of copper sulfate the body weight loss occurred even after the first administration, and by the 10^{th} day of the experiment the weight lagged by 6% - 8% compared to the control animals (**Figure 1**).

Body weight of 20-month-old animals also decreased by 3% - 5% compared to the control (**Figure 1**).

Consequently, the administration of LD_{33} of copper sulfate was accompanied by body mass loss, being expressed to a greater extent in younger growing animals.

The copper sulfate administration to the experimental animals was accompanied by insignificant decrease of body temperature by 0.6˚C - 0.7˚C and it was expressed to a greater extent in old animals (**Figure 1**).

The body temperature is known to decrease along with age [26]. If the difference between the control groups of young and old animals is turned to be 0.6˚C - 0.7˚C, then after triple copper sulfate injection this age difference increased till 0.8˚C - 1.5˚C (**Figure 1**).

It can be assumed that physiological changes will have an effect on the adaptive capacity of these animals to the subsequent or new stressors.

To evaluate this, young and old animals with copper-induced fibrosis were subjected to psychical and physical stress—swimming with load in cold water (14˚C).

The old control animals (20-month-old) lost the capacity to stay on the water six times faster (swimming with load) compared to the young control animals (**Figure 2**).

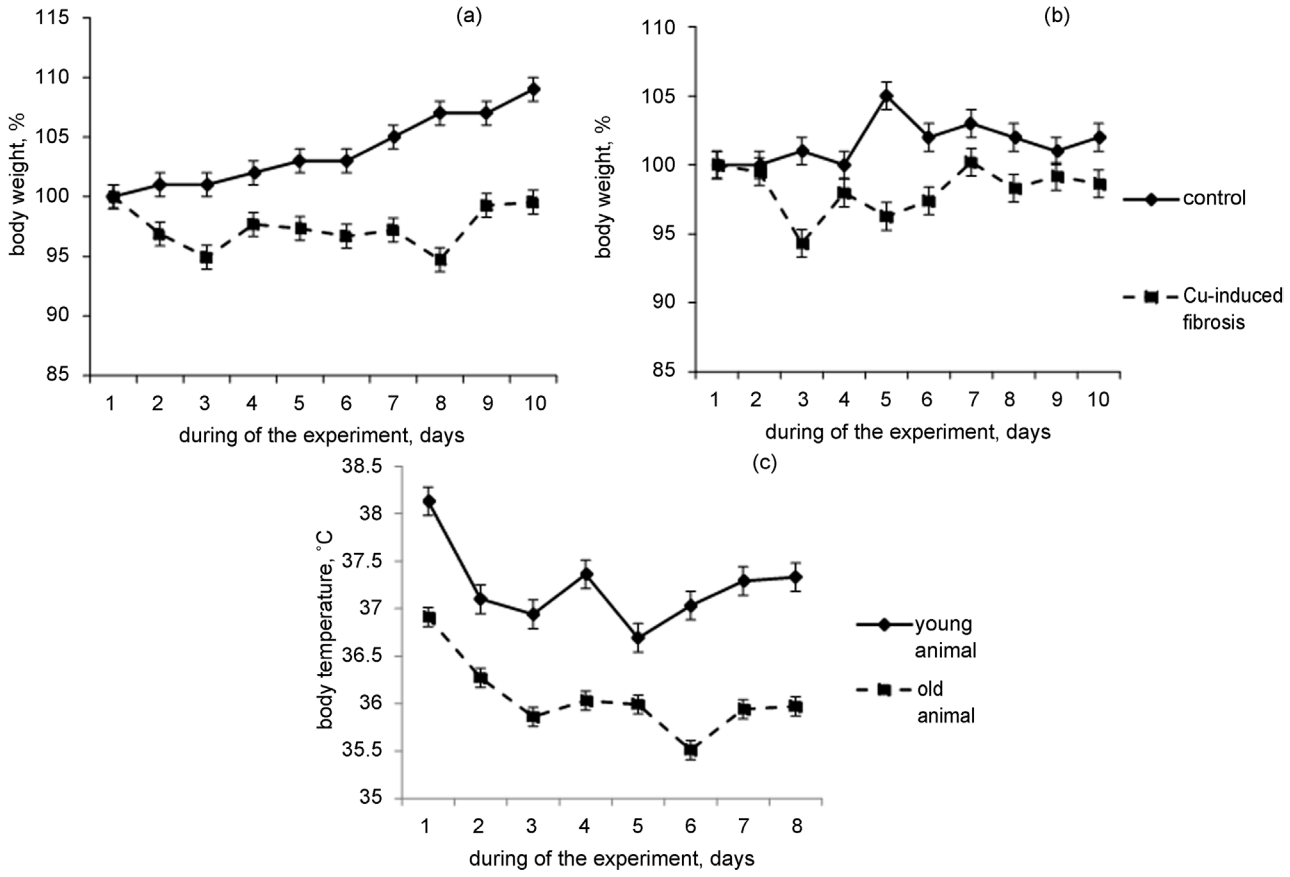

Figure 1. The dynamics of body weight young (a) and old (b) animals of the control group and animals with Cu-induced fibrosis. The body temperature experimental animals of different age (c).

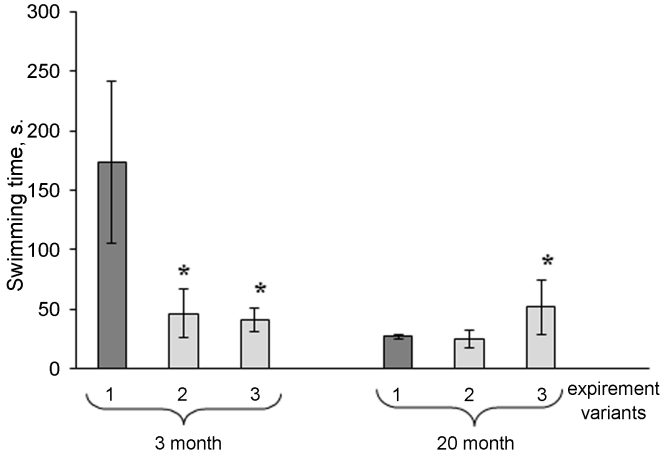

Figure 2. Swimming time 3 and 20 months rats of control group (1), 24 hours after the last administration of copper sulfate 1 mg/100 g of liver weight (2) and repeated swimming of these animals 24 hours after the first swimming (3). *P ≤ 0.05 compared with the same age control.

At the same time, with the progression of fibrosis in 3-month-old (young) animals their ability to perform a physical activity decreased in 3.7 times compared to the control (Figure 2), while the fibrosis progression in 20-month-old animals didn't affect their ability to keep on water (Figure 2).

Moreover, if swimming was repeated in 24 hours the young animals swam the same time, while old animals swam twice longer compared to the first swimming (Figure 2). So, old animals become adapted to this influence faster than young ones.

Consequently, even at the start of copper-induced fibrosis development (five days after the beginning of copper sulfate administration) the growth retardation, performance ability loss and insignificant decrease of rectal temperature were revealed. These changes had complex age-dependent character; the old animals lost body weight less and recovered faster at the background of higher body temperature decline.

Morphological changes in liver after three successive injections of copper sulfate

In five days after the first copper sulfate administration at a dose of 1 mg/100 g of body weight the was significantly lower than in the control, and the amount of the total collagen was increased (Figure 3). It should be noted that the shape of the liver lobes was changed, and all of them were joint. The development of the capsule was different in different animals that meant an individual variability of this index (Figure 3). In addition, liver color was dark gray and such liver was perfused badly indicating its poor circulation.

In liver histology of control group the capsule was thin, compact and tightly adjoint to liver parenchyma (Figure 4(a)).

Around the central veins of the liver lobules there is thin collagen matrix, radially divergent to the portal tracts (Figure 4(b)). Portal tracts presented the classical triad (hepatic artery, portal vein, bile duct) with a strong surrounding component of collagen (Figure 4(c)). The hepatocytes morphology was typical, cell nuclei were moderately hyperchromatic, sometimes binucleated (Figure 4(b)).

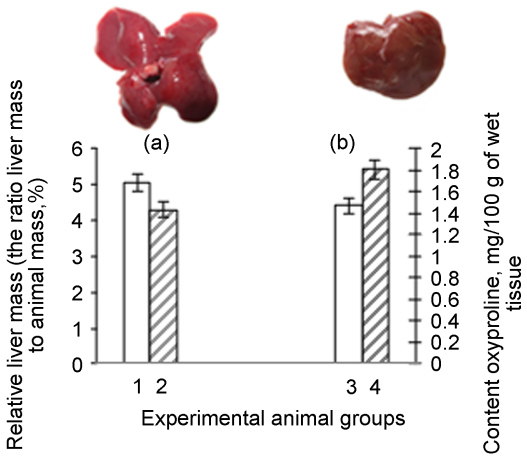

Figure 3. Morphology of liver of control rat group (a) and a group of rats who were administered three times successively copper sulfate at a dose of 1 mg/100 g body weight every 48 hours between doses (b). The relative liver weight in control rats (1) and rat liver treated with copper sulfate (2), and collagen content of control (3) and receiving the copper ions (4) rats. The parameters were determined 24 h after the last administration. *significant differences between the control and experimental animals at $P < 0.05$.

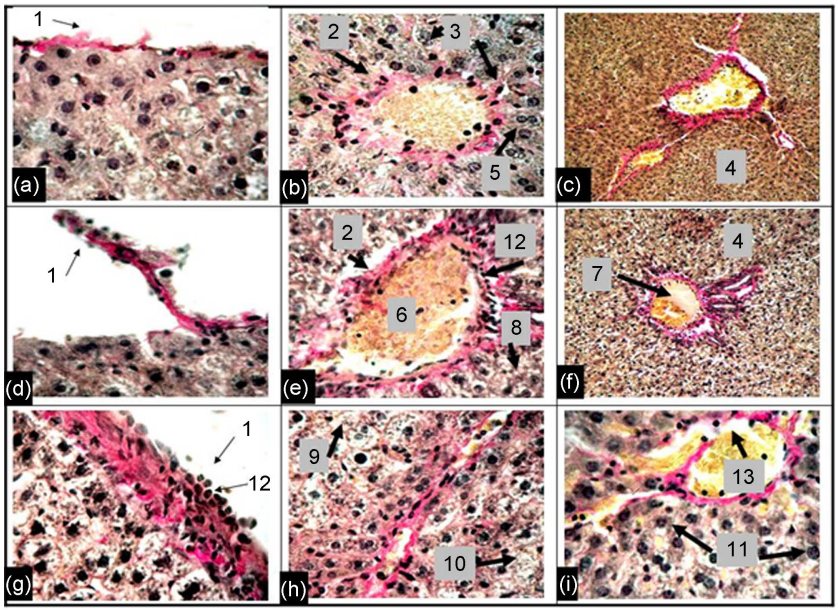

Figure 4. Photomicrographs liver preparations control ((a)-(c)) and experimental animals ((d)-(i)). Notes in the text. Van Gieson staining. Increased ×100 ((c), (f)) and ×400 ((a), (b), (d), (e), (g), (h), (i)). Legend: 1. Kapsula; 2. Central viena; 3. The collagen matrix; 4. The portal tracts; 5. Two-nuclear forms of hepatocytes; 6. Venous congestion; 7. "Separation" of formed elements; 8. Cells with "foamy" cytoplasm; 9. "Shadows" of the nuclei; 10. The cells have lost the nucleus; 11. "Many nucleolar" cells; 12. The infiltration of mononuclear elements; 13. Damage of the vascular endothelium.

In 24 hours after copper sulfate administration liver morphology differed from the control group.

There was an expressed venous congestion in parenchyma both in the central vein and in the portal veins (**Figure 4(e)**). In some branches of portal vein the "separation" of blood cells from plasma was revealed suggesting the venous stasis (**Figure 4(f)**). Throughout the parenchyma there were hepatocytes with hydropic dystrophy (with "foamy" cytoplasm), with necrobioses features (nucleus shadows) and necrotic (without nucleus) (**Figure 4(e)** and **Figure 4(h)**). The cell integrity maintenance suggests an unusual necrosis of hepatocytes according to the apoptotic type. The morphologically preserved hepatocytes often have as high as 3 nucleoli indicating the proliferative adaptive activity of cells (**Figure 4(i)**). The most pronounced liver morphology changes in the experimental animals were revealed in capsule structure. Differed from the control, it was in some places friable, not tightly adjoint to the parenchyma, sometimes with complete detachment from the parenchyma (**Figure 4(d)** and **Figure 4(g)**). There were a large number of fibroblasts, lymphocytes, reticulocytes, monocytes (**Figure 4(g)**). The infiltrate of mononuclear elements was also observed in portal tracts stroma (**Figure 4(e)**). These phenomena suggested the inflammatory process in connective tissue. In some instances the vessels endothelium was damaged that was accompanied by bleeding in parenchyma (**Figure 4(i)**).

Some indices of prooxidant-antioxidant system activity in young and old animals after three successive copper sulfate administrations

Blood serum

In 24 hours after the last administration of copper sulfate lipid hydroperoxides (LHP) amount in young and old animals was increased two times compared to the corresponding control values (**Figure 5(a)**).

It should be noted that the amount of LHP in old control animals was 44% less than in young ones and these differences remain unchanged against the fibrosis background (**Figure 5(a)**).

Consequently, after the consequent administration of copper sulfate the oxidative stress progressed and it was manifested both in old and in young animals.

The increase of lipid hydroperoxides amount by 22% - 23% occurred against the decrease of glutathione peroxidase activity to the same extent in young and old animals (**Figure 5(b)**). It is considered that oxidative stress was caused by the inhibition of antioxidant enzymes.

Mitochondria

Amount of LHP in mitochondria at copper-induced fibrosis in young animals increased by 30% compared to the control, while it increased two times in the old animals (**Figure 6**).

Aconitase activity in mitochondria at liver fibrosis decreased by 90% in young and old animals (**Figure 6**). In young animals with fibrosis glutaredoxin, glutathione peroxidase and glutathione reductase activity in mitochondria was by 29%, 24% and 23% respectively lower than in the control. In old animals, glutaredoxin and glutathione reductase were by 54% и 26 % respectively lower than in the control, while glutathione peroxidase did not change (**Figure 6**).

Thus, prooxidant-antioxidant system in liver and blood serum responds "actively" to successive administrations of copper sulfate at a dose LD_{33}, and quantitative changes in their indices are dependent on the of age.

Some parameters of cellular and humoral immunity after three successive administrations of copper sulfate in young and old animals

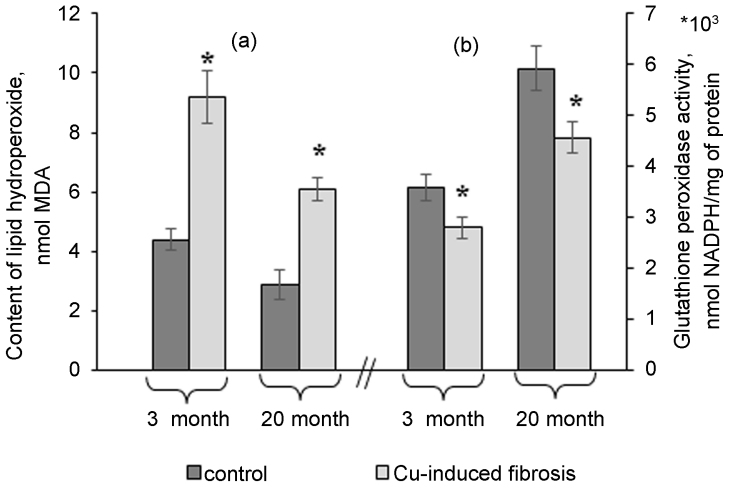

Figure 5. The contents of lipid hydroperoxide in 3 months control and in animals with Cu-induced fibrosis, the same 20 months control and in animals with Cu-induced fibrosis in blood serum (a) and glutathione peroxidase activity in these same animals (b). *P ≤ 0.05 compared with the same age control.

The number of phagocytic cells capable to trap microbial cells (phagocytic index-PI) in these animals after three successive administrations of copper sulfate was twofold decreased compared to the control, both in young and in old animals (Figure 7(a)). It suggests the immunodeficiency manifestation in animals with copper-induced fibrosis, regardless of their age.

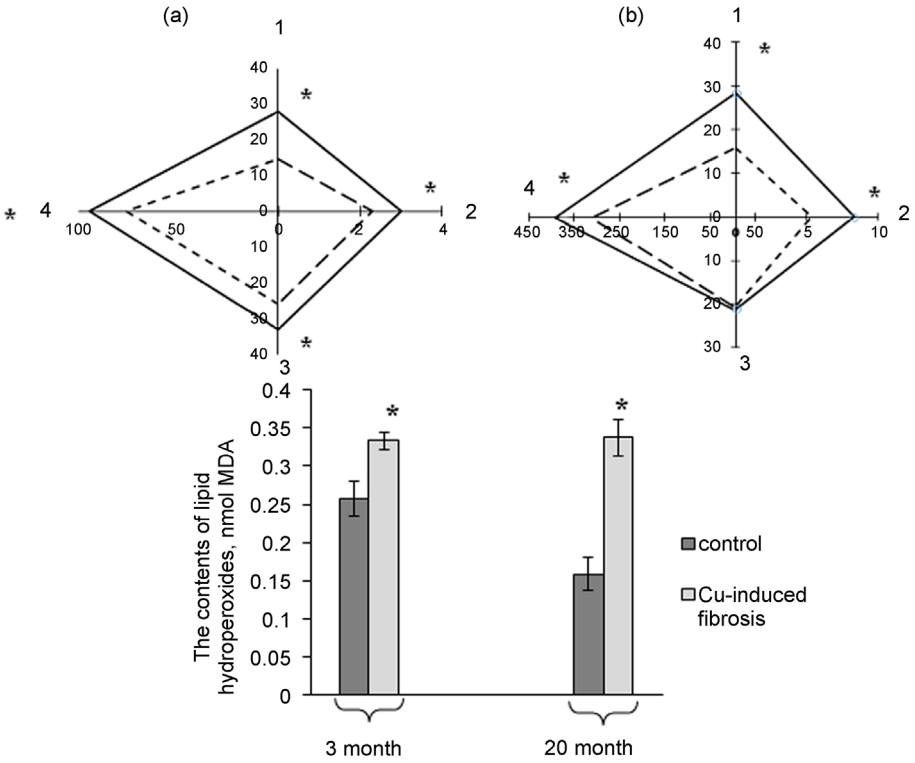

Figure 6. The contents of lipid hydroperoxides in liver mitochondria in the control group 3 months and 20 months, groups with Cu-induced fibrosis at 3 monthsand 20 months (histogram) and aconitase activity (1) and glutaredoxin (2), glutathione reductase (3) and glutathione (4) in the control (—), and animals with Cu-induced fibrosis (—) at 3 months. (a) and 20 months. (b) animals, respectively. *P ≤ 0.05 compared with the same age control.

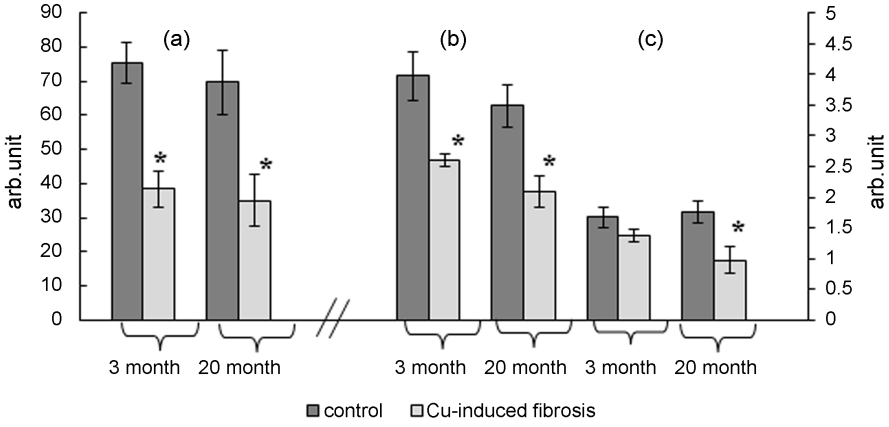

Figure 7. The phagocytic index (a), phagocytic number (b) and completeness index of phagocytosis (c) in control group 3-month-young and 20-month-old; group with Cu-induced fibrosis at 3 month and 20 month. *p ≤ 0.05 compared with the same age control.

The absorption capacity of granulocytic neutrophils, *i.e.* an average number of microbial cells absorbed by phagocytic cells (phagocytic number-PN) was 35% lower than in the control in young animals and 40%—in old animals with copper-induced fibrosis (Figure 7(b)).

The index of completeness of phagocytosis in young animals with copper-induced fibrosis did not differ from the control level, and in old animals it was 45% lower than in the control (Figure 7(c)).

Therefore, the progression of copper-induced liver fibrosis, even at the earliest stages, was accompanied by the formation of immune deficiency in cellular immunity and was similar in young and old animals. Only the completeness of phagocytosis was lower in old animals compared to the young ones.

The amount of PAMW amount, including the peptides of 500 to 5000 Da, in blood serum didn't change in both age groups of animals after copper sulfate administration (Table 1).

The amount of serum ceruloplasmin in animals with copper-induced fibrosis was the same as in the control, both in young and in old animals (Figure 8(a)).

The haptoglobin is well-known to bind the hemolysis products, and the hem-haptoglobin complex acts in organism as antioxidant [26]. The amount of haptoglobin did not differ between young and old control animals (Figure 8(b)). Induction of hepatic fibrosis did not influence the amount of haptoglobin in the blood serum of young rats. At the same time, its amount in old animals increased 2 times compared to the control (Figure 8(b)).

Table 1. The content of molecules of average weight in the serum blood of experimental animal of different age.

Group of animals	The content of molecules of average weight (arb. unit)	
	3-month-young	20-month-old
Control	0.190 ± 0.010	0.150 ± 0.020
Cu-induced fibrosis	0.170 ± 0.001	0.140 ± 0.010

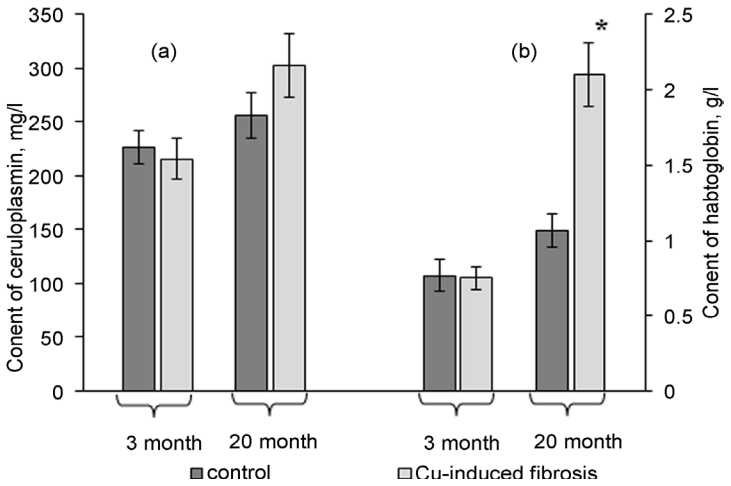

Figure 8. The content of ceruloplasmin (a) and haptoglobin (b) in the serum blood in 3 month control group and 20 month; group with Cu-induced fibrosis at 3 month and 20 month. *p ≤ 0.05 compared with the same age control.

4. Discussion

Considering the mechanism of Cu-induced fibrosis, it should be noted that after three successive copper sulfate injections copper ions was detected in blood serum, and they were accumulated in the liver in large quantities [7].

The results of the present study indicate that copper ions accumulation in liver and in other tissues in high concentrations induced the system changes.

Thus, at the molecular level, copper ions inhibited the activity of enzymes of the Krebs cycle (aconitase—twofold decrease), antioxidant enzymes (glutathione peroxidase, glutathione reductase, glutaredoxin—by 40% - 50%), and that was accompanied by an increase in the amount of the products of free radical reactions.

Along with this, copper ions exerted a pronounced cytotoxic effect on the membranes of liver cells [27]. Ultimately, this was manifested in the initiation of necrosis and possible apoptosis of hepatocytes. Morphological disorders of hepatocytes were well detected on histological specimens. Decline in performance ability and animal body weight was also demonstrated.

The liver is known by its vast range of functions, and any changes in its state lead to disturbance in the most body systems. The liver takes part in the removal of removal of CD8+ -T- cells by antigen-mediated cell death [28]. The inhibition of detoxification, which occurred under the prolonged action of copper ions [29], can be accompanied by an increase in amount of autoantibodies inhibiting the liver function even more. At the same time, production of autoantibodies is both pathogenetic and physiological [30].

The key factor in liver fibrogenesis is the transforming growth factor (TGF-β1) [31] [32].

The products of free radical reactions, cytokines, and hepatocyte degradation products were shown to induce the synthesis of TGF-β1 in stellar cells, providing in such away their transition into activated stellar cells and their transformation into fibroblast-like cells which actively synthesize collagen and other elements of connective tissue [31] [32].

The importance of the formation of connective tissue on the background of hepatocyte destruction can be evidenced by the fact that at this time other types of cells (even hepatocytes) begin to produce connective tissue [33].

However, after the "elimination" of the threat of further destruction of hepatocytes and the "production" of a sufficient amount of connective tissue, which may coincide with the consumption of fatty inclusions in stellar cells, the reduction of retinoids and other metabolites in them, stellar cells begin to produce metalloproteinases [34].

Matrix metalloproteases are a family of extracellular zinc-dependent endopeptidases that are capable of destroying all types of extracellular matrix proteins [35]. At present, about 30 enzymes of this family are isolated. It has been shown that they participate in tissue remodeling, angiogenesis, proliferation, migration and cell differentiation, apoptosis and tumor growth inhibition [34] [36].

Consequently, the process of fibrosis induction can be divided into several

stages. At the first stage, genotoxic compounds accumulate in the body and, above all, in the liver, and they are distributed over different compartments of cells and tissues (Figure 9(I)) At the next stage, a specific metabolic pattern of inflammatory mediators, cell degradation products and free radical products is formed (Figure 9(II)). Further, these products activate stellar cells and fibrogenesis, as an urgent reaction to eliminate liver degradation. At this stage, the proliferation of hepatocytes is negligible and subsequently ends with the synthesis of a large amount of connective tissue (Figure 9(III)). And, finally, at the fourth stage, the activation of matrix metalloproteinases and the choice of strategies for further adaptation take place.

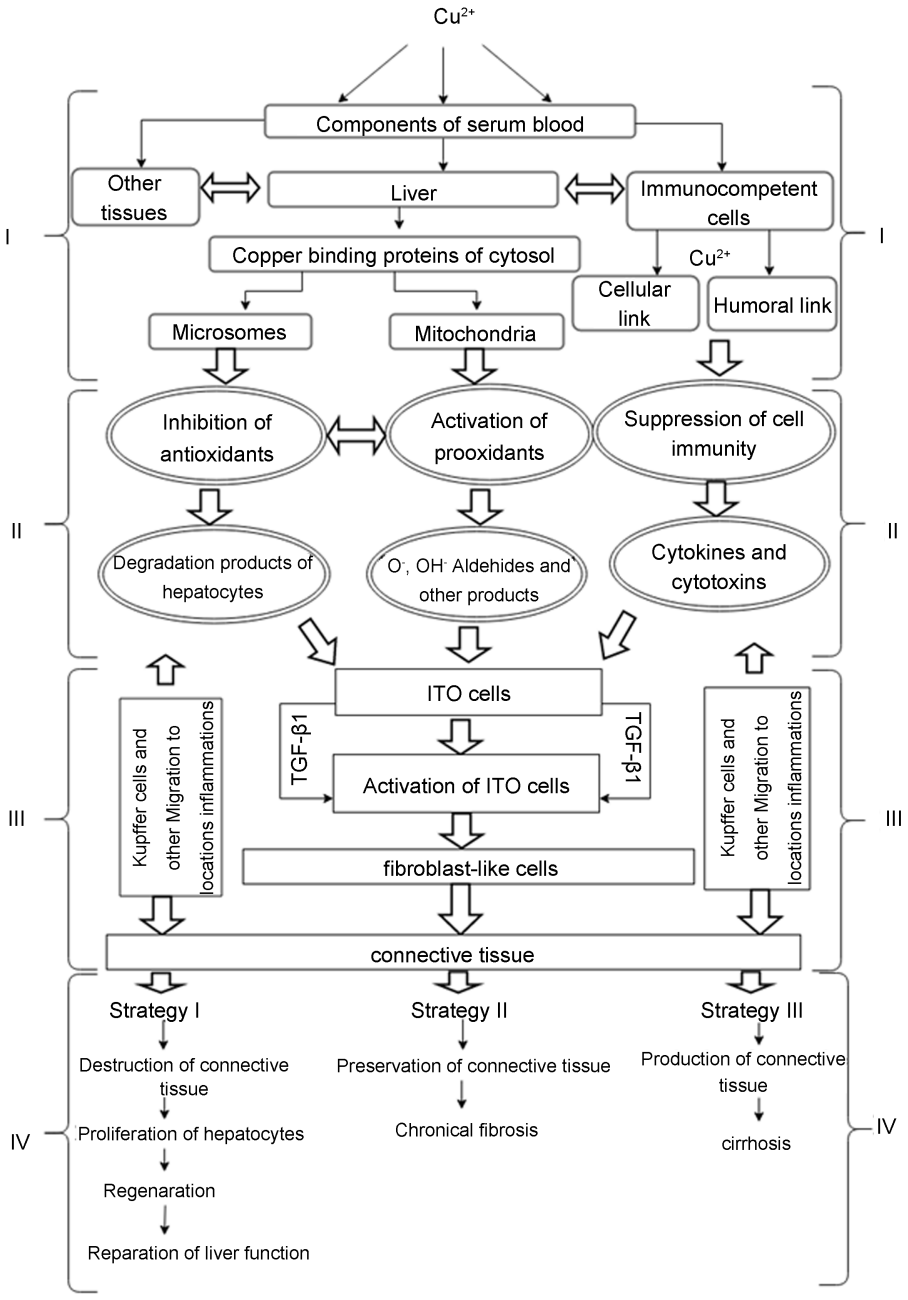

Figure 9. Schema which demonstrated hierarchy of formation Cu-induced liver fibrosis.

In such a metabolic situation, several strategies for further adaptation can be launched and implemented.

Strategy I—increased proliferation of hepatocytes, destruction of connective tissue by metalloproteinases, *i.e.* true regeneration of the liver with the further restoration of its functions. At the physiological level, this manifests itself as a recovery (**Figure 9**).

Strategy II—increased production of connective tissue that replaces the damaged hepatocytes, but physiologically it is manifested in a chronic fibrosis (**Figure 9**).

Strategy III—for some reason unknown to us, the process of true liver regeneration is not carried out, and, on the contrary, the degradation of hepatocytes, which are replaced by a connective tissue, continues, and physiologically it manifests as cirrhosis (**Figure 9**).

It can be stated that all molecular and cellular processes occurring at this time in the liver have an adaptive character. The evidence of the adaptive and not pathogenetic function of the described changes is the manifestation of a pronounced hormesis effect to the lethal doses of copper sulfate in these animals [37].

If these arguments are correct, then another question arises. Why adaptive reactions (useful in some cases) can lead to the development of pathologies and increase the probability of death?

We believe that the choice of a strategy for further adaptation will take place at the moment of reaching such a metabolic state of the system when it has a high (the highest) degree of heterogeneity of the factors determining (dominant) in the choice of the metabolic strategy.

For this model, this is a fairly high ratio between factors with the opposite direction (factors of activation of proliferation and factors of activation of fibrogenesis regulation). The moment of the onset of a high degree of variability of the determining factors is a point of instability or a point of bifurcation. Probably, when the bifurcation point is reached, the behavior of the biological system becomes unpredictable in the sense that it makes a choice between potentially possible states. Among the possible states, as noted, three strategies can be implemented (**Figure 9**).

The dominance of the factors determining strategic choice will depend not on one factor, but on a complex set of various factors, and, first of all, on the character of the dynamics of metabolic patterns, or rather the characteristics of the "centers of attraction" of interrelated metabolic cycles.

Comparative analysis of changes of metabolic parameters studied in young and old animals with Cu-induced fibrosis showed relatively small age-dependent differences at this level in comparison with the corresponding age control.

Thus, in old animals the most apparent changes were in LHP amount in mitochondria (increase of 112%), in the index of completeness of phagocytosis (decrease of 45%) and in haptoglobin content (increase of 96%).

At the same time, in old animals the development of fibrosis did not cause

such a pronounced inhibition of performance ability in comparison with young animals, they lost less body weight than young ones. Moreover, assessing the survival of young and old animals for a month after Cu-induced fibrosis showed that all old animals survived while 10% of young experimental animals died (in each age group there were 50 animals).

5. Conclusion

In conclusion, it should be noted that the accumulation of copper ions in the liver cells after three successive injections induces fibrotic changes in the liver. They are characterized by: 1) the formation of a specific adaptive metabolic pattern with a pronounced shift in the prooxidant-antioxidant system towards prooxidants; 2) systemic changes and in particular, inhibition of the cell link of immunity, morpho-functional reconstruction of the liver, and changes in physiological characteristics; 3) inhibition of total metabolism, which manifested itself in lower body temperature, loss of body weight and loss of performance ability; 4) the relationships between induced metabolic patterns and physiological characteristics were age-dependent; 5) the formed specific adaptive metabolic pattern is unstable and can later be realized in one of three possible adaptive strategies, the choice of which is influenced by the age of the animals.

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