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Stem cells take part in regulation of prooxidant activity and immunity at liver fibrosis

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Abstract: It has been explored the possibility of correction of prooxidant-antioxidant parameters at rats with experimental liver fibrosis and immunological status of these animals using xenogeneic embryonic stem cells. It is shown that the threefold sequential administration of copper sulfate in dose 1 mg / 100 g of body weight, which amounted to 30% of the lethal dose in 48 hour intervals between administrations induced liver fibrosis in 24 hours after the last administration to 3 month animals. It was found that the development of fibrosis, was accompanied 50 per cent increase in TBA-active products in the mitochondria and 25% of the increase in the microsomes of liver cells. Content of Ig A, IgM, circulating immune complexes (CIC) and the average molecular weight peptides was increased to 2-3 times in the same time. Introduction of xenogenic embryonic stem cells in animals with experimental fibrosis accompanied by decrease in the amount of TBA-active products to almost intact control. It coincided with the 60% increase in the activity of glutathione peroxidase in the mitochondria. Introduction of stem cells to experimental animals provide a normalization of the content of Ig A, Ig M, the CiC and the average molecular weight of the peptides.

Keywords: Embryonic Stem Cells, Fibrosis, Prooxidant-Antioxidant System, Immune Parameters

1. Introduction

The free radical hypothesis of aging, expressed by D. Harman in 1974 stimulated the study of the mechanisms of free radical formation and the role of products of free radical reactions in the development of pathologies and mechanisms of natural aging. Numerous studies in this area have shown a strong correlation between the development of a number of diseases, including cancers [1], Alzheimer's disease [2], cardiovascular disease [3], and oxidative stress. The overlap between the low activity of the antioxidant system and life span was discovered [4-6]. Moreover, it is shown that a change of the content of the free radical products (oxidation of lipids, proteins carbonyls, formation of endogenous aldehydes) is observed when changing diets [7, 8], and physical activity [9]. All this suggests that the pro-oxidant-antioxidant system is one of the metabolic systems of the cell and the organism as a whole, which actively responds to a wide range of changing endogenous and exogenous factors and is an important indicator of the functional state of the organism.

It is supported by numerous data on the physiological regulatory role of diverse products of free radical reactions [10-11].

We understand that free radical processes on the one hand underlie the regulation of physiological functions, and are able to induce various pathologies on the other - and thereby participates in the regulation of the life span of the individual.

At the same time, in recent years there is a large number of critical works against free radical theory of aging [12-15], and Barja offered a new perspective on this issue [16].

We believe that pro-antioxidant system (PAS) is one of the

regulatory systems, which is "interacting" with other regulatory systems, and above all with the immune system, providing control of physiological processes. In case of violation of these systems pathology can develop. For biological systems not the absolute values of the parameters are important but the relationship between the functional systems. Unfortunately, the studies of the relationship of PAS with other regulatory systems are extremely deficient. In this regard, in this paper we present the results of studies of some indicators of PAS and characteristics of the immune status of intact animals and animals with induced liver disease, and the impact of exogenous embryonic stem cells to these indexes.

It has previously been shown previously that a triple sequential administration of copper sulfate to experimental animals at doses of 1 mg/100 g of body weight, which amounted to about 30% of the lethal dose, was accompanied by marked changes in liver function [17, 18]. It can be assumed that the chronic administration of copper will affect the rate of PAS and the immune system, and this model can be used to investigate the relationship of these systems and their role in the elimination of induced pathologies.

Mitochondria are well-known to be a major site of free radical reactions in the cell [19]. Along with this, endoplasmic reticulum (microsomes) makes some contribution to the formation of free radical products in the cell. It seems reasonable to determine the contribution of mitochondria and microsomes in the PAS in the model of toxic action of copper ions.

It is known that the ratio of pro- and antioxidant components can be regulated by various compounds and factors [20-21]. However, the cell technologies currently are of particular interest currently. It has been shown that the administration of stem cells into experimental animals act multifunctionally [22-25].

In this regard, the content of TBA-active products, glutathione peroxidase activity and glutathione-S-transferase in mitochondria and the liver of control animals and animals with induced hepatic fibrosis, and also the content of Ig A, M, G, circulating immune complexes, and peptides of the average molecular weight of the peptides in the serum of these animals were investigated.

2. Materials and Methods

The experiments were done conducted on three-months-old Wistar rat males. All the animals were divided in three groups, by 5 animals in each group. The first group: the intact control – wasn't affected; the second group – the experimental fibrosis. To induce the fibrogenesis the copper sulfate was introduced intraperitoneally by 1mg/100 g of body weight sequentially three times to each animal. The interval between copper sulfate administrations was 48 hours. Twenty four hours after the last copper sulfate administration the animals were taken in the experiment.

The stem cells were introduced to intact animals and to the animals with fibrosis induced by copper ions. The embryonic stem cells (ESC) were obtained by the specialists of the firm "Embriotech" (Ukraine) from the embryos on the 6th week of gestation. The cells were tested on the sterility, vitality and clusters of differentiation CD (CD90, CD34, CD38, CD45), the density of leucocytes antigens of HLA class by immunofluorescent method by usage of monoclonal antibodies and conjugation reaction with FITC ("Sorbent", Moscow, Russia) were determined.

The obtained and selected cells were cryopreserved.

Before using the cells were depreserved and their vitality was estimated by usage of trypan blue. The embryonic stem cells (ESCs) with the vitality index 80-90% were used in the experiment.

The xenogeny ESCs were introduced to the animals of the 3rd group intraperitoneally by one time in 1 ml of physiological solution with 2x10^5 of cells per 250 g of body weight by the scheme (Fig.1).

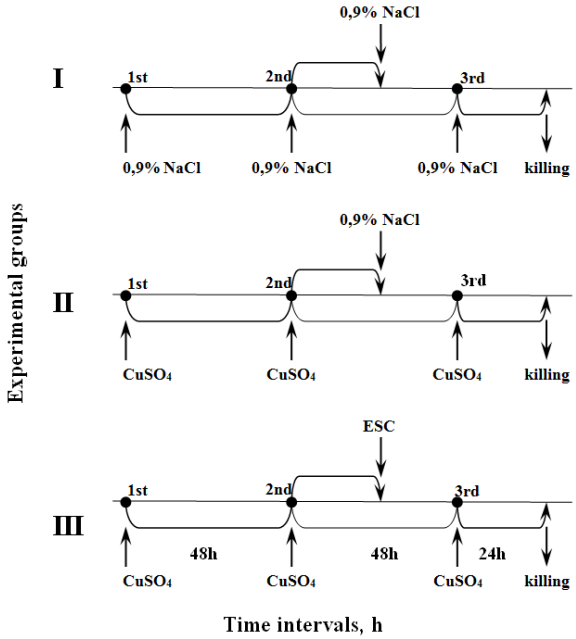

Figure 1. *The scheme of copper sulfate and embryonic stem cells introduction to the experimental animals: I – control group of animals to whom physiological solution was introduced; II – the experimental group of animals with induced fibrosis; III – experimental group of animals to whom ESCs were introduced against of fibrogenesis.*

Twenty four hours after the copper sulfate addition of 1 mg/100 g was introduced to the experimental animals of the 3rd group and 24 hour after the animals of all three groups were anesthetized (Fig. 1). After slaughtering of the anesthetized animals the blood was collected and the serum was obtained.

The liver was perfused by physiological solution, taken out and weighed, in liver aliquot the content of collagen was determined [26], the rest of liver was in 100 mM of Tris-HEC buffer, pH 7,4, at 4 C. Mitochondria and microsome fractions were obtained by differential centrifugation [27].

The TBA-active products of LP in mitochondria and microsomes were determined by the method [28]. The absorption spectrum of the colored product was recorded on

double-beam spectrophotometer Specord UV VIS (Germany), measuring the difference in extinction at 535 and 520 nm. The HPL content was expressed in equivalent amounts of malonic dialdehyde (MDA) using a molar extinction coefficient of $1.56 \cdot 10^5 \cdot M^{-1}cm^{-1}$.

The copper ion content in mitochondra and microsomes fractions was determined after lipid extraction by the atomic adsorption photometry (SELMIC – 600. Ukraine).

Glutathione peroxidase activity (GP, EC 1.11.1.9) was determined in cytosolic fractions, and liver mitochondria serum by spectrophotometer at 340 nm with the help of the method of [29] in 50 mM K^+, Na^+- phosphate buffer (pH 7.4) containing 1 mM EDTA, 0.15 mM NADPH, 1 unit of yeast glutathione reductase, 0.2 % Triton X-100 and 3 mM Na azide to inhibit KAT. 1.2 mM cumene hydroperoxide and 0.4 mM hydrogen peroxide were added. Incubation temperature was 37 °C. The activity was expressed in nmol NADPH/min per mg of protein or ml of serum considering a molar extinction coefficient $6.22 \cdot 10^3 \cdot M^{-1} cm^{-1}$.

The glutathione-S-transferase activity (GT EC 2.5.1.18) was measured in liver cytosol and mitochondria spectrophotometrically at 340 nm [30] in a medium containing 0.1 M K^+ phosphate buffer, pH 6.5, 1 mM 1-chloro-2,4-dinitrobenzene, 5 mM GSH, 0.2 % Triton X-100. The incubation temperature was 37 °C. The activity was calculated using a molar extinction coefficient $9.6 \cdot 10^3 \cdot M^{-1}cm^{-1}$.

In the serum the contents of immunoglobulins A, IgM, IgG. The reagent kit for determining the concentration of serum immunoglobulin production by Human, Germany. Circulating immune complexes and peptides of average molecular mass [31] were determined.

All the experiments were repeated no less than three times and the data obtained were analyzed statistically by Wilcoxon-Mann-Witney test.

3. Results

Induction of pro-oxidant activity in the liver cells and characterization of the immune status after exposure to copper ions. Triple sequential administration of copper sulfate in 1 mg/100 g body weight at 48 hour intervals between administrations to experimental animals was accompanied by a decrease in liver weight relative to the body weight (Fig. 1).

In this period (7 days after the first administration of copper sulfate) morphology of the liver lobes changed, all lobes "fused" at the expense of connective tissue, the collagen content increased, i.e. at this time fibrosisdeveloped (Fig. 2).

In the animals with induced liver fibrosis content of TBA-active products into the mitochondria was increased by 53% compared to control (Table 1). The content of TBA-active products in the microsomal fraction was also increased, but only by 25% compared with the control (Table 1).

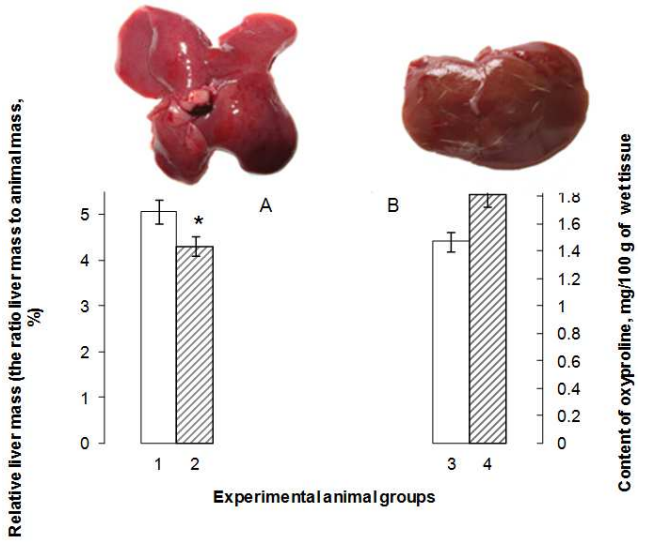

Figure 2. *Morphology of liver of control rat group (A) and a group of rats who were administered three times successively copper sulfate at a dose of 1 mg / 100g body weight every 48 hours between doses (B). The relative liver weight in control rats (1) and rat liver treated with copper sulfate (2), 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.*

Consequently, repeated injections of copper sulfate to the experimental animals was accompanied by the formation of fibrosis during activation of pro-oxidant system, i.e. in this case oxidative stress was manifested. Thus pro-oxidant system in mitochondria was 2-fold more active as compared with that of microsomes.

It has previously been shown that exogenous copper ions distributed unequally in the liver cell compartments [34], which may explain the observed differences in the induction of TBA-active products.

The content of endogenous copper ions was found to be the same in mitochondria and microsomes of control animals (Table 1).

Table 1. *Contents of TBA-active products (MDA nmol/mg protein) in control mitochondria and microsomes of rat liver 24 hours after three consecutive administrations of copper sulfate 1 mg/100 g body weight of animal.*

Fraction	The content of TBA-active products nmol MDA / mg protein		The content of the cupper ions, µg/mg of protein	
	Control	Fibrosis	Control	Fibrosis
Mitochondria	0,591± 0,031	0,914± 0,045*	0,011 ± 0,005	0,190 ± 0,01*
Microsomes	0,690 ± 0,080	0,862 ± 0,058*	0,011 ± 0,006	0,060± 0,002*

The average values of 5 independent experiments are presented.
* - the difference at between the control and experiment P <0.05

After 24 h after three repeated administrations of copper sulfate, copper ion content in the mitochondria was increased 17 times and in the microsomal fraction - 5.4 times compared with the control (Table 1). Consequently, 3 times more of exogenous copper ions were associated with mitochondria compared with microsomes. There is no direct correlation between the content of copper ions in the mitochondria and microsomes and induction of TBA-active products in these fractions of liver cells. It can be explained not only so much by the difference of degree of association of copper ions with microsomes and mitochondria, but by the difference in the prooxidant activity of enzymes in the mitochondria and microsomes.

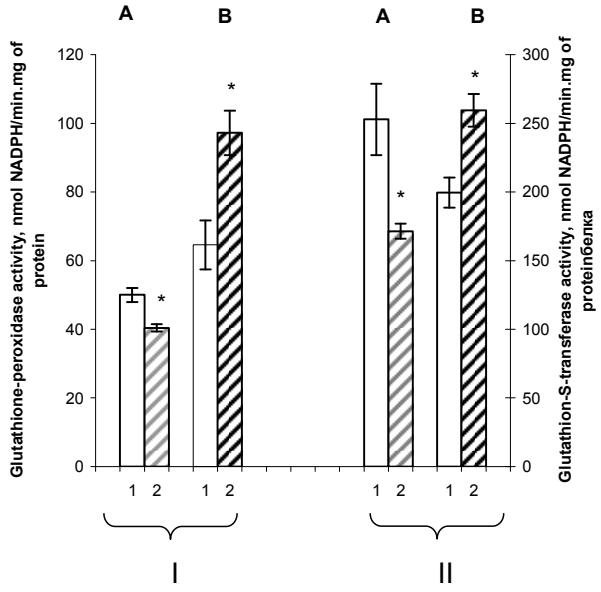

Figure 3. *Activity of glutathione peroxidase (I) and glutathione-S-transferase (II) in the mitochondria (A) and microsomes (B) in control rat liver (1) and after 24 after three repeated administrations of copper sulfate (2). * - significant differences between the intact and experimental animals at P <0.05.*

The evaluation of the antioxidant system glutathione peroxidase (GP) and glutathione-S-transferase are of the greatest interest, as they play a key role in the metabolism of glutathione; their activity is induced by various factors and they are polyfunctional.

It has been found that against the multiple increase of the copper ions content in mitochondria, the GP activity therein was reduced by only 20% compared with the control (Fig. 3). At the same time the activity of GP in the microsome, on the contrary, was increased by 50% compared to the control (Fig. 3).

The injection of copper ions into experimental animals was accompanied by an inhibition of the activity of G-S-T in mitochondria by 32% compared with the control (Fig. 3). At the same time, the enzyme activity in microsomes on the contrary copper ion content was increased by 30% (Fig. 3).

Consequently, against the background of the copper ions action GP and G-S-T activity was significantly decreased in the mitochondria, but in microsomes on the contrary, ontrary, these enzymes was elevated compared with the untreated control. This suggests that at the development of fibrosis there were the opposite changes in antioxidant enzyme activity of mitochondria and microsomes.

The evaluation of some parameters of the immune system in animals with fibrosis revealed that the content of Ig A and Ig M in serum was increased 3.9 and 2.0 fold respectively compared to control level (Table 2).

At the same time, the content of Ig G decreased by 27% compared to control (Table 2). Content of circulating immune complexes (CIC) in animals with induced fibrosis, which was also characterized by oxidative stress, was increased 1.8-fold (Table. 2). The content of peptides the average molecular weight (PAMW) was increased at 2.2 times in blood serum of experimental animals (Table 2).

Table 2. *The content of immunoglobulins and circulating immune complexes and the peptides of average molecular weight in the serum of experimental animals 24 h after last administration of copper sulfate.*

Experimental variant	Parameter				
	Ig A, g/l	Ig M, g/l	Ig G, g/l	CIC, Conventional units	PMMW, Conventional units
Intact liver	1,3± 0,02	0,73± 0,024	6,2± 0,39	78,0 ± 3,7	0,23 ± 0,03
Liver fibrosis	5,1 ± 0,17*	1,5 ± 0,30*	4,5± 0,22*	142,0 ± 4,5*	0,50± 0,05*

The average values of three independent experiments are presented.
* - significant difference between the control and experiment at P <0.05

Consequently, liver fibrosis and oxidative stress were accompanied by not only change of the PAS, but also by marked inhibition of the activity of the immune system. Experimental animals with induced fibrosis on the ground of against oxidative stress are a good model to study the mechanisms of regulation of interaction of PAS and the immune system in liver pathologies.

Influence of embryonic stem cells (ESC) on the characteristics of the PAS and the immune system on the model of induced fibrosis. In the first experimental series the possible influence of the ESC on the activity of PAS of control animals was determined. It was found that a single administration of xenogeneic ESCs in control animals had no significant influence on the content of TBA-active products neither in the mitochondria nor in microsomes (Table. 3). The activity of GP in the mitochondria was 60% higher than in control, and G-S-T in the mitochondria was not significantly changed. Administration of ESC in control animals had no effect on the studied enzymes in the microsomal fraction (Table. 3).

Therefore, the administration of ESC has no effect on the activity of the PAS in control animals except for GP activity, which was significantly increased.

In the case where the ESC were administrated in animals with induced fibrosis, the content of TBA-active products in mitochondria and microsomes decreased by 26 and 19%, respectively, compared to the pathology and was not different from control (Table 4). The activity of GP in the mitochondria was increased by 60% compared with fibrosis, and in microsomes did not change, and coincided with the untreated control (Table. 3, 4). The activity of G-S-T in mitochondria and microsomes after administration of ESC responded to control values (Table. 4).

Table 3. *Content of TBA-active products and the enzyme activity in mitochondria and microsomes of liver of control animals and animals treated with ESCs.*

Fraction	The content of TBA-active products nmol MDA / mg protein		Enzymes activity			
			GP		G-S-T	
	Control	Fibrosis with ESC	Intact control	Intact control with ESC	Intact control	Intact control with ESC
Mitochondria	0,591± 0,031	0,613± 0,018	50 ± 0,010	80,4 ± 6,2*	252,8 ± 26,2	208,5±4,7
Microsomes	0,690 ± 0,080	0,740 ± 0,055	64,6 ± 7,1	76,2±3,2	199,5± 11,4	195,7 ± 21,7

The average values of 5 independent experiments are presented.
* - significant difference at P <0.05 between the control and experiment.

However, ESC does not have any impact on the content of Ig G in animals with experimental fibrosis (Fig. 3). Therefore, ESCs provide patterning immunological parameters and characteristics of the PAS close to the control variant.

Therefore, the introduction of ESCs led to the change of PAS parameters on the background of developing pathology, while there was not a proportional change of all investigated parameters but the other relations were formed between them.

Table 4. *The content of TBA-active products and the activity of enzymes in mitochondria and microsomes in liver of animals with induced fibrosis, which was used as a comparison group and animals with induced fibrosis after administration of ESCs.*

Fraction	The content of TBA-active products nmol MDA / mg protein		Enzymes activity			
			GP		G-S-T	
	Fibrosis	Fibrosis + ESC	Fibrosis	Fibrosis + ESC	Fibrosis	Fibrosis + ESC
Mitochondria	0,914± 0,045	0,680± 0,033*	40,4 ± 1,1	64,5 ± 1,8*	171,4 ± 5,5	204,1±24,0
Microsomes	0,862 ± 0,058	0,700 ± 0,080*	97,2 ± 6,5	79,1±4,0	259,4± 12,0	189,7 ±8,1

The average values of 5 independent experiments are presented.
* - significant difference between the control and experiment at P <0.05.

In a greater degree the ESCs influenced the ESC the immune system parameters. Thus, the content of Ig A and Ig M was reduced to 3.6 and 1.8 fold respectively compared to the experimental fibrosis and didn't differ from intact control (Fig. 3). Administration of ESC in animals with fibrosis completely normalized content of the CIC and PAMW (Fig. 4).

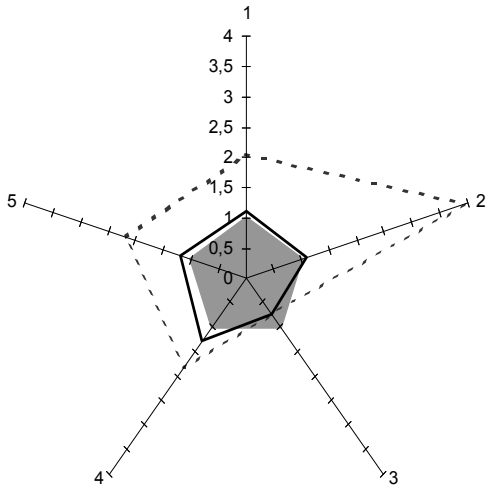

Figure 4. *Contents of IgM (1), IgA (2), IgG (3), CIC (4) and PAMW (5) in serum of control rats, which is taken as a unit (■) rats with experimental fibrosis (—) and rats with a experimental fibrosis, additionally treated with embryonic stem cells (---SC).*

4. Discussion

There is evidence that copper in the ionized form as a transition metal is pro-oxidant [32]. However, there are studies in which copper is shown to not exhibit the pro-oxidant activity [33].

In the present study, it was shown that only with such a significant increase (17-fold) of copper ions in the mitochondrial fraction the manifestation of pro-oxidant properties (increase of TBA-active products by 53%) was observed.

At the same time, the increase in the content of copper ions in the microsomes was accompanied by a slight increase in the content of TBA-active products in these organelles. The activity of the studied antioxidant enzymes, on the contrary, increased, i.e. there was the opposite directed effect on pro- and antioxidant elements of the system (Fig. 5).

Such stimulation of GP and G-S-T in microsomes by copper ions is rather non-specific response of the endoplasmic reticulum. If we compare the ratio of the relative characteristics of pro- and antioxidant elements in mitochondria and microsomes of the liver, the differences in their relationship could be noticed. This allow to assume that in these organelles antioxidant system are composed of various components and GP and G-S-T which operate in

mitochondria and microsomes perform several different functions. The polyfunctionality of these antioxidant enzymes is actively investigated.

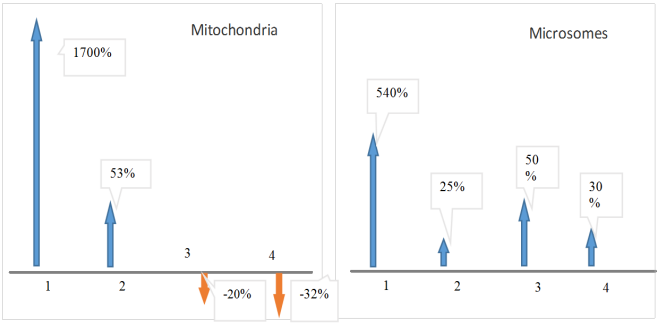

Figure 5. *Diagram shows the direction of change of the studied parameters in relation to baseline (—); the content of copper (1) TBA-active products (2) GP activity (3) and G-S-T (4) as a percentage of baseline liver mitochondria and microsomes of rats with experimental fibrosis.*

This suggests that the stability of the systems to the xenobiotic toxic effects will depends on the variety of options strategies of adaptive solutions. It should be noted an important fact of threefold excess of binding of copper ions by mitochondria compared to microsomes. It can be explained by a high speed of elimination of copper ions from the microsomes compared with mitochondria. It can be assumed that the mitochondria act as "traps" and microsomes as "transitors" of the excess of endogenous copper. Model of fibrosis induced by copper ions may be a good model to study the mechanisms of choosing of the strategy of adaptation to xenobiotics.

As it is known, hepatic fibrosis is forming of sclerosis of connective tissue with scarring in various organs which occur as a result of chronic inflammation. Inflammatory process that leads to fibrosis, can be induced by alcohol, drugs, xenobiotics, an excess of iron and copper in the body [34-37].

The rate of fibrogenesis depends on the degree of infection of the body, alcohol abuse, weakened immunity, obesity and fatty liver disease, diabetes, and its progression is completed by cirrhosis, liver failure, portal hypertension and require for liver transplantation.

Development of methods of fibrogenesis slowdown or its reversible change is an urgent task. In this model the fibrogenesis is triggered by an excessive accumulation of copper ions in the liver mitochondria. This process was accompanied by induction of free radical reactions to a subsequent breach of hepatocytes and activation of stellate cells (fibroblasts and myofibroblasts of bone marrow), which was accompanied by increased synthesis of collagen (Fig. 1).

The described events, along with the synthesis of connective tissue are accompanied by a sharp increase in inflammatory cytokines, and as a consequence, a multiple increase of the load on the immune system (Table. 3).

So fibrosis becomes a vicious circle, the manifestation of which is that the hepatocyte injury induced fibrosis and fibrogenesis aggravates further damage to hepatocytes. As a result it takes 4-5 steps from fibrosis to cirrhosis. It should be noted that in fibrosis the interrelated changes in many, if not all, functional systems occur. And as functional systems react to such adjustment by different ways, then various metabolic and functional patterns are formed. Nature of these metabolic patterns will determine the fate of the metabolic system: acceleration of fibrogenesis, slowing it or even reversible changes (such cases have been described).

The emerging metabolic, fibrosis-dependent pattern, which was the case in our experiment, can be influenced by changing the microenvironment formed (content of cytokine-specific low molecular weight proteins and other biologically active compounds). In our opinion the stem cells could be the best and promising option to change "microenvironment" in cell and tissue organization of the liver. The changes of the interrelated metabolic patterns - in our study a PAS and the immune system – as a result can occur, and, we will see a normalization, and rather forming of pattern close to the untreated control (Fig. 4).

5. Conclusion

1 Three-time administration of copper sulfate in a dose of 1 mg/100 g of body mass at intervals of 48 h induces fibrogenesis in liver.

2 The fibrogenesis was induced by an increase of copper ions content in liver mitochondria by 17 times and in microsomes by 5,4 times, at of the content of TBA-active products in mitochondria increased by 53% and in microsomes by 25%.

3 At the same time Ig A content increased by 3,9 times and Ig M content by 2 times, whereas Ig G content decreased by 27% compared to control. The content of CIC was increased 1,8-fold and PAMW– 2,2-fold in blood serum.

4 At fibrogenesis induced by copper ions mitochondria are more vulnerable compared to microsomes. The content of TBA-active products in mitochondria was increased by 53% and GP activity was decreased by 20%, and G-S-T – by 32%. In microsomes activity of GP was increased by 53%, and G-S-T – by 30%, at that the content of TBA-active products was increased only by 25%.

5 Administration of ESC to intact animals didn't influence the indicators of pro-oxidant system and increase GP activity only in microsomes by 60% compared to control.

6 Administration of ESC to animals with an experimental fibrosis led to decrease of TBA-active products content compared to control and TBA-active products didn't differ significantly from intact control. The indicators of immune system activity coincided with intact control except for Ig G which remained decreased. There were coincidences between indicators of PAS activity and immune systems against on the background of fibrosis and its reversal.

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