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PECULIARITIES OF CONNECTIVE TISSUE DEGRADATION IN RAT'S LIVER ON EARLY TERMS OF CHRONIC ALCOHOLIC HEPATITIS MODELLING

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The article deals with the peculiarities of connective tissue degradation in rat liver on early terms of chronic alcoholic hepatitis modelling. Modeling of chronic alcoholic hepatitis leads to the development of oxidative stress in rat liver tissues due to a decrease in the activity of antioxidant enzymes and an increase in the production of reactive oxygen species. As a result of the development of oxidative stress in the liver of rats in the first stages of modeling alcoholic hepatitis, the concentration of the anti-inflammatory heparin-heparan fraction of glycosaminoglycans decreases, the accumulation of keratan-dermatan and chondroitin fractions of glycosaminoglycans occurs. The processes of degradation of the fibrous components of the liver's connective tissue increase and the concentration of the sulfide anion decreases.
Keywords: rats, alcoholic hepatitis, connective tissue, sulfide anion, glycosaminoglycans, L-hydroxyproline

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ОСОБЛИВОСТІ ДЕГРАДАЦІЇ СПОЛУЧНОЇ ТКАНИНИ ПЕЧІНКИ ЩУРІВ НА РАННІХ ТЕРМІНАХ МОДЕЛЮВАННЯ ХРОНІЧНОГО АЛКОГОЛЬНОГО ГЕПАТИТУ

У статті розглядаються особливості деградації сполучної тканини печінки щурів на ранніх стадіях моделювання хронічного алкогольного гепатиту. Моделювання хронічного алкогольного гепатиту призводить до розвитку окисного стресу в тканинах печінки щурів внаслідок зменшення активності антиоксидантних ферментів та збільшення продукування активних форм кисню. В результаті розвитку окисного стресу в печінці щурів на перших етапах моделювання алкогольного гепатиту зменшується концентрація протизапальної гепарин-гепаранової фракції глікозаміногліканів, відбувається накопичення фракцій кератан-дерматану та хондроїтину глікозаміногліканів , процеси деградації волокнистих компонентів сполучної тканини печінки посилюються, а концентрація сульфідного аніона зменшується.
Ключові слова: щури, алкогольний гепатит, сполучна тканина, сульфідний аніон, глікозаміноглікани, L-оксипролін

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More than 240 million people worldwide are affected by the negative effects of alcohol abuse. Long-term alcohol abuse leads to the development of liver cirrhosis. Excessive intake of alcohol leads to the development of oxidative damage to hepatocytes with their subsequent replacement by connective tissue [8].

Individual components of the amorphous substance of connective tissue are able to control the effects of oxidative stress. Thus, a decrease in heparan sulfate production leads to a decrease in the intensity of autophagy processes, increases lifespan and decreases the intensity of the cellular response to oxidative damage [10]. Alcohol is able to alter the composition of glycosaminoglycans in the astrocytes of the brain, leading to the predominance of chondroitin-4-sulfate. Changes in the ratio of various glycosaminoglycans under the influence of alcohol are insufficiently described in the scientific literature.

Excessive alcohol consumption can alter antioxidant signaling through the Nuclear factor-erythroid 2 related factor 2 (NRF2) / H_2S axis and lead to depletion of the endogenous pool of H_2S [9]. Possible cross-interactions between NRF2 / H_2S and various sulfated fractions of glycosaminoglycans under conditions of excessive alcohol intake in the body require further study.

The purpose of the work was to determine the production of superoxide anion radical, superoxide dismutase and catalase activity, malondialdehyde concentration, sulfide anion concentration, free L-hydroxyproline concentration, concentration of heparin-heparan fraction, keratan-dermatan and chondroitin fractions of glycosaminoglycans.

Materials and methods. The study was carried out on 30 male Wistar rats weighing 200-260 g. Animals were divided into 2 groups: control group (n=6) and experimental group (n=24). In animals of the experimental group, chronic alcoholic liver damage was simulated by intraperitoneal injection of 16.5 % (v/v) ethanol solution in 5 % (mass/volume) glucose solution once every 2 days at a dose of 4 ml/kg [2]. Animals from the experimental group were removed from the experiment on the 1st, 3rd, 5th and 7th days under thiopental anesthesia. The object of the study was rat liver.

For biochemical studies, the liver was homogenized in a Tris-buffer solution (pH=7.4) to obtain 10 % (w/v) homogenate. All spectrophotometric measurements were performed on an Ulab 101 spectrophotometer.

To assess the intensity of superoxide anion radical (SAR) production, the color reaction of SAR with nitro blue tetrazolium was used [1]. The activity of superoxide dismutase (E.C. 1.15.1.1, SOD) was assessed by the degree of inhibition of the autooxidation reaction of adrenaline in an alkaline medium according to the methodological recommendations [1]. Catalase activity was assessed by the amount of decomposed hydrogen peroxide in the presence of 10% tissue homogenate. The concentration of hydrogen peroxide was determined using the colorimetric method with ammonium molybdate [1]. The concentration of free malondialdehyde (MDA) was determined from the concentration of the colored product formed in the reaction of MDA with 1-methyl-2-phenyl-indole [7].

To assess changes in the metabolism of connective tissue, we determined the concentration of free L-hydroxyproline and the concentration of heparin-heparan fraction (*Hp*), keratan-dermatan (*Ke/De*) and chondroitin fraction (*Ch*) glycosaminoglycans (GAGs). The concentration of free L-hydroxyproline was determined after its oxidation with chloramine-T by the amount of chromogen formed in the reaction of L-hydroxyproline with 4-dimethylaminobenzaldehyde [3]. To isolate individual fractions of glycosaminoglycans from tissue homogenate, we used the method of differential precipitation in propanol-2 solution [3]. The concentration of individual fractions was determined from the concentration of uronic acids in the resulting precipitates [3].

The content of sulfide anion (S^{2-}) in the liver homogenate was determined by a modified reaction with N-N-dimethyl-para-phenylenediamine sulfate [12].

The results obtained were amenable to statistical processing using the Microsoft Office Excel software package and the Real Statistics 2019 extension to it. The Mann-Whitney test was used to determine the statistically significant difference between the groups. The difference was considered statistically significant at $p<0.05$.

Results of the study and their discussion. The production of SAR in the liver of rats in the dynamics of the experiment increased on the 1st, 3rd and 7th days of modeling chronic alcoholic hepatitis, but decreased on the 5th day of the experiment, and were reaching values below the level of the control group of animals (table 1).

SOD activity was reduced on the 1st, 3rd and 7th day of modeling chronic alcoholic hepatitis, but increased on the 5th day of the experiment to the level of the control group. The peak decrease in SOD activity was observed on the 1st day of modeling chronic alcoholic hepatitis.

Catalase activity showed different dynamics. On the first day, no statistically significant changes in catalase activity in the rat liver were found. From the 3rd to the fifth day, the maximum decrease in catalase activity was observed, and on the seventh day, there was a tendency to an increase in its activity.

The concentration of MDA in the liver of rats increased from the first day of the experiment and remained elevated at all studied periods of modeling of chronic alcoholic hepatitis.

Table 1

Dynamics of oxidative damage to the liver of rats in the first days of modeling chronic alcoholic hepatitis (M±m)

Groups	SAR production, nmol/s per g tissue	SOD activity, c.u.	Catalase activity μkat/g tissue	Concentration of MDA, μmol/g tissue
Control, n=6	1.84±0.004	12.34±0.55	0.376±0.008	12.32±0.11
1st day of experiment, n=6	4.33±0.57*	1.41±0.09*	0.376±0.008	20.32±0.71*
3rd day of experiment, n=6	8.15±0.03*/**	6.82±1.33*/**	0.280±0.022*/**	32.68±4.11*/**
5th day of experiment, n=6	1.09±0.016*/**	13.07±0.50**	0.226±0.011*	20.93±0.98*/**
7th day of experiment, n=6	6.82±0.07*/**	5.39±0.40*/**	0.321±0.002*/**	32.49±1.59*/**

Note: * – the difference is statistically significant when compared with the control group ($p<0.05$); ** – the difference is statistically significant when compared with the previous period of the experiment ($p<0.05$).

The concentration of free L-hydroxyproline in the liver of rats increased on the 1st, 3rd and 5th day of the experiment, but returned to the values of the control group of animals on the 7th day of the experiment.

The concentration of *Hp* in the liver of rats increased on the 1st and 3rd day of the experiment, decreased to the level of control animals on the 5th day of the experiment, and decreased below the level of control animals on the 7th day of the experiment. The *Ke/De* concentration increased from the first day

and remained elevated in all studied terms of modeling chronic alcoholic hepatitis. It should be noted that the minimum increase in this fraction of glycosaminoglycans is noted on the 5th day of the experiment. The *Ch* concentration increased on the 1st day, sharply decreased on the 3rd day of the experiment, and progressively increased from the 5th to the 7th day of the experiment.

The concentration of sulfides in the liver of rats, which were simulated chronic alcoholic hepatitis, decreases from the first day of the experiment and remains low at all studied stages. The peak decline is observed on the 5th day of the experiment.

Table 2

Metabolism of connective tissue and the concentration of sulfides in rat liver in the first days of modeling chronic alcoholic hepatitis (M±m).

Groups	L-hydroxyproline concentration, μmol / g	*Hp* Concentration, μmol / l	*Ke / De* concentration, μmol / l	*Ch* concentration, μmol / l	S^{2-} concentration, μmol / g
Control, n=6	1.28±0.02	1.81±0.02	0.27±0.004	0.59±0.01	7.23±0.17
1st day of experiment, n=6	1.81±0.01*	2.41±0.10*	1.01±0.04*	1.99±0.08*	5.27±0.03*
3rd day of experiment, n=6	1.46±0.04*/**	2.32±0.14*	1.75±0.08*/**	0.15±0.01*/**	3.69±0.03*/**
5th day of experiment, n=6	1.78±0.04*/**	1.42±0.03**	0.42±0.01*/**	0.71±0.01*/**	1.10±0.03*/**
7th day of experiment, n=6	1.32±0.08**	0.70±0.11*/**	1.79±0.04*/**	2.78±0.13*/**	6.52±0.03*/**

Note: * – the difference is statistically significant when compared with the control group ($p<0.05$); ** – the difference is statistically significant when compared with the previous period of the experiment ($p<0.05$)

Increased SAR production during modeling alcoholic hepatitis may be associated with activation of the hepatic microsomal alcohol-oxidizing system (MAOS), the main alcohol-destructive component of which is the isoform of cytochrome 450 2E1 [13]. It was experimentally found that the blockade of cytochrome 450 2E1 during excessive intake of large doses of ethanol prevents the development of alcoholic steatohepatitis and normalizes the redox balance in the liver [13].

In addition to the abovementioned effect of alcohol on MAOS, it should also be noted the possibility of blockade of cytochromes of mitochondria with acetaldehyde, which is formed as a result of the first reaction of alcohol breakdown in the liver by alcohol dehydrogenase, by MAOS or catalase. Alcohol dehydrogenase type 2, which is localized in the liver mitochondria, leads to the formation of acetaldehyde inside the mitochondria. This reaction is accompanied by the reduction of NAD^+ to NADH and FAD^+ to FADH, which leads to a shift in the ratio of $NADH/NAD^+$ and $FADH/FAD^+$. This shift in the redox balance leads to the accumulation of Fe^{2+} ions inside the mitochondria, which, upon initiation of the Fenton reaction, can form a hydroxyl radical [5].

An increase in SOD activity, a decrease in SAR production and MDA concentration on the 5th day of the experiment may be associated with the activation of NRF2 in response to the most intense, among the observed periods, lipid peroxidation, which was observed on the 3rd day of the experiment. The lack of an increase in catalase activity can be associated with both its ability to convert alcohol into acetaldehyde, and with the possible use of hydrogen peroxide in the Fenton reaction to form a hydroxyl radical [5, 13]. The likelihood of such development of events is confirmed by the high, relative to the control group, concentration of MDA on the 3rd day of the experiment. It is obvious that the activation of transcription of antioxidant genes through NRF2 is an insufficient compensatory response, since it does not affect the processes of acetaldehyde formation. Longer alcohol administration can lead to the activation of NF-kB and an increase in the processes of oxidative damage to hepatocytes, which was observed on the 7th day of the experiment.

The connective tissue capsule of the liver is damaged throughout the entire period of the experiment, as evidenced by an increase in the concentration of L-hydroxyproline, as a marker of the intensity of collagenolysis processes. The destruction of the fibrous elements of the connective tissue of the liver during alcohol intoxication can be associated both with the ability of alcohol to increase the activity of matrix metalloproteinase-9 (MMP-9), and with increased production of reactive oxygen species (superoxide anion radical, hydroxyl radical and hydrogen peroxide) [11].

The increase in the concentration of *Hp* in the liver of rats on days 1 and 3 of the experiment may be associated with the anti-inflammatory properties of this class of sulfated glycosaminoglycans [6]. *Hp* is able to prevent the destruction of the connective tissue capsule by blocking neutrophil elastase and MMP-9; therefore, an increase in the concentration of *Hp* may be a protective response to increased damage to the connective tissue capsule in the first days (1st and 3rd) of the experiment. *Hp* are in antagonistic

relationship with the proinflammatory transcription factor NF-kB, which may explain the decrease in *Hp* concentration on the 7th day of the experiment [4].

The limitation of our study is the impossibility of complete separation of the keratan-dermatan fraction of GAGs, which complicates the interpretation of the results. An increase in the concentration of keratan sulfate may indicate a deficiency in the activity of N-acetylgalactosamine-6-sulfate sulfatase and be a sign of degenerative changes at the molecular level. The data on the dynamics of changes in the concentration of the chondroitin-sulfate fraction of GAGs is more indicative for assessing the degree of degradation of the amorphous substance of the connective tissue and the accumulation of unmetabolized products. An increase in the concentration of dermatan sulfate in liver tissues is dangerous, since dermatan sulfate is able to enhance the aggression of immunocompetent cells, being a powerful stimulator of the immune response through CD^{5+} B lymphocytes [15].

The role of the sulfide anion is also controversial. On the one hand, the endogenous sulfide anion has pronounced antioxidant properties, and on the other hand, the excessive formation of the sulfide anion from its donors, such as NaHS, can lead to the development of oxidative stress [14]. In our study, we did not use sulfide anion donators, but studied the pool of endogenous sulfide anion. Consequently, its decrease in all studied periods indicates its active use to compensate for excessive production of superoxide radical anion with excessive intake of alcohol. Sulfide anion is a potent NRF2 transcription factor stimulator and NF-kB inhibitor. Since the concentration of the sulfide anion was the lowest on the 5th day of the experiment, it can be assumed that the increase in the activity of antioxidants is more associated with the activation of NF-kB. Thus, the increase in the concentration of sulfide anion on the 7th day of the experiment can be explained by its lesser use for antioxidant protection, since the diet of animals did not change.

Conclusion

Modeling of chronic alcoholic hepatitis leads to the development of oxidative stress in rat liver tissues due to a decrease in the activity of antioxidant enzymes and an increase in the production of reactive oxygen species.

As a result of the development of oxidative stress in the liver of rats in the first stages of modeling alcoholic hepatitis, the concentration of the anti-inflammatory heparin-heparan fraction of glycosaminoglycans decreases, the accumulation of keratan-dermatan and chondroitin fractions of glycosaminoglycans occurs. The processes of degradation of the fibrous components of the connective tissue of the liver increase and the concentration of the sulfide anion decreases.

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