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The influence of phenformin on the extracellular matrix of the liver of rats under long-term administration of ethanol

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Experimental and clinical studies have revealed the influence of AMP-activated protein kinase (AMPK) signaling on the development of non-alcoholic liver fibrosis. Currently, the results of experimental studies demonstrate that inhibition of AMPK promotes fibrogenesis, while its activation prevents the development of liver fibrosis. The purpose of this work is to establish the effect of activation of AMP-activated protein kinase by the administration of phenformin on the content of glycosaminoglycans, oxyproline and sialic acids in the liver of rats under the conditions of long-term administration of ethanol. The study was conducted on 24 male Wistar rats. The animals were randomly divided into 4 groups of 6 animals each, on which we modeled ethanol-induced liver damage and administered phenformin hydrochloride at a dose of 10 mg/kg. The experiment lasted 63 days. In the liver of rats, the content of total glycosaminoglycans, the concentration of heparin-heparan, keratan-dermatan and chondroitin fractions of glycosaminoglycans, the content of free oxyproline and sialic acids were studied. Long-term alcoholization leads to a violation of the extracellular matrix of the liver of rats, which is evidenced by a decrease in the concentration of proteoglycans and a redistribution of their fractions in the direction of a decrease in anti-inflammatory and regenerative fractions. Chronic intake of alcohol increases the processes of desialylation of glycoconjugates and the intensity of collagenolysis. Activation of AMP-activated protein kinase by administration of phenformin under the conditions of simulating ethanol-induced liver damage leads to an increase in the concentration of glycosaminoglycans due to the growth of heparin-heparan and chondroitin fractions and reduces the intensity of desialylation of glycoconjugates and collagenolysis in the liver of rats.

Key words: liver; AMP-activated protein kinase; phenformin; extracellular matrix; ethanol; alcohol-associated liver disease; glycosaminoglycans; collagen; sialic acids; oxyproline.

INTRODUCTION

It is known that chronic alcohol consumption can lead to the development of steatohepatitis, fibrosis, cirrhosis and even hepatocellular carcinoma. The interaction of different liver cell types and the nature of cell and extracellular matrix (ECM) interactions is critical to understanding alcohol-mediated liver injury. Liver fibrosis is a complex pathophysiological process that consists of three phases, namely inflammatory injury, activation of hepatic stellate cells (HSC) and secretion of extracellular matrix biomolecules [1]. The fibrosis that results from this determines the degree of damage to the architecture of the liver after long-term alcohol consumption [2].

The ECM, which includes more than 300 macromolecules [3], performs various functions and is the main environment surrounding cells, which mutually affects their function and modulates various fundamental aspects of the cellular microenvironment. The ECM is a highly dynamic structure that is constantly undergoing a remodeling process that is indispensable in the remodeling of tissue architecture. Among the macromolecules of the ECM of the liver are glycoconjugates, such as proteoglycans, glycoproteins that form a physical framework for cells and provide structural support, tensile strength, compression resistance and elasticity [4]. In addition to its mechanical and biochemi-

cal properties, ECM helps maintain hydration and homeostasis and, interacting with cell surface receptors and matrix components, regulates cell differentiation, adhesion, proliferation, migration, and the cell cycle [5]. The ECM also controls cell morphogenesis and function through its ability to deposit growth factors and cytokines. Thus, the ECM creates a complex microenvironment that is particularly dynamic in nature and undergoes continuous remodeling not only during development, but also under conditions of liver injury and regeneration and repair [6]. Accordingly, well-coordinated regulation of ECM remodeling is essential to maintain homeostasis and prevent the onset and progression of alcoholic hepatitis [7].

Experimental and clinical studies have revealed a close relationship between AMP-activated protein kinase (AMPK) and liver fibrogenesis. AMPK attenuates TGF-β signaling in HSC and protects against TGF-β-induced inflammatory injury during liver fibrosis [8]. TGF-β is the first inducer of NF-κB activation during inflammatory injury phase in liver fibrogenesis. AMPK also counteracts NF-κB-associated inflammatory injury during liver fibrosis [9]. Overall, the results of experimental studies show that inactivation of AMPK promotes liver fibrosis, while its activation can inhibit this process and thus AMPK protects against inflammatory damage and delays or attenuates liver fibrosis.

In addition, AMPK induces HSC apoptosis and inhibits their proliferation by activating the iNOS/NO pathway [10]. Taken together, these data show that AMPK activation can positively influence the functional state of HSCs, leading to the inhibition of liver fibrosis. AMPK also suppresses ECM secretion and liver fibrosis [11].

Thus, summarizing the known facts about the effect of AMPK on liver metabolism, it is known that AMPK attenuates the intensity of the development of liver fibrosis by reducing the intensity of the primary inflammatory lesion, ECM secretion and HSC activation. The role of AMPK in liver ECM remodeling under conditions of chronic alcoholic hepatitis is insufficiently studied. It is known that hepatocytes, at an early stage of alcohol-induced liver damage, protect themselves by activating AMPK, whereas prolonged ethanol oxidation can destroy the repair system (including the AMPK signaling pathway and autophagy). Lu X. et al. [12] demonstrated that alcohol can induce high levels of p-AMPK at the initial stage of alcoholic liver disease, while p-AMPK levels can significantly decrease in the liver of mice after 2 months of alcohol administration.

The purpose of this work. To determine the effect of AMP-activated protein kinase activation on the content of glycosaminoglycans and their fractions, oxyproline and sialic acids in the liver of rats under conditions of chronic alcoholic hepatitis.

METHODS

The experiments were performed on 24 white, sexually mature male Wistar rats into 4 groups (6 animals each), weighing 180-220 g. The animals were divided into following groups: I - control group; II - group - animals, which received phenformin hydrochloride (phenformin, “Sigma-Aldrich” USA), as an activator of AMP-activated protein kinase, orally at a dose of 10 mg/kg [13] daily for 63 days (control + phenformin group); III group – animals, on which we simulated chronic alcoholic hepatitis (n = 6) by forced intermittent alcoholization for 5 days by intraperitoneal administration of 16.5% ethanol solution in 5% glucose solution, at the rate of 4 ml/kg body weight once a day. Afterwards, there was a break for two days. Then animals again received intraperitoneal administration of 16.5% ethanol solution in 5% glucose solution, at the rate of 4 ml/kg body weight a day for 5 days. Then they were converted to 10% ethanol as the only source of drink for 51 days, total experimental procedure lasted 63 days [14] (alcoholic hepatitis group). IV group - animals, on which we simulated chronic alcohol hepatitis as in group III, and administered phenformin

according to the scheme of group II (alcoholic hepatitis + phenformin group).

The control group included animals that were subjected to similar manipulations throughout the study period, but were injected with a physiological solution (0.9% sodium chloride). The conditions for keeping animals in the vivarium were standard. Animals were removed from the experiment on the 63rd day by blood sampling from the right ventricle of the heart under thiopental anesthesia.

Research was conducted in accordance with the standards of the Council of Europe Convention on Bioethics "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (1997), general ethical principles of experiments on animals approved by the First National Congress on Bioethics of Ukraine (September 2001) and other international agreements and national legislation in this area. The animals were kept in a vivarium accredited in accordance with the "Standard rules of order, equipment and maintenance of experimental biological clinics (vivarium)". Devices used for research were subject to metrological control. All experimental procedures were approved by Bioethical Committee of Poltava State Medical University (Record No. 197 from 23.09.2021).

To determine the total concentration of glycosaminoglycans (GAG), the carbazole method was used, which was based on determining the content of uronic acids after acid hydrolysis of GAG, based on their color reaction with carbazole [15]. To define the concentration of different fractions (heparin-heparan, keratan-dermatan and chondroitin) of GAG in tissue homogenate, we used method of sequential precipitation [15]. After sequential precipitation, we used carbazole method to determine concentrations of different fractions.

The concentration of free oxyproline was measured colorimetrically after the oxidation of oxyproline to pyrrole-2-carboxylic acid. Its concentration was estimated by the content of the colored product formed in the reaction of the product of the reaction of pyrrole-2-carboxylic acid with paradimethylaminobenzaldehyde [15].

The content of sialic acids was determined by the Hess method by measuring absorption on a Ulab 101 spectrophotometer at a wavelength of $\lambda = 540$ nm [16].

Statistical processing of the results of biochemical studies was carried out using a pairwise comparison by the non-parametric Mann-Whitney method. All statistical calculations were performed in the Microsoft office Excel program and its extension Real Statistics 2019. The difference was considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

The effect of AMP-activated protein kinase activation on the biochemical parameters of rat liver ECM. Statistical analysis of obtained results revealed that in case of presence of statistically significant difference between groups P values were lower than 0.01.

As a result of the conducted experimental research, we established that under the conditions of phenformin administration, the total concentration of GAG increased by 1.07 times due to the keratan-dermatan fraction, which increased by 2.04 times, and the chondroitin fraction of GAG, which increased by 1.15 times, and the concentration heparin-heparan fraction of GAG in the liver of rats decreased by 1.33 times compared to the control group (Table). Evaluating the metabolism of collagen proteins under the conditions of administration of the AMP-activated protein kinase activator, we noted a decrease in the content of free oxyproline by 1.42 times compared to the control. The concentration of sialic acids in the liver of rats increases by 1.87 times under the conditions of phenformin administration compared to the control group. Thus, the introduction of phenformin led to a decrease in collagenolysis and an increase in the catabolism of glycoproteins and proteoglycans of the extracellular matrix of the liver, with a change in the ratio of GAG frac-

tions: an increase in the keratan-dermatan and chondroitin fractions against the background of a decrease in the concentration of the heparin-heparan fraction.

The decrease in the intensity of the processes of collagen breakdown in the liver of rats that received phenformin solution orally for the purpose of activating AMPK can be associated with the ability of the AMPK cascade to reduce the activity of matrix metalloproteinases (MMP), in particular MMP-9 [17]. Excessive activity of MMP-9 is one of the pathogenetic mechanisms that lead to the development of fibrotic changes in the liver, so the probable decrease in its activity under the influence of phenformin is a positive effect of the drug [18]. According to the results of our study, the activation of AMPK by the introduction of phenformin led to an increase in the concentration of GAG due to the increase in keratan-dermatan and chondroitin fractions. Similar results were obtained by Shrikanth et al. [19], who established that after exposure to the AMPK activator of NRK-52E line cells under conditions of hyperglycemia, there was an increase in the concentration of keratan-dermatan and chondroitin fractions of GAG in these cells. Probably, the effect of the AMPK activator depends on the type of cells and the conditions in which this cell is. The increase in the concentration of sialic acids in the liver of rats under the influence of phenformin administration may be related to the inhibitory effect of phenformin on the synthesis of glycoproteins [20]. It may also be related to the activation of the AMPK cascade, as the literature shows that the AMPK activator berberine has neuraminidase activity [21]. The detailed mechanisms of effect of phenformin on proteoglycans and glycoproteins of liver ECM require further study.

Effect of long-term ethanol administration on biochemical parameters of rat liver ECM. Long-term alcohol intoxication of the body of rats led to increased collagenolysis and catabolism of glycoproteins of the extracellular matrix of the liver. Against the background of

Biochemical indicators of extracellular matrix of the liver of rats under conditions of chronic alcoholic hepatitis and phenformin administration (M ± m; n = 6)

Biochemical parameters	Group			
	control	control + phenformin	alcoholic hepatitis	alcoholic hepatitis + phenformin
Total concentration of glycosaminoglycans, μmol/l	2.62 ± 0.02	2.8 ± 0.01*	2.07 ± 0.02*/**	3.17 ± 0.01*/**/***
Concentration of heparin-heparan fraction, μmol/l	1.81 ± 0.02	1.36 ± 0.01*	0.78 ± 0.01*/**	2.03 ± 0.02*/**/***
Concentration of keratan-dermatan fraction, μmol/l	0.27 ± 0.004	0.55 ± 0.008*	0.84 ± 0.009*/**	0.41 ± 0.008*/**/***
Concentration of chondroitin fraction, μmol/l	0.59 ± 0.009	0.68 ± 0.007*	0.49 ± 0.006*/**	0.94 ± 0.006*/**/***
Concentration of free oxyproline, μmol/g	1.28 ± 0.02	0.9 ± 0.01*	2.88 ± 0.05*/**	1.18 ± 0.01*/**/***
Concentration of sialic acids, mg/g	1.26 ± 0.04	2.36 ± 0.07*	7.67 ± 0.02*/**	4.29 ± 0.04*/**/***

*P < 0.01 compared to the control group; **P < 0.01 compared to the control+ phenformin group; ***P < 0.01 compared to the alcoholic hepatitis group.

increased degradation of ECM proteoglycans of the rat liver, the ratio of individual GAG fractions shifted towards the predominance of the keratan-dermatan fraction, which was described in detail in our previous publications [22].

The effect of AMP-activated protein kinase activation on the biochemical parameters of rat liver ECM under the conditions of long-term ethanol administration. We found that the introduction of phenformin under the conditions of chronic administration of ethanol led to an increase in the total concentration of GAG in the liver of rats by 1.21 times compared to the control group and by 1.53 times compared to the group of animals with ethanol-induced liver damage (Table). The concentration of the heparin-heparan fraction of GAG in the liver of rats increased by 1.12 times under the conditions of phenformin administration against the background of long-term exposure to ethanol compared to the control group and by 2.6 times compared to alcohol hepatitis group. The concentration of the keratan-dermatan fraction of GAG in the liver of rats increased by 1.52 times compared to the control group and decreased by 2.05 times compared to the group of rats which were administered ethanol. The concentration of the chondroitin fraction of GAG in the liver of rats increased by 1.59 times under the conditions of administration of Phenformin against the background of chronic alcoholic liver damage compared to the control group and by 1.92 times compared to alcoholic hepatitis group.

Restoration of the concentration of the heparan-heparin fraction of GAG in the liver of rats under the conditions of activation of AMPK by phenformin may be associated with a decrease in oxidative damage to liver cells due to excessive intake of alcohol [23]. Reduction of oxidative cell damage during phenformin administration in chronic alcoholic liver injury simulation may be a consequence of AMPK-dependent activation of the antioxidant system, which is implemented through the AMPK/SIRT1 axis [24]. The increase in the chondroitin fraction of GAG in this group of animals may be related to the above-mentioned effects of phenformin [18].

Analyzing the metabolism of collagen proteins of liver ECM of rats which were administered phenformin and ethanol, we found that the concentration of free oxyproline decreased by 1.08 times compared to the control and by 2.44 times compared to the group of rats with ethanol-induced liver damage. Prolonged intake of ethanol can cause activation of MMP-2 and MMP-9, which underlies the development of alcohol-induced liver fibrosis and cirrhosis [25]. The decrease in the concentration of free oxyproline in the liver of animals with the introduction of phenformin and ethanol consists in the inhibition of the activity of MMP-9 through the activation of the AMPK cascade [17, 26].

The concentration of sialic acids in the liver of rats increased by 3.4 times under the conditions of administration of phenformin on the background of chronic alcoholic liver damage compared to the control group and decreased by 1.79 times compared to the group of rats that were administered only ethanol. In the mechanism of ethanol-induced liver damage, the leading role is attributed to the development of oxidative stress [27], which promotes an increase in neuraminidase-1 activity, which can explain the desialylation of glycoconjugates in animals under conditions of long-term ethanol administration [28]. A decrease in the concentration of sialic acids under the conditions of the administration of phenformin and ethanol may be associated with the activation of antioxidant protection during the stimulation of the AMPK cascade [24].

CONCLUSIONS

Long-term alcoholization leads to a violation of the extracellular matrix in the liver of rats, which is evidenced by a decrease in the concentration

of proteoglycans and a redistribution of their fractions in the direction of a decrease in anti-inflammatory and regenerative. Chronic intake of alcohol increases the processes of desialylation of glycoconjugates and the intensity of collagenolysis in the liver of rats.

Activation of AMP-activated protein kinase by the introduction of phenformin under the conditions of chronic alcoholic hepatitis modeling leads to an increase in the concentration of glycosaminoglycans due to the growth of heparin-heparan and chondroitin fractions and reduces the intensity of desialylation of glycoconjugates and collagenolysis in the liver of rats.

The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of co-authors of the article.

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ВПЛИВ ФЕНФОРМІНУ НА ПОЗАКЛІТИННИЙ МАТРИКС ПЕЧІНКИ ЩУРІВ ЗА УМОВ ТРИВАЛОГО ВВЕДЕННЯ ЕТАНОЛУ

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Сигналінг АМФ-активованої протеїн кінази (AMPK) впливає на розвиток неалкогольного фіброзу печінки. Нині дані експериментальних досліджень демонструють, що інгібування AMPK сприяє фіброгенезу, тоді як її активація попереджає розвиток фіброзу печінки. Метою нашої роботи було встановити вплив активації АМФ-активованої протеїн кінази введенням фенформіну на вміст глікозаміногліканів, оксипроліну та сіалових кислот в печінці щурів за умов тривалого введення етанолу. Дослідження проведено на 24 щурах-самцях лінії Вістар. Тварини були рандомізовано розподілені на 4 групи по 6 тварин, яким моделювали етаноліндуковане ушкодження печінки та вводили фенформін гідрохлорид в дозі 10 мг/кг. Експеримент тривав 63 доби. В печінці щурів досліджували вміст загальних глікозаміногліканів, концентрацію гепарин-гепаранової, кератан-дерматанової та хондроїтинової фракцій глікозаміногліканів, вміст вільного оксипроліну та сіалових кислот. Тривала алкоголізація призводила до порушення екстрацелюлярного матриксу печінки щурів про що свідчить зменшення концентрації протеогліканів та перерозподіл їх фракцій у бік зменшення протизапальної та регенераторної. Хронічне надходження алкоголю посилило процеси десіалізації глікокон'югатів та інтенсивність колагенолізу. Активація АМФ-активованої протеїнкінази за допомогою введення фенформіну за умов моделювання етаноліндукованого ушкодження печінки призводило до збільшення концентрації глікозаміногліканів внаслідок зростання гепарин-гепаранової та хондроїтинової фракцій і зменшувало інтенсивність десіалізації глікокон'югатів та колагенолізу в печінці щурів.

Ключові слова: печінка; АМФ-активована протеїнкіназа; фенформін; екстрацелюлярний матрикс; етанол; алкогольасоційована хвороба печінки; глікозаміноглікани; колаген; сіалові кислоти; оксипролін.

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