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INFLUENCE OF DOXORUBICIN ON THE DEVELOPMENT OF OXIDATIVE-NITROSATIVE STRESS IN THE LIVER OF RATS UNDER CONDITIONS OF CHRONIC ALCOHOLIC HEPATITIS

Introduction. *Chronic alcohol consumption leads to oxidative-nitrosative liver injury, which induces the release of cytokines and growth factors, leading to activation of hepatic stellate cells and fibrosis. Modern studies have revealed a close connection between AMP-activated protein kinase and fibrogenesis.*

The aim of the study *– to determine the effect of inhibition of AMP-activated protein kinase by the administration of doxorubicin on the development of oxidative-nitrosative stress in the liver of rats under conditions of long-term administration of ethanol.*

Research Methods. *Experiments were performed on 24 white, sexually mature male Wistar rats, weighing 180-220 g. Chronic alcoholic hepatitis was modeled by the method of forced intermittent alcoholization according to Yu.M. Stepanov (2017). Doxorubicin was administered at a dose of 1.25 mg/kg IV 4 times a week throughout the experiment, which lasted 63 days. The activity of NO-synthase isoforms, the concentration of nitrite and peroxynitrite, the activity of arginase, superoxide dismutase and catalase, the concentration of malondialdehyde, oxidation-modified proteins, nitrosothiols and sulfide anion, and the production of superoxide anion were determined in the homogenate of the liver of rats. The significance of the differences was assessed by the Mann-Whitney U-test at p<0.05.*

Results and Discussion. *Administration of doxorubicin under the conditions of chronic alcoholic hepatitis modeling reduced the activity of the inducible isoform of NO-synthase by 4 times, the activity of superoxide dismutase by 1.95 times and increased the activity of catalase by 1.77 times in the liver of rats compared to chronic alcoholic hepatitis. Under these conditions, the concentration of malonic dialdehyde in the liver increased by 1.71 times, the production of superoxide anion radical increased by 1.3 times, the concentration of peroxynitrite increased by 1.9 times, sulfide anion decreased by 2.11 times, while OMP in the liver of rats decreased by 1.98 times compared to chronic alcoholic hepatitis.*

Conclusion. *Administration of doxorubicin against the background of chronic alcoholic hepatitis limits the oxidative modification of liver proteins and the production of nitric oxide from the inducible isoform of NO-synthase.*

KEY WORDS: **liver; oxidative stress; nitrosative stress; chronic alcoholic hepatitis; AMPK; doxorubicin.**

INTRODUCTION. Fibrosis of the liver occurs as a result of chronic liver damage of various etiologies. Chronic alcohol consumption leads to oxidative-nitrosative liver injury [1], which induces the release of cytokines and growth factors such as transforming growth factor-β (TGF-β), leading to the activation of hepatic stellate cells (HSCs). Transformation of resting HSCs into activated myofibroblasts leads to increased alpha-smooth muscle actin (α-SMA) and deposition of extracellular matrix, which play a key role in the onset and development of liver fibrosis [2].

AMP-activated protein kinase (AMPK) has been shown to be involved in the pathophysiology of liver fibrosis, and up-regulation of AMPK phosphorylation contributes to the attenuation of liver fibrosis [3]. AMPK is the main molecule that prevents or delays the process of fibrogenesis. Current research has revealed a close relationship between AMPK and fibrogenesis. In the AMPKα1 knockout mouse, cardiofibroblast proliferation and migration are enhanced, whereas myodifferentiation is suppressed after myocardial ischemia. This response correlates with increased collagen expression and myocardial fibrosis [4]. Pioglitazone inhibits liver fibrosis in rats with nonalcoholic steatohepatitis by activating the AMPK/TGF-β1 pathway [5]. Eugenol, a cinnamate derivative and AMPK activator, alleviates fibrosis by downregulating SREBP1 gene expression through the AMPK/mTOR/p70S6K pathway, leading to attenuation of fibrosis [6]. These results suggest

that knockout or inactivation of AMPK promotes fibrogenesis, while activation of AMPK may be a possible treatment for fibrosis.

AMP-activated protein kinase is a heterotrimeric protein kinase that consists of a catalytic subunit (α) and two regulatory subunits (β and γ), each with multiple isoforms, and plays a central role in cellular energy homeostasis. In response to various metabolic stressors, AMPK is activated by the accumulation of AMP as a result of ATP depletion and in turn regulates multiple metabolic pathways to balance cellular energy. In addition to balancing cellular energy, AMPK also induces apoptosis through several tumor suppressors, including LKB1, TSC2, and p53. LKB1 is an activating kinase of AMPK, and TSC2 and p53 are direct substrates of AMPK [7]. Thus, AMPK has a dual role: protecting cells from hypoergosis and inducing cell apoptosis, but the underlying mechanisms by which AMPK determines cell fate remain poorly understood.

The purpose of the study is to determine the effect of inhibition of AMP-activated protein kinase by the administration of doxorubicin on the development of oxidative-nitrosative stress in the liver of rats under conditions of long-term administration of ethanol.

RESEARCH METHODS. The experiments were performed on 24 male Wistar rats, weighing 180-220 g. The animals were divided into 4 groups (6 animals in each): I – control group; II – group animals which received doxorubicin hydrochloride (doxorubicin, S.C. Sindan-Pharma S.R.L.), as inhibitor of AMP-activated protein kinase, at a dose of 1.25 mg/kg intraperitoneally four times a week for 63 days (doxorubicin) [8]; III group – animals, on which we simulated chronic alcoholic hepatitis for 63 days by method described in our previous work [9] (chronic alcoholic hepatitis). IV group – animals, on which we simulated chronic alcohol hepatitis as in group III and administered doxorubicin hydrochloride according to the scheme of group II (chronic alcoholic hepatitis with doxorubicin).

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 manipulations with laboratory animals were approved by bioethical committee of Poltava State Medical University (Record № 197 from 23.09.2021).

Removal of animals from the experiment occurred on day 63 by taking blood from the right ventricle of the heart under thiopental anesthesia. Serum and liver were studied.

Total NO-synthase activity was evaluated by the increase of nitrites after incubation of 10 % liver homogenate (0.2 ml) for 30 min in the incubation solution (2.5 ml of 0.1 M trisbuffer, 0.3 ml of 320 mM aqueous solution of L-arginine and 0.1 ml of 1 mM NADPH+H+ solution). To determine the activity of constitutive NOS (cNOS) 1 % solution of aminoguanidine hydrochloride was used and the incubation time was extended to 60 min [10]. The activity of inducible NOS (iNOS) was calculated by the formula: iNOS= gNOS-cNOS.

Adrenaline auto-oxidation reaction in an alkaline environment with the generation of superoxide was used to determine SOD activity [11]. The method of catalase activity estimation was based on the determination of colored products formed by the reaction of hydrogen peroxide with ammonium molybdate [12].

Free malonic dialdehyde specifically reacts with 1-methyl-2-phenyl-indole in a mixture of methanol and acetonitrile to form chromogen (carbocyanine dye) with a maximum light absorption at a wavelength of 586 nm [13]. Peroxynitrite concentration was measured by using its reaction with potassium iodide under pH 7.0 in 0.2 M phosphate buffer with the same pH, which yields I_3 with maximum absorbance at 355 nm wavelength [10].

The method for the determination of nitrosothiols was based on the determination of the difference in the concentration of nitrites (NO_2^-) using Griess reagent (modified by Ilosvay) before and after oxidation of nitrosothiol complexes (SNO) to nitrites with a solution of mercuric chloride ($HgCl_2$) [14]. Sulfides specifically react with N-N-dimethyl-paraphenylenediamine in the presence of Fe^{3+} ions and excess of hydrochloric acid to form a red-pink chromogen with a maximum light absorption at a wavelength of 667 nm [15].

The concentration of oxidized proteins was determined spectrophotometrically. The principle of the method is that 2,4-dinitrophenylhydrazine

reacts with the carbonyl groups of oxidized proteins to form dinitrophenylhydrazones which are then detected [16].

The method for estimation of superoxide anion radical production was based on Nitroblue tetrazolium (NBT) reduction by superoxide with the formation of diformazan, a dark blue insoluble precipitate [17].

Statistical processing of biochemical research results was performed using Kruskal–Wallis one-way analysis of variance with following pairwise comparison using the Mann-Whitney U-test. In order to avoid multiple comparison error we used correction by Bonferroni method. All statistical calculations were performed in Microsoft office Excel and its extension Real Statistics 2019. The difference was considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION.

Effect of inhibition of AMP-activated protein kinase on biochemical parameters of rat liver.

Administration of doxorubicin as an inhibitor of AMP-activated protein kinase in an experiment on rats made it possible to establish the following changes in the liver of animals: iNOS activity increased by 2.31 times, cNOS activity increased by 1.74 times, MDA concentration increased by 1.93 times, OMP increased by 3 times, peroxynitrite increased by 7.84 times, sulfide anion increased by 1.35 times, production of superoxide anion radical increased by 1.17 times compared to the control group of rats. The activity of catalase in the liver of rats under the conditions of administration of doxorubicin decreased by 1.05 times, the concentration of nitrosothiols decreased by 1.8 times and nitrites decreased by 1.76 times in relation to the control group of rats (Table).

Changes in the biochemical indicators of the liver of rats under the conditions of inhibition of AMP-activated protein kinase against the background of chronic alcoholic hepatitis modeling.

Administration of doxorubicin under the conditions of modeling chronic alcoholic hepatitis increased the activity of cNOS in the liver of rats by 1.48 times compared to the control group of animals (Table). iNOS activity decreased in relation to the group of animals that were injected with doxorubicin by 2.06 times, and in relation to the group of chronic alcoholic hepatitis by 4 times. The activity of SOD in the liver of a group of rats under the conditions of administration of doxorubicin against the background of simulation of chronic alcoholic hepatitis was reduced by 1.96 times in relation to the control, by 1.89 times in relation to the group of animals administered doxorubicin and by 1.95 times in relation to groups of animals with chronic alcoholic hepatitis. In contrast, catalase activity increased by 1.08 times in relation to the control, by 1.13 times in relation to the group of rats administered doxorubicin and by 1.77 times in relation to the group of rats with chronic alcoholic hepatitis. Under these conditions, the concentration of MDA in the liver increased by 2.2 times in relation to the control, by 1.71 times in relation to the group of rats with chronic alcoholic hepatitis. Production of the superoxide anion radical in the liver of rats under the conditions of administration of doxorubicin injection against the background of simulation of

Table – **Biochemical indicators in the liver of rats under the conditions of inhibition of AMP-activated protein kinase by doxorubicin against the background of chronic alcoholic hepatitis modeling (M±m)**

Biochemical parameters	Group			
	Control	Doxorubicin	Chronic alcoholic hepatitis	Chronic alcoholic hepatitis with doxorubicin
Inducible NO synthase activity, μmol/min per g of protein	0.16±0.02	0.37±0.04*	0.72±0.07*^	0.18±0.03^#
Constitutive NO synthases activity, μmol/min per g of protein	0.027±0.0003	0.047±0.002*	0.044±0.0009*	0.04±0.004*
Superoxide dismutase activity, c.u.	12.34±0.55	11.84±0.36	12.23±1.03	6.28±0.05*^#
Catalase activity, μkat/g	0.376±0.008	0.359±0.001*	0.23±0.01*^	0.406±0.002*^#
Malonic dialdehyde concentration, μmol/g	12.32±0.11	23.75±0.14*	15.91±0.32*^	27.15±1.92*#
Superoxide anion radical production, nmol/s per g	1.84±0.004	2.16±0.03*	2.71±0.03*^	3.51±0.04*^#
$ONOO^-$ concentration, μmol/g	0.45±0.01	3.53±0.03*	4.26±0.03*^	8.1±0.02*^#
S-NO concentration, μmol/g	0.36±0.019	0.2±0.03*	0.18±0.034*	0.11±0.02*^
NO_2 concentration, nmol/g	7.14±0.17	4.05±0.42*	5.67±0.34*^	5.27±0.56*
Sulfide anion, μmol/g	7.23±0.17	9.73±0.34*	15.01±0.32*^	7.13±0.65^#
OMP concentration, c.u.	0.04±0.002	0.12±0.001*	0.34±0.007*^	0.172±0.002*^#

Note: * – $p<0.05$ compared to the control group;
^ – $p<0.05$ compared to the doxorubicin group;
# – $p<0.05$ compared to chronic alcoholic hepatitis group.

chronic alcoholic hepatitis increased by 1.91 times in relation to the control, by 1.63 times in relation to the group of animals administered doxorubicin and by 1.3 times in in relation to the group of rats with chronic alcoholic hepatitis. In this group of rats, the concentration of peroxynitrite in the liver increased by 18 times compared to the control group, by 2.29 times in relation to the group of animals administered doxorubicin and by 1.9 times in relation to the group of rats with chronic alcoholic hepatitis, and the concentration of nitrosothiols decreased by 3.27 times in relation to the control and by 1.82 times in relation to the group of rats administered doxorubicin. The concentration of NO_2 in the liver of rats decreased by 1.35 times in the group of animals with AMPK inhibition under the conditions of chronic alcoholic hepatitis simulation compared to the control group. The concentration of HS^- in the liver of rats in the group of animals that were injected with doxorubicin under conditions of simulation of chronic alcoholic hepatitis was reduced by 1.36 times compared to animals that were injected with only doxorubicin and by 2.11 times compared with the group of rats with chronic alcoholic hepatitis. Under the conditions of AMPK inhibition against the background of chronic alcoholic hepatitis simulation, the concentration of OMP in the liver of rats increased by 4.3 times compared to the control, by 1.43 times in relation to the group of animals injected with doxorubicin and decreased by 1.98 times in relation to the group rats with chronic alcoholic hepatitis.

The toxicity of doxorubicin in most cases is due to its ability to inhibit AMPK activity [18]. Inhibition of AMPK is often accompanied by increased production of reactive oxygen species (ROS), which is consistent with our results in the doxorubicin administration group and the doxorubicin administration group on the background of chronic alcoholic hepatitis, where an increase in ROS production was found [19]. On the other hand, excessive production of ROS can stimulate AMPK activation in cases where ROS are products of a disrupted mitochondrial electron transport chain [20]. Therefore, increased production of ROS and increased intensity of lipid peroxidation processes in the liver in the groups of doxorubicin administration and doxorubicin administration against the background of chronic alcoholic hepatitis simulation is not a consequence of its effect on AMPK activity in liver cells, but is more related to the direct toxicity of doxorubicin.

The toxicity of doxorubicin for liver tissues is due to the peculiarity of its biotransformation in hepatocytes. The actual toxic effects of doxorubicin on liver tissues are mediated by its ability to reduce the activity of the tissue antioxidant system [21]. Research has also established that among the metabolites that accompany the toxic effect of doxorubicin on the liver, a special place is occupied by urea, the concentration of which often changes under the conditions of use of doxorubicin [22]. The introduction of doxorubicin is accompanied by a decrease in arginase 2 activity and an increase in iNOS activity in the heart, which is partially supported by the results of our study, which show an increase in iNOS activity in the group of animals that were injected with doxorubicin [23]. It was also established that doxorubicin inhibits the activity of arginase 1 [24]. Under the conditions of chronic persistent inflammation in liver tissues, its macrophages have the ability to form an immunomodulatory phenotype, which is accompanied by an increase in the expression of arginase 1 genes, which can explain the sharp decrease in iNOS activity in the group of a combination of long-term administration of ethanol and doxorubicin [25]. The absence of such an effect in the group of animals with chronic alcoholic hepatitis may indicate a much lower intensity of the inflammatory process in this group.

Enhanced formation of active forms of nitrogen (peroxynitrite) under the conditions of doxorubicin administration and when it is combined with long-term ethanol administration may be due to the activation of xanthine oxidase [26]. Alcohol can also increase the activity of xanthine oxidase by increasing the concentration of its substrate in liver tissues [27]. The combination of substrate activation of xanthine oxidase under the influence of alcohol and the influence of doxorubicin on its activity creates conditions under which the majority of nitric oxide can be formed independently of the NO-synthase mechanism, which also explains the decrease in iNOS activity in the group of the combination of doxorubicin administration against the background of modeling chronic alcoholic hepatitis.

CONCLUSIONS. 1. The introduction of doxorubicin against the background of chronic alcoholic hepatitis limits the oxidative modification of liver proteins and the production of nitric oxide from the inducible isoform of NO-synthase.

2. The combination of doxorubicin and chronic alcoholic hepatitis leads to an increase in lipid peroxidation in the liver of rats due to a decrease in antioxidant protection and an increase in the formation of reactive oxygen species, which is a manifestation of doxorubicin toxicity.

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ПОЛТАВСЬКИЙ ДЕРЖАВНИЙ МЕДИЧНИЙ УНІВЕРСИТЕТ

ВПЛИВ ДОКСОРУБІЦИНУ НА РОЗВИТОК ОКСИДАТИВНО-НІТРОЗАТИВНОГО СТРЕСУ В ПЕЧІНЦІ ЩУРІВ ЗА УМОВ ХРОНІЧНОГО АЛКОГОЛЬНОГО ГЕПАТИТУ

Резюме

Вступ. *Хронічне вживання алкоголю призводить до оксидативно-нітрозативного ушкодження печінки, яке індукує вивільнення цитокінів і факторів росту, що призводить до активації зірчастих клітин печінки та фіброзу. Сучасні дослідження виявили тісний зв'язок між АМФ-активованою протеїнкіназою і фіброгенезом.*

Мета дослідження *– встановити вплив інгібування АМФ-активованої протеїнкінази шляхом введення доксорубіцину на розвиток оксидативно-нітрозативного стресу в печінці щурів за умов тривалого введення етанолу.*

Методи дослідження. *Експерименти виконано на 24 білих статевозрілих щурах-самцях лінії Вістар масою 180–220 г. Хронічний алкогольний гепатит моделювали методом примусової перивистої алкоголізації за Ю. М. Степановим (2017). Доксорубіцин вводили у дозі 1,25 мг/кг внутрішньочеревно 4 рази на тиждень протягом усього експерименту, який тривав 63 доби. У гомогенаті печінки щурів визначали активність ізоформ NO-синтаз, концентрацію нітритів та пероксинітриту, активність аргіназ, супероксиддисмутази і каталази, концентрацію малонового діальдегіду, окисномодифікованих протеїнів, нітрозотіолів та сульфід-аніона, продукцію супероксид-аніон-радикала. Достовірність відмінностей оцінювали за U-критерієм Манна – Уїтні при p<0,05.*

Результати й обговорення. *Введення доксорубіцину за умов моделювання хронічного алкогольного гепатиту знизило активність індуцибельної ізоформи NO-синтази в 4 рази, активність супероксиддисмутази – в 1,95 раза, підвищило активність каталази в 1,77 раза в печінці щурів щодо групи тварин із хронічним алкогольним гепатитом. За цих умов концентрація в печінці малонового діальдегіду зросла в 1,71 раза, продукція супероксид-аніон-радикала збільшилася в 1,3 раза, концентрація пероксинітриту підвищилася в 1,9 раза, сульфід-аніона – знизилася в 2,11 раза, окисномодифікованих протеїнів – у 1,98 раза стосовно групи тварин із хронічним алкогольним гепатитом.*

Висновки. *Введення доксорубіцину на тлі хронічного алкогольного гепатиту обмежує окисну модифікацію протеїнів печінки та продукцію оксиду азоту від індуцибельної ізоформи NO-синтази.*

КЛЮЧОВІ СЛОВА: **печінка; оксидативний стрес; нітрозативний стрес; хронічний алкогольний гепатит; АМФ-активована протеїнкіназа; доксорубіцин.**