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EEXPRESSION OF mRNA iNOS AND mRNA eNOS IN THE LIVER OF RATS WITH CHRONIC ALCOHOL INTOXICATION AND WITH THE INTRODUCTION OF ACHILLEA MICRANTHOIDES KLOK HERB EXTRACT. ET KRYTZKA.

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A few studies have established that ethanol changes the activity and expression of NOS in target organs, leads to overproduction of NO and its cytotoxic forms [1,2]. All this causes the initiation of nitrosating stress, apoptosis, and changes immunocompetence. This is due to the nature of ethanol effect on various NOS isoforms. Some researchers have shown that ethanol increases the expression of iNOS, the trigger of nitrosative stress indirectly via the activation of IL-1b [3-5]. The above theoretically and experimentally substantiates the use of NOS modulators and NO scavengers in the neuroprotective treatment of alcoholic disease. However, the mechanism of pharmacological regulation of the nitroxidergic system during alcohol intoxication is not completely clear. Our works established the antioxidative modulation possibility of the expression of various NOS isoforms in ischemia of the myocardium and brain, as well as in alcoholic lesions of these organs [6-8]. We also established a pronounced antioxidant effect of the lipophilic extract of the herb Achillea micranthoides Klok. Et Krytzka (LEYH) in a model of chronic alcohol intoxication [9]. It was found that the antioxidant effect of LEYH significantly exceeds the reference drug Hepabene in such indicators as a decrease in the level of CPH and nitrotyrosine in the liver of rats with CAI.

Ehe hepatoprotective and antioxidant effect of LEYH is realized due to the bioflavonoids-catechins, leucoanthocyanidins, chalcones, flavanols contained in them, which exhibit the properties of scavengers of reactive oxygen species and NO, inhibit the lipoperoxidation of hepatocyte cell membrane phospholipids, increase the expression of glutathione peroxidase, inhibit free radical-induced synthesis of pro-inflammatory factors and apoptosis. The obtained results make it possible to further study the mechanisms of the hepatoprotective action of LEYH in conditions of alcoholic liver damage.

Aim of the study: to study the effect of LEYH on the character of iNOS and eNOS mRNA expression in rat brain after chronic alcohol intoxication.

Materials and methods. We obtained a lipophilic extract of the herb Achillea micranthoides Klok. Et Krytzka (LEYH). The raw material was the herb Achillea micranthoides Klok. Et Krytzka (Yarrow), harvested during flowering (July-September). Drying was carried out in a Termolab SNOL 24/350 oven (Ukraine) at a temperature of 35° C for 12 hours. Used pre-ground grass (d = 0.3 mm). The LEYH was obtained by extraction of corn oil (1: 5) on an ultrasonic device "UZDN-A1200T" at an operating frequency of 50 Hz (t = + 40°C) for 2 hours. The extraction was repeated 2 times under the same conditions. The obtained extract was filtered, the raw materials were squeezed out, the grist was separated. The extract was sedimented in a cool place, the precipitate was filtered. The resulting lipophilic extract contains essential oil, vitamin K 1, fatty acids, polyphenolic compounds, organic acids, amino acids, tannins, macro- and microelements.

All studies were carried out in accordance with the "Guidelines for the submission of documentation for medicines to the Pharmacological Center of the Ministry of Health of Ukraine". Studies have been performed on a sufficient number of experimental animals. All manipulations were carried out in accordance with the

regulation on the use of animals in biomedical experiments (Strasbourg, 1986, as amended in 1998) and the "General ethical principles of experiments on animals" (Kiev, 2001), which are consistent with the provisions of the European Convention for the Protection of vertebrates that are used for experimental and scientific purposes. " The protocols of experimental studies and their results were approved by the decision of the Bioethics Commission of the ZSMU (protocol No. 33 dated 10.26.2018).

The experiments were performed on 40 white outbred rats of both sexes, weighing 180-190 g., obtained from the nursery of the Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine. The duration of the quarantine (acclimatization period) for all animals was 14 days. During quarantine, each animal was examined daily (behavior and general condition), animals were observed in cages twice a day (morbidity and mortality). Before the start of the study, animals meeting the criteria for inclusion in the experiment were divided into groups using the randomization method. Non-eligible animals were excluded from the quarantine study. Cages with animals were placed in separate rooms. Light mode: 12 hours - light, 12 hours - darkness. The air temperature was maintained within 19-25 ° C, relative humidity - 50-70%. Temperature and humidity were recorded daily. The ventilation mode was established, providing about 15 room volumes per hour. Experimental animals were kept on the same rations, under ordinary conditions of vivarium. Animals were housed in standard cages - 5 animals per cage. Diet - feed grain, bread, root crops (beets, carrots).

Chronic alcoholic hepatitis was modeled by intragastric administration with a metal probe of 20% ethanol at a dose of 8 g / kg IG for 60 days.

After ethanol introduction, the studied oils were administered intragastrically for 30 days using a metal probe at a dose of 20 mg / kg as well as reference drug Hepabene (MerckleGmbH / Ratiopharm International GmbH, Germany) 100 mg / kg. Mortality was recorded daily. On the 90th day of the experiment, 1 hour after the drugs injections, animals were tested for the duration of thiopental sleep in order to determine the detoxifying function of the liver. For this, animals of all groups were injected intraperitoneally with thiopental sodium (40 mg / kg). At the end of the test, animals with signs of awakening were taken out of the experiment by decapitation. The liver was taken for research.

In this series of experiments, there were five groups of animals:

1) intact (10 rats);
2) control - untreated with chronic alcoholic hepatitis, receiving saline (10 rats);
3) animals with chronic alcoholic hepatitis treated with LEYH (10 rats);
5) animals with chronic alcoholic hepatitis treated with Hepabene (10 rats).

At the end of the experiment, animals were removed from the experiment after 2-4 minutes. after injection of sodium etaminal (40 mg / kg) (until the straightening reflex is lost) in order to minimize metabolic changes. The liver was quickly seized from the animals and placed in a Buen fixative for a day and after standard histological processing the tissue was paraffin-embedded [10]. Slices of the liver 5 microns thick were made on a rotary microtome. The method of polymerase chain reaction with reverse transcription in real time (RT-PCR) was used to assess the state of expression of iNOS and eNOS mRNAs. Molecular-genetic research included several stages. Tissues were dewaxed by incubation in two successive xylene baths for 5 minutes each, and then in two successive baths of 100% ethanol for 5 minutes each. After dewaxing and centrifugation, the precipitate was dried in air to remove residual ethanol. Total RNA was isolated from rat tissue using the Trizol RNA Prep 100 kit (IZOGEN, Russia), which contains the following reagents: Trizol reagent and ExtraGene E. RNA was isolated according to the kit protocol. For reverse transcription (synthesis to DNA), we used the "Reagent kit for reverse transcription (OT-1)" (SINTOL, Moscow). The preparation and conduct of the reaction were carried out according to the set protocol.

Real-time polymerase chain reaction. To determine the level of expression of the studied genes, we used a CFX96 ™ Real-Time PCR Detection Systems amplifier (Bio-Rad Laboratories, Inc., USA) and a set of reagents for PCR-RT in the presence of SYBR Green R-402 (Syntol, Russia) The final reaction of the amplification mixture included SYBR Green dye, SynTaq DNA polymerase with antibodies that inhibit enzyme activity, 0.2 μl of direct and reverse specific primers, dNTP-deoxynucleosidetriphosphates, 1 μl of matrix (cDNA). The reaction mixture was brought to a total volume of 25 μl by the addition of deionized H2O.

Specific primer pairs (5'-3 ') for analysis of the studied and reference genes were selected using PrimerBlast software (www.ncbi.nlm.nih.gov/tools/primer-blast) and manufactured by ThermoScientific, USA. Amplification occurred under the following conditions: initiated denaturation 95°C - 10 min; further 50 cycles: denaturation - 95°C, 15 sec., annealing of primers - 58-63°C, 30 sec., elongation - 72°C, 30 sec. The fluorescence intensity was recorded automatically at the end of the elongation stage of each cycle via the SybrGreen channel automatically. Actin beta (Actb) was used as a reference gene to determine the relative value of the change in the expression level of the studied genes.

Significance of differences among the experimental groups was performed using the nonparametric Mann-Whitney U-test. Differences with a significance level of more than 95% ($p < 0.05$) were considered significant. The research results were processed using the statistical package of the licensed program "STATISTICA for Windows 6.1" (StatSoft Inc., No. AXX R712D833214SAN5), as well as "SPSS 16.0", "Microsoft Excel 2003".

Results. Analyzing the data presented in tables 1 and 2 characterizing the expression of eNOS and iNOS mRNA in the liver of rats undergoing chronic alcoholization, the following was established. Expression of eNOS mRNA in treated groups is higher than control values, but lower than intact values, and iNOS mRNA expression in treated groups is lower than or approaches control (intact) values, significantly lower than control values. Thus, it can be concluded that on the 30th day after violent 60-day alcoholization the nature of the expression of NOS mRNA in the liver changes - the expression of endothelial nitric oxide mRNA is inhibited and the expression of inducible NO synthase mRNA is significantly increased. The data obtained are in line with the modern concept of liver damage in alcoholism, which is consistent with our previous studies, which demonstrate the formation of endothelial dysfunction of the blood vessels of the liver in chronic alcohol alcoholization [11-13]. The obtained data on the increase in the expression of iNOS mRNA in rat liver on the 30th day after chronic alcoholization does not contradict the data of other researchers, and our previous studies, which show an increase in the expression of this NOS isoform in target organs: heart, kidneys, brain in case of alcohol disease [14-16]. An increase in the activity and expression of iNOS in response to alcohol damage to target organs occurs on the first day and is associated with activation of oxidative stress and inflammation [15].

Table 1

Indicators of eNOS mRNA expression in rat liver after 60-day CAI and 30 days of treatment

Group of animals	ENOS mRNA expression, c.u.
Intact	1,00±0,0271
Control (CAI)	0,01±0,00112*
CAI+ LEYH	0,222±0,011*[1]
CAI+ Hepabene	0,021±0,00182*[+]

Note: * - p≤0.05 in relation to intact;
1 - p≤0.05 in relation to the control
+ - p≤0.05 in relation to the Hepabene group

Table 2

Indicators of iNOS mRNA expression in rat liver after 60-day CAI and 30 days of treatment

Group of animals	INOS mRNA expression, c.u..
Intact	1,00±0,271
Control (CAI)	5,127±0,0115*
CAI+ LEYH	3,112±0,0022*1+
CAI+ Hepabene	4,775±0,0122*

Note: * - p≤0.05 in relation to intact;
1 - p≤0.05 in relation to the control
+ - p≤0.05 in relation to the Hepabene group

Course administration of LEYH to animals with HAI led to a significant decrease in the liver of iNOS mRNA expression relative to control values by 39,3%. However, iNOS mRNA values in the liver of rats receiving LEYH were 3 times higher than intact values. Also, the introduction of LEYH led to an increase in the expression of eNOS mRNA in the liver of rats with CAI by 222% relative to the control group. However, the values of eNOS mRNA in the liver of rats receiving LEYH were 4.5 times lower than the values of intact. The introduction of Hepabene had a less pronounced effect in relation to the effect on the indices of iNOS mRNA and eNOS mRNA. An increase in the expression of endothelial synthase of nitric oxide and a decrease in the expression of inducible synthase of nitric oxide can be regarded as a manifestation of the hepatoprotective effect of LETP. It is known that a low level of NO due to insufficient eNOS activity under conditions of alcoholization leads to the formation of liver vascular endothelial dysfunction, a decrease in the detoxifying function of the liver, and inhibition of proliferation and reparative regeneration [11]. Increased expression of iNOS mRNA can be considered as a manifestation of NO-dependent mechanisms of alcohol damage to the liver [16]. It is known that chronic alcohol intoxication in rats leads to the activation of oxidative and nitrosating stress, as evidenced by the increase in protein nitrosylation products [6,16,17]. Free radicals attack proteins along the entire length of the polypeptide chain, disrupting not only the primary, but also the secondary and tertiary structure of proteins, which leads to aggregation or fragmentation of the protein molecule [18]. A similar effect of LEYH on eNOS mRNA iNOS mRNA indices is associated with antioxidant properties and the ability to inhibit hyperproduction of ROS by mitochondrial bioenergy systems, and also, possibly, by interrupting ROS-dependent mechanisms of IL-1b and TNF-a expression [14].

Conclusions.

1. The course administration of LEYH led to a change in the expression of iNOS mRNA and eNOS mRNA to varying degrees of severity in the liver of rats with chronic alcohol intoxication.
2. The detected decrease in the expression of iNOS in animals with HAI when using LEYH indicates the implementation of the antioxidant properties of the drug.
3. The detected increase in the expression of eNOS with the course of administration of LEYH can be regarded as a manifestation of the endothelioprotective effect of the drugs.
4. The degree of influence on the expression of iNOS mRNA and eNOS mRNA LEYH significantly exceeds the reference drug Hepabene.

References:

1. Peter J. S. Alcohol and Nitric Oxide Production by Cells of the Brain / J. S. Peter // Alcohol. - 1998. - № 16(2). - P. 159 – 165.
2. Deng X.S. Ethanol metabolism and effects: nitric oxide and its interaction / X.S. Deng, R.A. Deitrich // Curr. Clin. Pharmacol. - 2007. № 2 (2). - P. 145 - 153.

3. Egorov A.N. The role of nitrosating stress in the mechanisms of neurodegradation under conditions of prenatal alcohol intoxication and ways of pharmacocorrection of emerging disorders / A.N. Egorov, I.F. Belenichev, E.P. Sokolik, L.I. Kucherenko // Zaporizhzhya Medical Journal. - 2012. - No. 5. - p. 25 - 28.

4. Inoue M. Cross-talk between NO and oxyradicals, a supersystem that regulates energy metabolism and survival of animals / M. Inoue, E.F.Sato, A.M. Park // Free Radic. Res. - 2000. - Vol. 33. - P. 757 - 770.

5. Kharchenko O. Long-term alcohol consumption provokes oxidative and nitrosative stress in albino rats brain / O. Kharchenko // Pharmacology and oxicology. - 2015. - No. 3. - p. 49 - 54.

6. Belenichev I. F. Nitrosing stress in the brain of prenatally alcoholized rats: effects of Tiocetam / I. F. Belenichev, L. I. Kucherenko, A. N. Egorov, I. A. Mazur, N. V. Bukhtiyarova, E P. Sokolik // Pathology. - 2012. - No. 3. - p. 121.

7. Belenichev I.F. Thiol-disulfide system: role in endogenous cyto- and organoprotection, pharmacological modulation pathways / Belenichev I.F., Feroz Shah., Chekman I.S., Nagornaya E.A., Gorbacheva S.V., Gorchakova N.A. , Bukhtiyarova N.V., Reznichenko Yu.G. - Kiev: "LLC Publishing House " Euston ", 2020.-232p.

8. I.F. Belenichev, Influence of thiol antioxidants on the state of nitrosating stress in the brain of rats subjected to chronic alcohol intoxication / I.F. Belenichev, T.V. Kucher // Pharmacology and drug toxicology. - 2016. - No.2 (48). - p. 24 - 29.

9. Duyun I.F. Experimental study of hepatoprotective and antioxidant activity of Achillea micranthoides Klok herb extract. Et Krytzka / I.F. Duyun, A.V. Mazulin, I.F. Belenichev, A.V. Abramov // Pharmacology and drug toxicology.-2019.-Vol.13, № 1.-P.51-57.

10. Korzhevsky D.E. Fixation of material for histological examination. In: Morphological diagnostics: preparation of material for morphological examination and electron microscopy. Ed. Korzhevsky D.E. St. Petersburg; 2013; P. 10-25

11. Michel Th. Cellular signaling and NO production / Th. Michel, P. M. Vanhoutte // Pflugers Arch. - 2010. - № 459. - P. 807 - 816.

12. Zenkov N.K. NO synthase is normal and with pathology of different genesis. / N.K. Zenkov, E.B. Menshchikov, V.P. Reutov // Bulletin of the Academy of Medical Sciences of Ukraine. - 2000. - № 3. - p. 30 - 35.

13. Förstermann U. Nitric oxide synthases: regulation and function / U. Förstermann, W.C. Sessa // Eur. Heart J. - 2012. - Vol. 33, № 7. - P. 829 - 837.

14. Belenichev I.F. Neuroprotection and neuroplasticity: Monograph / I.F. Belenichev, V.I. Cherny, E.A. Nagornaya, S.V. Pavlov, N.V. Buchtiarova. - Kiev: Logos, 2015. - 512 p.

15. Kimura K.A. Hippocampal nitric oxide synthase in the fetal guinea pig: effects of chronic prenatal ethanol exposure / K.A. Kimura, J.F. Brien // Brain Res. Dev. Brain Res. - 1998. - № 106(1-2). - P. 39 - 46.

16. Spanagel R. The neuronal nitric oxide synthase gene is critically involved in neurobehavioral effects of alcohol / R. Spanagel, S. Siegmund [et al.] // J. Neurosci. - 2002. - № 22(19). - P. 8676 - 8683.

17. David M. L. Synaptic Effects Induced by Alcohol / M. L. David, R. Marisa // Curr Top BehavNeurosci. - 2013. - № 13. - P. 31 - 86.

18. Zhang F., Lau S. S., Monks T. J. The cytoprotective effect of N-acetyl-L-cysteine against ROS-induced cytotoxicity is independent of its ability to enhance glutathione synthesis //Toxicological Sciences. – 2011. – T. 120. – №. 1.- C. 87-97

19. Ikeda M. Neuronal nitric oxide synthase is resistant to ethanol / M. Ikeda, T. Komiyama, I. Sato [et al.] // Life Sci. - 1999. - №64(18). - P. 1623 - 1630.

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