Research Article

Correction of metabolic disorders in experimental chronic ethanol intoxication

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ABSTRACT

Purpose: The purpose of the study was to study the corrective effects of pharmacological agents and allogeneic hepatocyte proteins (AHP) on metabolic disorders caused by chronic ethanol intoxication (CAI). **Materials and Methods:** Studies were conducted on 110 male Wistar rats. CAI was caused through intragastric daily administration of 20% ethanol solution at a dose of 3 ml/kg within 60 days. Allogeneic hepatocyte proteins (AHPs) were isolated from the culture fluid of hepatocytes obtained from newborn animals by M.N. Berry, D.S. Friend method. The obtained AHPs were intraperitoneally administered 5 times (with a 24-h interval) to the rats with CAI at the rate of 5 mg/kg of protein starting from the 55th day of ethanol intoxication. Pharmacological preparations with immunomodulatory, antioxidant, and hepatoprotective activity were administered in accordance with the instructions for their use. **Results:** Due to CAI, the following signs developed: Cytolytic, cholestatic, cytotoxic, immune-inflammatory syndromes, insufficiency of synthetic processes, oxidative stress, imbalance of the functional and metabolic activity of erythrocytes, and neutrophils of peripheral blood. The combined introduction of Longidasa, Mexicor, and Essentiale Forte H normalized to 11.1% and corrected 88.9% of the studied laboratory parameters. The combined use of Glutoxim, Mexidol, and Heptral normalized and corrected 22.2% and 77.8% of the indicators, respectively. The most effective one turned out to be the introduction of AHP – normalized and corrected 70.4% and 29.6% of the altered laboratory parameters, respectively. **Conclusion:** A promising direction for further research is the purification of the culture fluid proteins of allogeneic hepatocytes with the release of the protein essence of the active substance, aimed at creating a medicine on this basis.

KEY WORDS: Allogeneic hepatocyte proteins, Chronic ethanol intoxication, Metabolic disorders

INTRODUCTION

According to the World Health Organization data, 5.3% of deaths in the world are directly related to alcohol. This means that every year the effects of alcohol consumption kill up to 3 million people, and 1 of 20 people dies of the pathology associated with alcohol abuse.[1]

Prolonged administration of ethanol is known to result in toxic damage to organs and systems, mainly the liver, pancreas, immune, and cardiovascular systems, which is manifested by numerous metabolic disorders.[2-4]

Proportional relationship between the duration of ethanol intake and changes in the erythrocyte membrane proteins, which are responsible for the structure formation, stabilization, shaping, membrane flexibility, and intracellular metabolism of circulating erythrocytes, was revealed. Changes in the content and ratio of lipid fractions responsible for the lipid framework and ordering of protein macromolecules of erythrocyte membrane lead to significant dysfunction of erythrocyte properties.[5]

Prolonged ethanol introduction increases the sensitivity to peroxidation processes, which ultimately leads to changes in the physicochemical properties and dysfunction of cells in various organs and systems, but hepatocytes have the highest sensitivity to the effects of alcohol since they are the first in the way of the toxicant and responsible for ethanol metabolism. The formation of a highly reactive acetaldehyde intermediate contributes to the activation of free radical processes resulting in damage to their cell membranes.[4]

Considering the above mentioned, problems of reducing the effects of metabolic disorders due to

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Website: jprsolutions.info | ISSN: 0975-7619

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Received on: 07-07-2019; Revised on: 11-08-2019; Accepted on: 15-09-2019

chronic ethanol intoxication by means of pathogenetic correction methods require further study, both in clinic and in the experiment.[6,7]

The aim of the study was to study the corrective effects of pharmacological preparations and allogeneic hepatocyte proteins on metabolic disorders caused by chronic ethanol intoxication.

MATERIALS AND METHODS

The experiments were carried out on 85 healthy adult male Wistar rats weighing 150–200 g. Besides, 25 allogeneic hepatocyte donors were used in 5–6 days after birth. All the studies were performed at the same time from 8 to 12 o'clock. The animals were kept and slaughtered in accordance with the principles set forth in the Convention for the Protection of Vertebrate Animals used for experimental and other purposes (Strasbourg, France, 1986), and according to the rules of laboratory practice of the Russian Federation (The Decree of the Ministry of Health of the RF No. 267 from 06/19/2003). Chronic alcohol intoxication (CAI) was modeled by forced intragastric introduction of 20% ethanol solution at a dose of 3 ml/kg (2.92 g/kg) in 24 h within 60 days.

During the experiment, the animals were divided into five groups: the 1st group (control) – healthy rats; the 2nd group – CAI; the 3rd group – CAI and the introduction of "Longidasa" (100 IU, in 48 h, No. 10), "Mexicor" (50 mg/kg, intraperitoneally, in 24 h, No. 15), and "Essentiale Forte H" (5 mg in terms of phosphatidylcholine, dissolved in 1 ml of olive oil, intragastrically, in 24 h, No. 15); the 4th group – CAI and administration of "Glutoxim" (20 mg/kg, intramuscularly, No. 10), "Mexidol" (50 mg/kg, intramuscularly, No. 15), and "Heptral" (760 mg/kg, intraperitoneally, No. 10); and the 5th group – CAI and the introduction of allogeneic hepatocyte proteins (AHPs).

Dosages calculation for the introduction to experimental animals was performed with the use of dose conversion factors (mg/kg per mg/m^2) for rats and humans depending on body weight.

The excretion of allogeneic hepatocytes from animals in 5–6 days after birth was performed according to M.N. Berry, D.S. Friend method,[1] for this purpose, after the liver sampling, it was fragmented, hepatocytes were removed from tissue by squeezing with the use of a glass homogenizer in culture medium 199. The obtained cell suspension was washed out twice by centrifugation for 10 min at 400 g, diluted in medium 199 and the number of cells was counted. Their viability was determined in a test with trypan blue, while in further experiments, cell suspensions containing more than 90% of viable cells were used. During all the manipulations with the cell suspension, the temperature of the used medium 199 was 36–37°C.[8,9]

To obtain the culture fluid of allogeneic hepatocytes in medium 199, 5×10^7 cells were cultured in 3 ml of medium within 6 h. When the incubation period expired, the cells were precipitated by centrifugation (15 min at 400 g). To obtain proteins from 50 to 100 ml of culture liquid, they were precipitated with an equal volume of 10% triacetic acid, and the precipitate formed was isolated by centrifugation for 20 min at 1500 g. When the supernatant was isolated, the precipitate was diluted in 0.9% sodium chloride solution and dialyzed in two shifts in phosphate-buffered saline pH 7.2–7.4 for 18 h. Having determined the protein concentration and bringing it with 0.9% sodium chloride solution to 5 mg/ml, the resulting solution was filtered through 0.2 μm sterilization membranes, packed in sterile vials by 2 ml and lyophilized by means of VIRTIS freeze dryer. The obtained AHPs were administered 5 times (with a 24-h interval) intraperitoneally to the rats with CAI at the rate of 5 mg/kg of protein starting with the 55th day of ethanol intoxication.[10,11]

The rats were drawn out of the experiment in 24 h following the last injection of ethanol preparations or AHP.

Blood sampling in experimental animals was carried out under anesthesia, by intracardiac injection. Plasma was obtained from heparinized blood through centrifuging for 5 min at 400 g. To assess the liver function in the plasma, the activity of aspartate and alanine aminotransferase (AST and ALT), alkaline phosphatase (ALP), gamma-glutamine transpeptidase (GGT), bilirubin, fibrinogen, prothrombin index (PTI), and thymol sample was determined. The values of all these indicators were assessed by unified methods using standard reagent kits. Liver enzymes activity was evaluated on an automatic biochemical analyzer Vitalab Flexor E (Netherlands) with Analyticon® Biotechnologies AG reagents (Germany). Fibrinogen content was determined on a semi-automatic analyzer of hemostasis indicators STart4 (France) with Diagnostica Stago reagents (France).[12,13]

Lipid peroxidation (LPO) intensity was assessed by acylhydroperoxides (AHP) and malondialdehyde (MDA) content in plasma and erythrocytes using the TBK-Agat kit ("Agat-Med" Russia) with Apel-330 spectrophotometer (Japan) at a wavelength of 535 nm and 570 nm. By the method of direct/competitive heterogeneous enzyme-linked immunosorbent assay with the detection of reaction products in 405–630 wavelength range, the state of the antioxidant system was evaluated using the following kits: Superoxide dismutase (SOD) activity by "Bender Medsystems" (Austria) and catalase by Cayman Chemical (USA).

Total antioxidant activity was determined by the method based on the degree of ascorbate inhibition and tween-80 ferro-induced oxidation to MDA. The level of nitric oxide stable metabolites was detected using the "R&D" kit (England) for solid-phase EIA. Registration of all EIA results was carried out using a microplate photometer "Sunrise," Tecan (Austria).

After plasma isolation, packed red blood cells were settled in 20 ml of 10 mM Na-phosphate buffer (pH = 7.4) containing 0.9% sodium chloride and 3% dextran T-500 for 30 min at 37°C. After centrifugation, the supernatant liquid was removed by aspiration, and packed red blood cells were subjected to additional purification on a chromatographic column through Hemoglobin-cellulose, after which the sorption capacity of erythrocytes (SCE) and sorption capacity of their glycocalyx (SCG) were determined.[14]

Neutrophils isolation from the obtained blood was performed on Ficoll-Urografin density gradient (ρ = 1.078). Their phagocytic activity was assessed by phagocytic index (PI), phagocytic number (FN), and phagocytes activity index (PAI). The activity of oxygen-dependent neutrophil systems was assessed by the reduction reaction of nitroblue tetrazolium (NBT test), spontaneous, and zymosan stimulated (NBT-sp. and NBT-st.).[5]

Statistical processing of the research results was carried out with the calculation of mean values (M), mean arithmetic error (m) using the computer software package Microsoft Excel, 2010. The significance of the differences was evaluated by Mann–Whitney U-test. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Prolonged supply of ethanol in experimental animals causes the development of biochemical syndromes of the liver damage: Cytolysis (increased AST and ALT activity), toxic damage (increased ratio of AST/ALT enzymes [De Ritis] and GGT/AST), intracellular cholestasis (increased activity of ALP, GGT, and bilirubin concentration), insufficiency of synthetic processes (decrease in PTI and fibrinogen concentration), and immunoinflammatory (increase in thymol sample). Combined introduction of "Longidasa," "Mexicor," and "Essentiale Forte H" corrects toward the control animals, but not to their values, all the studied parameters. The combined use of "Glutoxim," "Mexidol," and "Heptral" in comparison with the previous group does not affect the activity of ALP, normalizes the level of fibrinogen, and corrects to a greater extend the other laboratory parameters. The introduction of AHP in the presence of CAI turned out to be the most effective one, as in comparison with pharmacological preparations, it

Table 1: Functional activity of hepatocytes under prolonged ethanol intoxication; correction of disorders (M±m)

Indicators	Units of measure	1	2	3	4	5
		control	Ethanol intoxication			
			-	Introduction of "Longidasa," "Mexicor," and "Essentiale Forte H"	Introduction of "Glutoxim," "Mexidol," and "Heptral"	Introduction of AH proteins
AST	U/p•l	20.9±1.6	41.9±3.1*[1]	31.4±2.17*[1,2]	34.73±2.1*[1-3]	24.3±2.1*[1-4]
ALT	U/p•l	19.8±1.1	32.2±2.9*[1]	25.7±2.2*[1,2]	24.16±1.9*[1-3]	23.9±1.2*[1-4]
De Ritis ratio, AST/ALT		1.06±0.05	1.3±0.03*[1]	1.18±0.05*[1,2]	1.44±0.03*[1-3]	1.02±0.2*[2-4]
GGT	U/p•l	4.7±0.3	19.9±2.4*[1]	12.8±1.42*[1,2]	12.05±1.4*[1,2]	8.7±1.4*[1-4]
GGT/AST		0.23±0.01	0.46±0.03*[1]	0.42±0.03*[1,2]	0.35±0.03*[1-3]	0.36±0.03*[1-4]
ALP	U/p•l	241.2±17.1	338.8±13.0*[1]	336.6±13.8*[1,2]	358.07±13.8*[1-3]	252.6±19.1*[2-4]
Bilirubin	mcmol/l	5.6±1.2	13.5±1.2*[1]	8.81±1.03*[1,2]	8.02±0.6*[1-3]	7.7±0.4*[2-4]
PTI	%	64.7±1.7	62.3±1.8*[1]	58.0±3.66*[1,2]	59.62±3.06*[1-3]	65.0±2.8*[2-4]
Fibrinogen	g/l	3.2±0.1	2.7±0.1*[1]	3.8±0.2*[1,2]	3.22±0.18*[2,3]	3.1±0.2*[2,3]
Thymol test	U. S-H	0.59±0.02	2.2±0.1*[1]	3.13±0.02*[1,2]	3.09±0.03*[1-3]	0.94±0.03*[1-4]

In this table and in Tables 2-3, the gear indicates significant differences in arithmetic means ($P<0.05$); the numbers next to the gear show in relation to the indicators of which group these differences are given

normalizes the De Ritis ratio, the activity of ALP, PTI, and fibrinogen concentration and corrects the other studied parameters of hepatocytes functional activity as much as possible [Table 1].

60-day-long force introduction of ethanol caused in experimental animals; the activation of LPO processes (increased concentrations of MDA and AHP in blood plasma) and the development of oxidative stress (a decrease in antioxidant protection factors: TAS, catalase activity, and SOD). Besides, a decrease in CM_{NO} level was revealed. The combined use of "Longidasa," "Mexicor," and "Essentiale Forte H" normalizes TAS and brings to norm, but not to the parameters of control animals, the other studied biochemical parameters of LPO, oxidative stress, and CN_{NO} content. The introduction of "Glutoxim," "Mexidol," and "Heptral" turned out to be more effective in comparison with the previous preparations, as it corrected to a greater extent the concentration of LPO products, catalase activity, and CM_{NO}. The use of AHP, in comparison with the previous group, additionally normalized LPO products and catalase activity [Table 2].

When evaluating the metabolic activity of circulating blood erythrocytes in group with CAI there was an increase in POL products (MDA, AHP), a decrease in the activity of antioxidant protection enzymes (SOD, catalase) and sorption properties of red blood cell membrane (SCE, SCG) in comparison with the control group. The administration of "Longidasa," "Mexicor," and "Essentiale Forte H" normalizes catalase activity and corrects the other studied parameters of red blood cell metabolic activity towards the norm. Combined introduction of "Glutoxim," "Mexidol," and "Heptral" if compared with "Longidasa," "Mexicor," and "Essentiale Forte H," normalized the level of MDA and corrected erythrocytes sorption capacity (SCE and SCG) to an even greater degree. The use of AHP in comparison with pharmacological preparations corrected to a greater extent the concentration of AHP and normalized the rest of studied erythrocytes parameters [Table 3].

As for functional metabolic activity of peripheral blood neutrophils, a decrease in their phagocytic activity (decrease in PI, PN, and PAI) was observed with the activation of oxygen-dependent metabolism (increase in NBT spontaneous and zymosan stimulated). The use of "Longidasa," "Mexicor," and "Essentiale Forte H" normalizes PN and brings closer to the parameters of control animals, but not to their level, the other indicators of neutrophils functional metabolic activity. Combined introduction of "Glutoxim," "Mexidol," and "Heptral" additionally normalized the NBT-sp. and corrected the other neutrophil parameters studied. AHP proved to be the most effective ones since their introduction normalizes both the phagocytic- and oxygen-dependent activities of circulating blood polymorphonucleocytes [Table 3].

Thus, long-term intoxication with ethanol leads to the shift in the normal parameters in 100.0% of the studied metabolism indicators. The obtained data allow us to conclude that in animals with CAI, the development of the main biochemical syndromes of the liver damage is observed: They are cytolytic, intracellular cholestasis, toxic damage by immunoinflammatory type, and insufficiency of synthetic processes. Besides, under these conditions, "oxidative stress" and impaired functional-metabolic activity of erythrocytes and peripheral blood neutrophils develop. When the combination of "Longidasa," "Mexicor," and "Essentiale Forte H" was introduced, 11.1% of the studied laboratory parameters were normalized and 88.9% were corrected. The use of "Glutoxim," "Mexidol," and "Heptral" turned out to be more effective, as it is normalized and corrected 22.2% and 77.8% of the indicators, respectively. AHP proved to be the most effective ones since their administration normalized and corrected 70.4% and 29.6% of the altered laboratory parameters, respectively.

CONCLUSION

Relying on the obtained data, the introduction of various combinations of preparations with immunomodulatory, antioxidant, and hepatoprotective

Table 2: Correction of metabolic disorders in parameters of blood serum under prolonged ethanol intoxication (M±m)

Indicators	Units of measure	1	2	3	4	5
		Control	Ethanol intoxication			
			-	Introduction of "Longidasa," "Mexicor," and "Essentiale Forte H"	Introduction of "Glutoxim," "Mexidol," and "Heptral"	Introduction of AH proteins
MDA	micromole/l	2.35±0.05	6.2±0.3*[1]	4.24±0.1*[1,2]	3.0±0.17*[1-3]	2.4±0.2*[2-4]
AHP	RU	0.27±0.02	0.71±0.03*[1]	0.42±0.02*[1,2]	0.31±0.02*[1-3]	0.26±0.02*[2-4]
TAS	%	44.2±2.0	39.1±1.1*[1]	42.7±1.2*[2]	43.67±1.1*[2]	44.7±2.2*[2]
SOD	RU/ml	11.3±0.6	6.9±0.3*[1]	8.9±0.8*[1,2]	9.33±0.7*[1,2]	8.9±0.8*[1,2]
Cat.	mkat/l	14.2±0.7	9.4±0.5*[1]	12.0±0.4*[1,2]	13.4±0.3*[1-3]	14.7±0.6*[2-4]
CM_{ON}	micromole/l	6.9±0.3	4.1±0.2*[1]	4.7±0.3*[1,2]	5.6±0.2*[1-3]	5.7±0.5*[1-3]

Table 3: Functional metabolic activity of erythrocytes and polymorphonucleocytes in the presence of long-term intoxication with ethanol; correction of disorders (M±m)

Indicators	Units of measure	1	2	3	4	5
		Control	Ethanol intoxication			
			-	Introduction of "Longidasa," "Mexicor," and "Essentiale Forte H"	Introduction of "Glutoxim," "Mexidol," and "Heptral"	Introduction of AH proteins
MDA	micromole/l	0.45±0.06	0.98±0.05*[1]	0.7±0.03*[1,2]	0.44±0.02*[2,3]	0.49±0.04*[2,3]
AHP	RU	0.13±0.01	0.43±0.03*[1]	0.21±0.02*[1,2]	0.22±0.03*[1,2]	0.17±0.02*[1-4]
SOD	RU/ml	29.1±1.3	17.6±1.3*[1]	21.2±0.9*[1,2]	22.1±1.3*[1,2]	30.3±1.3*[2-4]
Catalase	mkat/l	10.9±0.6	6.2±0.2*[1]	10.5±0.7*[2]	11.3±0.7*[2]	10.8±0.7*[2]
Sorption capacity of erythrocytes	%	53.5±1.1	27.2±2.1*[1]	41.5±2.2*[1,2]	48.9±1.3*[1-3]	49.2±2.3*[2,3]
Sorption capacity of their glycocalyx	10^{12}g/er	2.8±0.1	1.3±0.1*[1]	2.0±0.08*[1,2]	2.5±0.04*[1-3]	2.9±0.1*[2-4]
Phagocytic index	%	74.6±1.7	56.2±3.0*[1]	67.6±1.4*[1,2]	70.8±1.3*[1-3]	72.5±2.1*[2,3]
PN	abs.	2.9±0.08	2.0±0.03*[1]	2.83±0.1*[2]	2.85±0.1*[2]	3.0±0.1*[2]
Phagocytes activity index	—	2.16±0.04	1.12±0.02*[1]	1.91±0.05 *[1,2]	2.0±0.02*[1-3]	2.18±0.02*[2-4]
NBT-sp.	mOD	0.74±0.03	1.85±0.8*[1]	0.93±0.03*[1,2]	0.78±0.03*[2,3]	0.72±0.04*[2,3]
NBT-st.	mOD	1.35±0.4	2.8±0.1*[1]	1.88±0.03*[1,2]	1.76±0.02*[1-3]	1.28±0.02*[2-4]

activity, as well as the administration of culture fluid proteins of allogeneic hepatocytes to the recipients with CAI, limits free radical oxidation processes; systemic inflammatory response at the level of innate immune mechanisms has significant positive effects on restoration of functional activity of hepatocytes and endoglobular metabolism. Taking into account, the revealed disorders under chronic ethanol intoxication (membrane-damaging effects, oxidative stress, activation of LPO, disturbance of innate immunity mechanisms, and metabolic activity of circulating erythrocytes), to other more effective combinations of immunomodulatory agents, antioxidants, and membrane protectors are possible in future to correct the damage. The most promising continuation, in our opinion, is the further purification of culture fluid proteins of hepatocytes with the release of a concentrated source of protein-based nature followed by further development of some preparation and its preclinical testing on various experimental models, first of all, liver pathology.[15,16]

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Source of support: Nil; Conflict of interest: None Declared