

Denefil O. V. The rates of middle molecules in the blood in rats with different motor activity in alcoholic and non alcoholic experimental hepatitis. Journal of Education, Health and Sport. 2021;11(12):385-395. eISSN 2391-8306. DOI <http://dx.doi.org/10.12775/JEHS.2021.11.12.032> <https://apcz.umk.pl/JEHS/article/view/JEHS.2021.11.12.032> <https://zenodo.org/record/5834338>

The journal has had 5 points in Ministry of Science and Higher Education parametric evaluation. § 8. 2) and § 12. 1. 2) 22.02.2019.
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The authors declare that there is no conflict of interests regarding the publication of this paper.

Received: 12.11.2021. Revised: 30.11.2021. Accepted: 28.12.2021.

THE RATES OF MIDDLE MOLECULES IN THE BLOOD IN RATS WITH DIFFERENT MOTOR ACTIVITY IN ALCOHOLIC AND NON ALCOHOLIC EXERIMENTAL HEPATOSIS

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Abstract

Alcohol use disorders affect millions of individuals worldwide. The impact of these facts lies in the elevated social and economic costs. Liver metabolizes 75-98 % of ethanol that enters the organism. If the level of alcohol in the liver cells exceeds its degradation rate, alcoholic liver disease develops.

The aim of the study was to determine the peculiarities of hepatotoxicity after simulation of of ethanol hepatitis and non-alcoholic steatohepatitis of high and low-motor active rats.

Material and methods of investigation. The experiments were performed on on 72 white outbred male rats. The animals were divided into three groups: control, non-alkoholic hepatitis (NAH), ethanol hepatitis (EH). Each of group was subdivided – animals with high and low-motor activity (HA and LA). Contents of middle-mass molecules (MMM) were determined in the blood serum.

Results. The analysis of the results shows that the levels of MMM in blood serum are increasing. Dystrophic changes that appear in a consequence of hypoxia are noted in NAH and EH. Both morphological and biochemical changes were more significant in HA animals. The degree of MMM accumulation depends on the severity of the pathology and motor

activity of the animals. Less activity of MMM₂₃₈ is observed in HA rats, which can be explained by the development of multi-organ pathology.

Conclusions. During our investigations it was found the significant increasing of MMM levels in blood serum in rats with ethanol hepatosis and nonalkoholic hepatitis. The accumulation of MMM is not only a marker of endotoxication, they also increase the course of the pathological process, acquiring the roles of secondary toxins, affect the viability of all organs and systems. The degree of MMM accumulation depends on the moto activity of the animals and simulated pathology and is more significant in highly active animals, compared with low-active in the ethanol using.

Less activity of MMM₂₃₈ is observed in HA rats with EH, which can be explained by the development of multi-organ pathology, in particular, renal impairment. Morphological investigations showed that the grade of liver injury was more significant in HA rats.

Key words: ethanol; liver; middle-mass molecules; rats; motor activity.

Introduction. Alcohol use disorders and alcohol dependency affect millions of individuals worldwide. The impact of these facts lies in the elevated social and economic costs. Alcoholic liver disease is caused by acute and chronic exposure to ethanol which promotes oxidative stress and inflammatory response. Chronic consumption of ethanol implies liver steatosis, which is the first morphological change in the liver, followed by liver fibrosis and cirrhosis [1].

The ICD-10 (International Classification of Disease 10th Revision) has reported eight causes of death related to alcohol consumption (mouth and oropharynx cancer, alcohol use disorders, ischemic heart disease, liver cirrhosis, road traffic accidents, poisonings, falls, intentional injuries). Most of these causes are related to the amount and time of exposure to alcoholic beverages ¹. According to the World Health Organization, the average alcohol consumption per capita in adults is 5.1 liters per year, though mean consumption varies worldwide. While in some countries, like Saudi Arabia, Iran, Somalia alcohol consumption (liters per capita per year) is 0.0, in other countries such as Luxembourg the consumption reaches 17.54, in Uganda is 19.47 [2].

Long-term and regular use of alcoholic beverages remains one of the problems for humanity. In recent years alcohol addiction (worldwide and particullary in Ukraine) is characterized by considerable spread and rejuvenation. A trend towards lowering the age of people, who consume alcohol, feminization (increasing the number of teenage girls,

consuming alcoholic beverages) and the potentially severe effects of alcohol consumption are nowadays relevant to society.

WHO experts consider the situation as “dangerous” if the dose of alcohol per per capita exceeds 8 liters per year. However, the total score of consumption of alcoholic beverages in our country is 12-15 liters per capita [2]. Liver works as a huge detoxification center in the body since it metabolizes 75-98 % of ethanol that enters the organism. If the level of alcohol in the liver cells exceeds its degradation rate, alcoholic liver disease develops [3].

Alcoholic liver disease includes a broad spectrum of disorders, such as simple steatosis, cirrhosis, acute alcoholic hepatitis with or without cirrhosis, and hepatocellular carcinoma as a complication of cirrhosis. Alcoholic liver disease can also run in conjunction with other common liver diseases, including nonalcoholic liver disease and hepatitis C virus infection, accentuating their prevalence and severity. A large French study looked at 1,604 biopsies on patients admitted for alcoholism or alcoholic liver disease and found normal liver, 14%; steatosis without fibrosis, 29%; some fibrosis ± steatosis, 20%; steatohepatitis without cirrhosis, 8%; cirrhosis total, 43%; cirrhosis and steatohepatitis, 13% [4].

Liver biopsy remains a useful tool to establish the diagnosis and to exclude other liver diseases remaining the only instrument, capable to provide prognostic information by staging and grading of these diseases. Alcoholic steatohepatitis is a progressive form of alcoholic liver disease and can evolve into cirrhosis. The currently accepted minimum diagnostic criteria for steatohepatitis include steatosis, lobular inflammation, and hepatocellular injury, but fibrosis must be absent. Histologically, hepatocellular injury in fatty liver disease usually occurs in the form of ballooning, but it might also be accompanied with apoptotic (acidophilic) bodies and lytic necrosis [5]. When there is a good quantity of neutrophils, diagnostic points out to an alcoholic etiology. Another mechanism seems to be sinusoidal collagen formation [1].

Alcoholic hepatitis is histologically characterized by hepatocellular injury with ballooned hepatocytes. Some of them contain steatosis, while others may contain intracellular, amorphous, eosinophilic inclusions, known as Mallory's bodies, which are often surrounded by neutrophils [6].

The characteristic pattern of fibrosis in non-cirrhotic patients with steatohepatitis follows a pericellular/perisinusoidal architecture as the result of collagen deposition in the space of Disse [7]. Perivenular fibrosis, periportal fibrosis, and cirrhosis, are typical features of alcoholic fibrosis and often coexist with the findings of alcoholic hepatitis. Biomarkers are

useful in advanced liver injury but almost useless in the diagnosis of earlier tissular changes [6].

In spite of the same alcohol consumption, the degree of liver damage and the development of alcoholic liver disease may vary from person to person. Obviously, this depends on the individual's reactivity. It is well-known that women are more vulnerable than men, children and adolescents – then adults [8]. Individual sensitivity is also present in males and it can be related to the behavior of the person, congenital emotional sensitivity, and increased anxiety [9]. Multi-year researches of ethanol consumption and effects have not fully explained most of the mechanisms of pathological processes associated with individual reactivity of the organism [1]. One of the contributing factors of individual reactivity is behavioral reactions.

Nonalcoholic fatty liver disease is a fatty infiltration of the liver in the absence of other causes of steatosis, such as alcohol consumption. It is characterized by excessive fat accumulation in the liver (hepatic steatosis). Nonalcoholic steatohepatitis is a subgroup of nonalcoholic fatty liver disease characterized by steatosis with additional findings of liver cell injury and inflammation. Hepatic steatosis and steatohepatitis can be distinguished only by liver biopsy and histology [10].

To establish the emotional sensitivity in the experiment in small animals, open field test can be used.

The open field test (OFT) is a common measure of exploratory behavior and general activity in both mice and rats, where both the quality and quantity of the activity can be measured. Principally, the open field is an enclosure, generally square, rectangular, or circular in shape with surrounding walls that prevent escape. The most basic and common outcome of interest is “movement”; however, this can be influenced by motor output, exploratory drive, freezing or other fear-related behavior, sickness, relative time in circadian cycle, among many other variables. Distance moved, time spent moving, rearing, and change in activity over time are among many measures that can be tabulated and reported. Some outcomes, particularly defecation, center time, and activity within the first 5 minutes, likely gauge some aspects of emotionality including anxiety. The OFT is also commonly used as a mechanism to assess the sedative, toxic, or stimulant effects of compounds. Thus, the OFT measures a number of facets of behavior beyond simple locomotion. As such, the test has a number of uses and is included in almost every thorough analysis of rodent behavior [11].

To determine the degree of damage, including but not limited to intoxication, biochemical analysis is used. In recent years it has been collected a significant amount of data,

indicating that in the pathogenesis of many diseases, including alcoholic intoxication, medium mass molecules (MMM) appear in blood plasma. They are of different nature, but most scientists believe that the main components of this group of compounds are peptides, glycopeptides, nucleopeptides, endorphins, derivatives of glucuronic acid, kinins, enkephalins, collagen fragments, serotonin, some humoral regulators – insulin, adrenocorticotrophic hormone, vasopressin, oxytocin, angiotensin, calcitonin, lipofuscin (intracellular complexes of lipids and proteins), atherogenically oxidized lipoproteins, some vitamins and others [12, 13].

Deep changes of metabolism, interfering biogenesis, structure and function of cells are present in the pathogenesis of alcoholism. The level of MMM in the blood has a great diagnostic value for the severity of the pathological process. The methods of MMM determination as an objective criteria for evaluation of the degree of intoxication in the organism [12] and the reflection of the level of pathological protein metabolism [13] are used in the clinical practice more and more widely. The accumulation of MMM is not only a marker of endotoxication, but it further enhances the course of the pathological process, acquiring the roles of secondary toxins, affecting the vital activity of all systems and organs [13].

Determination of the level of low-molecular fractions of proteins, so-called medium molecules, in blood serum of animals with different emotional resistance can be helpful in further investigations of the mechanisms of destructive effect of ethanol on the liver, depending on the emotional reactivity.

The aim of the study was to determine the peculiarities of hepatotoxicity after simulation of ethanol hepatitis and non-alcoholic steatohepatitis of high and low-motor active rats.

Material and methods of investigation. Experiments were carried out on 72 outbred male rats 5.5-7 months of age. The animals were divided into three experimental groups: control, non-alcoholic hepatitis (NAH) and ethanol hepatitis (EH), each of which was subdivided into two subgroups – animals with high and low-motor activity (HA and LA).

Motor activity was determined by the method of “open field”, which allows to assess the physiological response to the new situation, to identify the dynamics of changes in different states, to obtain multidirectional information about motor, research and emotional activity of animals [3]. Coincidentally with the motor component (the number of crossed squares, vertical positions with and without leaning against the wall) autonomic activity (the number of defecations, urinations, washings) and research activity (the number of viewings

into the hole, movings to the center of the field, vertical positions, and others) are also taken into account. In order to avoid artifacts animals were put in a dark, soundproofed room 10 minutes before testing.

A square arena 80x80 cm in size, with a division on 16 identical squares, with a height of side walls of 40 cm was used during the investigation. To the group of high-motor activity experimental animals we classified those that had high horizontal and vertical activity, intensively assessed all openings at the bottom of the test chamber (holes). And vice versa, to the group of low-motor activity animals were assigned those with low motor activity [3].

The animals of control group were kept on standard vivarium diet with free access to drinking water during the entire period of simulation of pathological processes.

For simulation of EH [14] the rats were put under quasi-forced alcoholization by giving 10% ethyl alcohol solution as the only drinking source diluted with 5% glucose solution for 60 days, preliminary adapting animals to ethyl alcohol by giving 5% ethanol solution (diluted with 5% glucose solution) in water troughs, as the only source of drinking for 7 days without any restrictions in food. Non-alkohol hepatitis animals were drinking only 5% glucose solution for 67 days. Animals were kept in vivarium under standard conditions with free access to food.

All experiments on experimental animals were carried out during the first half of the day in a special room at a temperature of 18-22 °C, a relative humidity of 40-60% and illumination intensity of 250 lux. The experiments were conducted in compliance with the norms of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purpose (Strasbourg, 18-March-1986), first national congress of bioethics (Kyiv, 2001) and the Order of the Ministry of Public Health of Ukraine No 690 from 23-Sep-2009.

Euthanasia of rats was performed by total bloodletting from the heart after previous thiopental-sodium anesthesia (60 mg·kg⁻¹ of body weight by intraperitoneal injection).

The levels of MMM in the blood serum at 238, 254, 260, and 280 nm wavelengths were determined by the method of Nikolaychik [15]. The principle of method is to fractionate the acid-soluble fraction of medium molecules with the subsequent detection of a ten-fold diluted supernatant at wavelengths of 254 and 280 nm [15]. All the received digital values are statistically processed, the difference between the repetitive values was determined using Student's coefficient. Liver samples were taken for the morphological investigations. Microscopic sections of the liver were painted with hematoxylin and eosin and examined under a light microscope.

Statistical processing of digital data was performed using the program "STATISTICA" 6.0 ("Statsoft", USA) [16].

Results and discussion. After ethanol injury of the liver the corresponding types of damage were detected steatohepatosis (Fig. 1, 2). The grade of liver damage was more significant in HA rats.

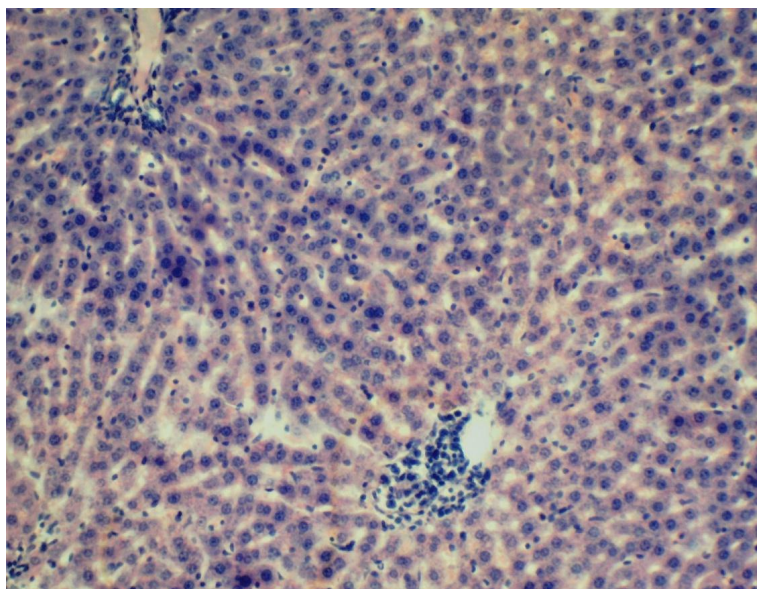


Figure 1. The liver of a rat that received 5% glucose for 67 days. The characteristic histological structure of the lobes is preserved. Some hepatocytes and their small groups contain fatty vacuoles, lymphocytic infiltration. Staining with hematoxylin and eosin. $\times 100$.

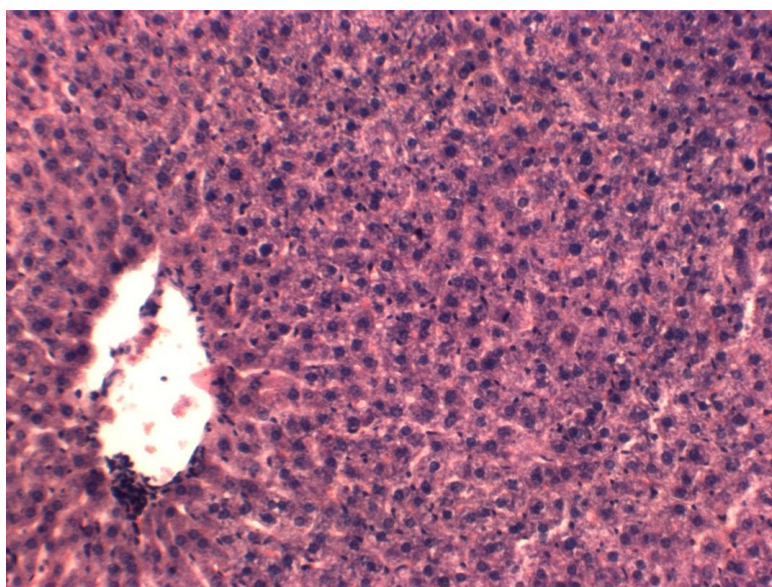


Figure 2. Fragment of the liver of a rat with low motor activity with ethanol hepatosis. Disorders of the beam-radial structure of the lobules, vacuolar and dystrophy and fatty degeneration of hepatocytes. Staining with hematoxylin and eosin. $\times 100$.

During the investigations of medium molecules rates it was observed that in control group of HA rats the levels of MMM₂₃₈ were 57.2 % (p<0.001) higher, then the levels of MMM₂₃₈ of LA, and 39.1 % (p<0.001) higher the levels of MMM₂₅₄, but 2.5 times lower (p<0.001) the levels of MMM₂₆₀ (Table).

Table. Changes of MMM parameters in blood serum of rats with hepatitis, hepatosis, fibrosis and cirrhosis of the liver, caused by ethanol in high-emotional and low-emotional rats (M±m, n=12)

Group	Parameter			
	MMM ₂₃₈	MMM ₂₅₄	MMM ₂₆₀	MMM ₂₈₀
High-active rats				
Control	0.282 ± 0.005	0.365 ± 0.008	0.107 ± 0.001	0.163 ± 0.007
Non-alcoholic steatohepatitis	0.118 ± 0.002*	0.208 ± 0.005*	0.244 ± 0.006*	0.383 ± 0.007*
Ethanol hepatosis	0.500 ± 0.010*	0.848 ± 0.005*	0.957 ± 0.005*	0.640 ± 0.009*
Low-active rats				
Control	0.121 ± 0.002 #	0.222 ± 0.002 #	0.269 ± 0.005#	0.179 ± 0.007
Non-alcoholic steatohepatitis	0.275 ± 0.004*,#	0.343 ± 0.007*,#	0.120 ± 0.002*,#	0.167 ± 0.004#
Ethanol hepatosis	0.500 ± 0.015*	0.709 ± 0.021*,#	0.870 ± 0.009*,#	0.668 ± 0.011*
Note: 1. * – parameters are statistically significant in comparison with control; 2. # – parameters are statistically significant in comparison between high-active and low-active animals.				

In simulation of nonalcoholic hepatitis significant in HA rats decrease in 2.3 times (p<0.001) of MMM₂₃₈ and in 1.8 times (p<0.001) MMM₂₅₄, increasing in 2.3 times (p<0.001) of MMM₂₆₀ and in 2.3 times (p<0.001) MMM₂₈₀ fractions comparing to the control group. In simulation of nonalcoholic hepatitis significant in LA rats increase in 2.3 times (p<0.001) of MMM₂₃₈ and in 1.5 times (p<0.001) MMM₂₅₄, decreasing in 2.4 times (p<0.001) of MMM₂₆₀ fractions comparing to the control group. So, in HA rats comparing to the LA group the levels of MMM₂₃₈ was lower in 2.3 times (p<0.001), the levels of MMM₂₅₄ were lower in 1.7 times (p<0.001), the levels of MMM₂₆₀ were 2 times higher (p<0.001), and the levels of MMM₂₈₀ – in 2.3 times higher (p<0.001).

In ethanol hepatosis it was recognized significant increasing of all MMM fractions in comparison to control group. Thus, in HA rats with EH the levels of MMM₂₃₈ increased in 1.8 times (p<0.001), the levels of MMM₂₅₄ – in 2.3 times (p<0.001), the levels of MMM₂₆₀ – in 8.9 times (p<0.001) and the levels of MMM₂₈₀ – in 3.9 times (p<0.001). In LA animals – in

4.1 times ($p < 0.001$), in 3.2 times ($p < 0.001$), in 3.2 times ($p < 0.001$) and in 3.7 times ($p < 0.001$) respectively. Comparing MMM parameters between study groups the levels of MMM₂₅₄ and MMM₂₆₀ were considered as higher in HA rats (in 16.4 % ($p < 0.01$) and 9.1 % ($p < 0.05$)) respectively.

The analysis of the results shows that the levels of medium molecules in blood serum are increasing. This indicates to the development of endogenous intoxication. Increasing of the amount of endotoxins can occur due to their production, with increased catabolism, or in violation of their disposal, detoxification and withdrawal.

There are three interconnected components of endogenous intoxication: microbiological, biochemical and immunological [17]. It was found that the significant immunological changes, primarily associated with the activation of polymorphonuclear leukocytes, macrophages, complement components, the synthesis of cytokines, and the appearance of autoantigens take place under endogenous intoxication. The abnormalities related to immunological disorders are confirmed by morphological changes. Thus, the presence of inflammatory infiltrates in hepatic parenchyma, the release of lymphocytes outside the hepatic tubules are noted in liver samples, taken in rats with hepatitis.

The main pathogenetic mechanism of endogenous intoxication is the development of generalized microcirculatory disturbances due to the impact of cytokines, released from activated polymorphocytic leukocytes and macrophages, prevalence of catabolic processes, the abnormalities of oxygen transport and its utilization by tissues. As a result of tissue damage and hypoxia the development multiple organ failure takes place. Dystrophic changes, that appear in a consequence of hypoxia are noted in hepatitis, hepatosis. Both morphological and biochemical changes were more significant in highly active animals in ethanol using.

When analyzing the most significant changes in HA and LA animals, it is evident that the level of MMM₂₈₀ is the highest in rats with ethanol hepatosis. Since the nucleotides and aromatic amino acids are determined in $\lambda = 280$ nm, the increasing of this parameter indicates to the marked cells destruction, decreasing of coenzymes, enzymes and hormones synthesis, which is more significant in HA rats.

In ethanol hepatosis in the HA rats, all changes are unidirectional: the highest increasing of MMM₂₆₀ levels is present indicating to the highest accumulation of nucleotides. The levels of MMM₂₃₈ increase with nonalcoholic hepatitis in LA, equal to control group in animals, and decrease in HA. They reflect the content of low molecular weight peptides. The obtained data indicate that with the aggravation of the pathological process the abnormalities

of filtration activity of kidneys occur while in HA the toxins accumulate in cells as a result of the development of renal failure.

In LA rats with ethanol hepatitis, maximal increasing of MMM₂₃₈ is observed, which indicates on the accumulation of low molecular weight peptides. Obviously, hepatocyte destruction is present, which is noted during morphological investigations.

Conclusions

During our investigations it was found the significant increasing of MMM levels in blood serum in rats with ethanol hepatitis and nonalcoholic hepatitis. The accumulation of MMM is not only a marker of endotoxication, they also increase the course of the pathological process, acquiring the roles of secondary toxins, affect the viability of all organs and systems. The degree of MMM accumulation depends on the moto activity of the animals and simulated pathology and is more significant in highly active animals, compared with low-active in the ethanol using.

Less activity of MMM₂₃₈ is observed in HA rats with EH, which can be explained by the development of multi-organ pathology, in particular, renal impairment. Morphological investigations showed that the grade of liver injury was more significant in HA rats.

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