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EFFECTS OF ETHANOL AND THE AMINO ACIDS MIXTURE ON PATHOPHYSIOLOGICAL PROCESSES IN RATS WITH ALLOXAN-INDUCED DIABETES

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The aim of the research was to evaluate the effects of ethanol and the amino acids mixture (GABA, glutamine, β-alanine) on alloxan-induced diabetes in laboratory rats, study of changes in animal behavior with "Open field" test, investigate the white blood cells differential count (the blood differential test), which are indicators of pathophysiological processes.

The "Open field" test showed that alloxan decreases the motor, orientation-exploratory activity of animals, suppresses emotions, and causes depression. Single intraperitoneal injection of ethanol, as well as the amino acid mixture to alloxan-induced diabetic rats have a hypoglycemic effect, significantly ($p<0,001$) reducing the blood glucose level by 26% and 33% respectively. However, these substances did little to improve the motor-exploratory behavior of animals, as well as changes in the differential count of lymphocytes. These data serve as the basis for studies with long-term daily use of the above mentioned substances.

Diabetes – alloxan – "Open field'' test – leukocytes differential count

Հետազոտության նպատակն էր ուսումնասիրել էթանոլի և ամինաթթուների (ԳԱԿԹ, գլուտամին, β-ալանին) խառնուրդի ազդեցությունը լաբորատոր առնետների մոտ ալլոքսանով հարուցված շաքարախտի վրա, հետազոտել կենդանիների վարքագծային փոփոխությունները «Բաց դաշտ» թեստով, ուսումնասիրել նրանց արյան լեյկոցիտների դիֆերենցիալ հաշվարկը՝ լեյկոֆորմուլան, որոնք պաթոֆիզիոլոգիական պրոցեսների ցուցիչներ են:

«Բաց դաշտ» թեստը ցույց է տվել, որ ալլոքսանի ազդեցությամբ նվազել են կենդանիների շարժողական, կողմնորոշիչ-հետազոտական ակտիվությունը և հուզականությունը, առաջացել է ընկճված վիճակ: Մեր հետազոտության արդյունքում հայտնաբերվել է, որ էթանոլի, ինչպես նաև ամինաթթուների խառնուրդի միանգամյա ներորովայնային ներարկումն ալլոքսանով շաքարախտ առաջացրած կենդանիների մոտ ունի հիպոգլիկեմիկ ազդեցություն, հավաստիորեն ($p<0,001$) իջեցնելով ծայրամասային արյան գլյուկոզի մակարդակը 26 % և 33 %, համապատասխանաբար: Սակայն վերջիններս էապես չեն նպաստել կենդանիների շարժիչ-ճանաչողական վարքագծի բարելավմանը, ինչպես նաև լեյկոցիտների դիֆերենցված հաշվարկի փոփոխմանը: Այս տվյալները հիմք են վերոհիշյալ նյութերի երկարատև ամենօրյա օգտագործմամբ նոր ուսումնասիրությունների համար:

Շաքարախտ – ալլոքսան – «Բաց դաշտ» թեստ – լեյկոցիտների դիֆերենցված հաշվարկ

Целью исследования было изучение влияния этанола и смеси аминокислот (ГАМК, глутамин, β-аланин) на аллоксан-индуцированный диабет у лабораторных крыс, исследование изменений поведения животных с использованием теста "Открытое поле", изучение дифференциального подсчета лейкоцитов крови – лейкоформулы, которые являются индикаторами патофизиологических процессов.

Тест "Открытое поле" показал, что аллоксан снижает двигательную, ориентировочно-исследовательскую активность животных, подавляет эмоции, вызывает депрессию. В

результате наших исследований установлено, что однократное внутрибрюшинное введение этанола, а также смеси аминокислот крысам с диабетом оказывает гипогликемический эффект, значительно ($p < 0,001$) снижая уровень глюкозы в периферической крови на 26 % и 33 % соответственно. Однако эти вещества незначительно способствовали улучшению двигательно-исследовательского поведения животных, а также изменению дифференциального подсчета лейкоцитов. Эти данные служат основанием для проведения исследований с длительным ежедневным применением вышеупомянутых нами соединений.

Диабет – аллоксан – тест "Открытое поле" – дифференциальный подсчет лейкоцитов

Diabetes Mellitus (DM) is one of the most common diseases of the century and it has not been fully studied yet. Over 400 million people on our planet suffer from diabetes and most of them consume alcoholic beverages. DM causes a variety of disorders characterized by hyperglycemia, altered metabolism of lipids, carbohydrates, and proteins, as well as complications associated with vascular injuries [18], heart disease, stroke, kidney disease, retinopathy, neuropathy, ulceration, and gangrene of the extremities [21]. It is known that DM weakens the body's antioxidant defenses [3,4,15], which, together with hyperglycemia and changes in lipid exchange causes a decrease in transparency of phospholipid membrane of peripheral tissue cells and, consequently damage to β-cells of the islets of Langerhans in the pancreas.

Alloxan, which is commonly used for the study of type I diabetes, is a breakdown product of uric acid, formed in the body in certain metabolic disorders selectively acting on pancreatic islet cells. Alloxan degrades the β-cells in the islets of Langerhans of the pancreas, inducing insulin deficiency [2]). According to some authors, this is due to the generation of cytotoxic hydroxyl radicals [13], which results in the formation of activated macrophage and lymphocytic infiltrate and, consequently, the appearance of the inflammatory foci [7].

Emerging evidence suggests that some amino acids may be important in the prevention of diabetes and diabetes-associated complications. Noteworthy, the resent publications in the field of the experimental diabetes give evidence about the protective abilities of gamma-aminobutyric acid (GABA) [1, 20, 23, 17]. Another amino acid, Beta-alanine (BA), a dietary supplement, has been experimentally shown to augment muscle carnosine and exercise capacity in healthy individuals and have evaluated its effectiveness in individuals with T2DM [11, 16].

There is some evidence that diabetes leads to depression [6]. As it has been proven that ethanol has a detrimental effect on all the physiological and mental functions of the body, of particular interest was to study the effect of ethanol on the motor and exploratory activity and depression of experimental rats.

It is known, that ethanol metabolism is accompanied by the generation of free radicals, the target of which are primarily the mitochondrial membranes. This occurs due to the depletion of oxidative phosphorylation, the separation of oxidation and phosphorylation, and loss of energy of the mitochondrial matrix [9]. On the other hand, there is some literature on the antioxidant properties of ethanol due to the neutralization of ·OH radicals. Ethanol is used both in the perfusion prophylaxis of heart damage and in reducing the harmful effects of ionizing radiation [10]. A comparable observation in type 1 diabetic patients has been described with lower blood glucose levels during the night and protracted hypoglycaemia the next morning after the consumption of 6 units of alcohol at 21.00 h [24]. For this reason, it is of great interest to study the action of

ethanol on behavior and the blood formed elements of animals with diabetes as well as on mitigation of the harmful effect of alloxan.

This study may help our understanding of pathophysiological events associated with the effects of ethanol on behavior and the blood formed elements of animals with diabetes as well as on mitigation of the harmful effect of alloxan.

Materials and methods. The study was performed on 32 healthy male white rats weighing 150-240 g. Animals were kept in the vivarium of the Institute of Biochemistry under 12-hour daylight / 12-hour darklight regime with standard diet and water *ad libitum* [8]. All animal experiments were approved by the Ethics Committee of the Institute of Biochemistry (IORG 0009782).

The animals studied were divided into four groups each consisting of 8 rats: 1) control rats; 2) alloxan-induced diabetic rats; 3) diabetic rats treated with ethanol; and 4) diabetic rats treated with AM.

On the first day of the experiment, each animal was weighed. The model of diabetes was induced by intraperitoneal injection of 0,5 ml alloxan at a dose of 150 mg / kg body weight. An appropriate amount of physiological saline was injected instead of alloxan in the control rats. In 3 days after the injection of alloxan (receiving well established diabetes) rats of 3rd and 4th groups were treated with a single i/p injection of 25% v/v ethanol 2.5 g/kg and AM (100 mg/kg GABA, 50 mg/kg glutamine, 100 mg/kg β-alanine) in 0,5 ml of physiological saline, respectively. The solutions were prepared freshly just prior to injection.

To study the behavior of the animals the "Open field'' test was used [5] with a radius of 1 m made from white plastic with a wall height of 50 cm and round bottom. To record the visual activity of animals, the floor was divided into 3 rows with the same area. Central part of the field had 1 section, middle and external parts – 8 and 16 sections, respectively. The illuminated lamp was installed at a distance of 1 m above the bottom.

Marked animals were placed in the central section of the field, two cameras recorded the movement, the orientation-research activity, and the emotions of the animals for 5 minutes. After testing each animal, the field was cleaned and disinfected with alcohol to eliminate odor. The following indicators were recorded: for the horizontal motor activity- retention time, section crossings; for the vertical activity- rearing and freezing; and for the emotions of the animals- sniffing, grooming, number of urinations and defecations (feces). The results were presented in a table format with the respective indicators.

The amount of glucose in peripheral blood (mmol/L) was determined by sequential snipping of the tail and taking a drop of blood. PKK-03 Satellite Express glucometer and the appropriate capillary electrochemical test layers were used to measure the blood glucose levels.

Over the next 2 days (in 4th and 5th days after the alloxan injection), the behavior was recorded, animals were weighed, and blood glucose was measured.

Then the animals were anesthetized for 5 minutes with the 97.5% diethyl ether, after which the blood samples were collected from the tail vein to perform the investigations needed.

Standard methods were used to prepare blood smears. Differential calculation of the percentage content of leukocytes (Leukocyte formula) was performed using Romanovski-Giemsa histological staining. Dried blood smears were fixed 10' in 96% ethanol. Stain was used at a 1:20 ratio. To produce a 1:50 dilution of Giemsa stain, 1 ml stock stain solution was added to 49 ml phosphate buffer (pH 6.4) solution. The smears were stained for 10-15', rinsed with tap water, left to dry in air [19] and investigated under a microscope with oil immersion. Leukocyte formula was determined on the basis of differential counting of 200 leukocytes in a stained smear and subsequent calculation of their percentage.

All values are presented as mean ± standard error (MEAN ± SEM). Data were statistically analyzed by the Sigma Stat program. A statistically significant comparison test was performed with ONE WAY ANOVA. The significance of the difference between the means was considered and accepted, when $p<0.05$.

Results and Discussion. Stable hyperglycemia with 5-fold increase of the blood glucose level in the experimental rats was observed 3 days after the single intraperitoneal injection of alloxan. The experimental rats demonstrated the following symptoms unlike

the control animals: increased daily water use (more than 120 ml), frequent urination, sudden weight loss, hair loss, and depression. In one day after the single intraperitoneal injection of ethanol (group 3) and AM (group 4), which corresponds to day 4 of the study, glucose level was reduced by 24.2% in group 3 and by 32.4% in group 4 and on day 5 of the experiment by 26.3% and 33%, respectively (fig. 1).

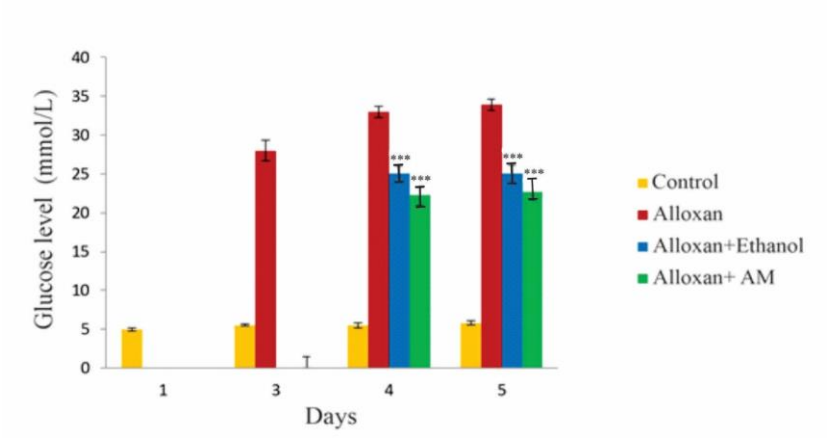

Fig.1. The glucose level in the peripheral blood of the control and experimental rats.
Data are presented as Mean ± SEM, $^{NNN}p<0.001$

The effects of alcohol on carbohydrate metabolism are complex and not all completely understood. Some are directly related to the influence of alcohol or its metabolic products, acetaldehyde and acetate, but others to an alcohol-induced increase within the liver cell of the NADH/NAD ratio (NAD for nicotinamide adenine dinucleotide). This socalled redox shift is the result of the oxidation of alcohol to acetaldehyde and of acetaldehyde to acetate by dehydrogenases [14]. The consequence of this shift is inhibition of the activity of the citric acid cycle and of β-oxidation of fatty acids, while the conversion of pyruvate to lactate is favored. The increase of the NADH/NAD ratio and of the lactate/pyruvate ratio contributes to inhibition of gluconeogenesis. After the consumption of 48 g alcohol (≈4 glasses), hepatic gluconeogenesis decreases about 45% [22, 12]. Alcohol influences glucose metabolism in several ways in diabetic patients as well as in non-diabetic patients. Since alcohol inhibits both gluconeogenesis and glycogenolysis, its acute intake without food may provoke hypoglycaemia, especially in cases of depleted glycogen stores and in combination with sulphonylurea [26]. Moderate drinking is associated with a reduced all-cause mortality, which is largely attributable to a favorable effect on atherosclerotic disorders, especially coronary heart disease (CHD). Mechanisms suggested to be involved are changes in lipid metabolism, hemostatic balance, blood pressure and insulin sensitivity [25].

The action of the mixture of neuroactive amino acids is likely to be due to the fact that GABA is involved in carbon and protein metabolism in the β-cells of the pancreas, thereby improving the state of these cells.

The "Open-field" test used for studying the animal behavior in 3 days after the alloxan injection has shown dramatic decrease of the motor and orientation-research activity, as well as the suppression of emotions (tab. 1).

Table 1. The study of the behavior of control and experimental animals by "Open field" test

	1 day	3 day	4 day			5 day		
	Control	Alloxan	Alloxan	Alloxan+ Ethanol	Alloxan+ AM	Alloxan	Alloxan+ Ethanol	Alloxan+ AM
Retention time (minute)	0,38±0,08	0,03±0,02	-	-	-	0,22±0,06	0,42±0,41	0,57±0,33
Section crossing	19,5±4,13	0	0	0	0	0,3±0,25	0,3±0,25	1±0,70
Rearing	12,2±1,95	0,1±0,08	0	0	0,3±0,25	0	0,3±0,25	0,8±0,48
Urination	0,6±0,15	0,7±0,25	1,3±0,63	0,8±0,25	1,0±0,41	0,5±0,29	0,5±0,29	0
Defecation	2,3±0,30	0,7±0,30	0,8±0,48	0,8±0,25	1,0±0,41	0,5±0,29	0	0,5±0,29
Freezing	6,7±0,84	4,3±0,92	6,2±0,80	2,5±0,87	3,0±1,68	3,0±1,08	4,0±0,71	11,2±5,34
Sniffing	9,0±0,50	4,0±0,85	6,5±1,94	3,2±0,63	3,0±2,12	2,0±0,71	3,7±0,75	4,2±1,65
Grooming	2,7±0,40	0	0	0	0	0,2±0,21	0,2±0,25	0,7±0,48

On the 4th day of the experiment the animals almost do not leave the central section, there was no section crossing and rearing. A decrease in the frequency of urination, defecation, and sniffing, which serves as an indicator of irritation of the vegetative nervous system, demonstrated the depressed state of the animals and their activity decline.

Table 2. Differential calculation of the percentage of leucocytes elements

	Control	Alloxan	Alloxan+ Ethanol	Alloxan+AM	Norm
Neutrophils (banded)	7.000 ± 2.52	1.500 ± 0.65	2.750 ± 0.85	4.333 ± 0.33	10-50
Neutrophils (segmented)	12.000 ± 1.73	32.250 ±9.97	23.500 ±2.63	20.333 ± 0.88	
Lymphocytes	72.667 ± 4.26	59.500 ±8.57	66.000 ±2.27	69.333 ± 2.60	50-70
Monocytes	3.667 ± 1.20	3.750 ± 1.03	3.750 ± 1.70	3.333 ± 1.20	0-10
Basophils	0.333 ± 0.33	0.250 ± 0.25	0.000 ± 0.00	0.667 ± 0.33	0-1
Eosinophils	3.333 ± 1.20	2.250 ± 1.93	2.750 ± 0.85	1.333 ± 0.88	0-5
Atypical monocytes	1.000± 0.58	0.000 ± 0.00	1.250 ± 1.25	0.667 ± 0.67	0-1

A decrease in behavioral indicators and an increase of depression indicate an extremely negative effect of alloxan on motor, orientation-exploratory activity and emotional state. Against this strong background, a single intraperitoneal injection of ethanol or AM had a little effect, which would probably be observed with prolonged use of these substances.

Examination of the leucocytes count of peripheral blood samples from all groups of animals showed a quantitative change of its elements within the normal range. However, the number of the segmented neutrophils was increased in the blood of the animals exposed to alloxan only, indicating an inflammatory process in the organism (tab. 2). This parameter gradually returns to norm under the influence of ethanol and AM.

This correlates with positive effect of these two components on the glucose levels in the blood.

Conclusions

1. The intraperitoneal injection of ethanol as well as the AM to alloxan-induced diabetic rats have a hypoglycemic effect, significantly ($p<0,05$) decreasing the blood glucose level by 26% and 33% respectively.

2. The "Open field" test has shown that alloxan is dramatically impacting animal behavior-characterized by the depressed state of animals. A single injection of ethanol and AM showed almost no impact, which would probably be observed with prolonged use of these substances.

3. Examination of the peripheral blood lymphocytes differential count showed a quantitative change of the elements within the norm. Long-term research may give a different picture.

Thus, the amino acid mixture and ethanol used in this study had a definite therapeutic effect in experimental diabetes.

This study could influence our understanding of the pathophysiological phenomena associated with the effects of ethanol on the behavior and hematopoietic elements of diabetic animals, as well as identify ways of mitigating the harmful effects of alloxan using the amino acid mixture (GABA, glutamine, β-alanine) to save life of the millions patients suffering from Diabetes.

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