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Ketosis Level as a Factor Determining Addictive Behavior of Alcoholized Rats

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A group of 20 rats was subjected to long-lasting alcoholization (90-day-long forced consumption of 10% aqueous ethanol solution, ES). Every day at 17.00, the ES volume consumed by the animals per day was measured, and the level of ketone bodies in the urine was estimated in points using a semiquantitative quick ketone test. Then, rats were divided into two equivalent groups, experimental and control ones (n = 10 in each group). During the subsequent three-day-long main stage of the experiment, animals of the experimental group were preliminarily (at 17.00 of the preceding day) subjected to peroral administration of 1.0 ml of solution of unithiol, i.e., an agent neutralizing ketone bodies. Within three days of the mentioned period, the level of ketonuria was estimated within a 9.00–17.00 observation period with one-hour-long intervals. This time, free access of rats to water or ES was provided. We found that rats of the experimental group consumed more than one-third of the daily ES norm within the first hour of observation; i.e., within the interval where the level of ketonuria was minimum (0.17 points, on average). After this, the amount of the consumed ES decreased, while the level of ketonuria increased significantly in a parallel manner. In control rats throughout the observation period, hourly consumption of ES and the level of ketonuria demonstrated no significant dynamics. We hypothesize that there is a causal relationship between the ketosis level and the behavioral reaction of alcohol consumption (the lower the ketosis, the higher the consumption). Under conditions of alcoholization resulting in the development of hypoglycemia, ketone bodies begin to be used in the brain as an energy substrate, and the brain becomes dependent on the level of ketonemia to a significant extent. These level is a rather important factor determining alcohol addiction; direct factors initiating a situational drive for alcohol consumption are hypoketonemia episodes.

Keywords: alcohol addiction (dipsomania), dynamics of alcohol consumption, ketonemia, ketonuria, energy metabolism in the brain.

As is known, the treatment of chronic alcoholism (dipsomania) remains rather ineffective. According to various evaluations, recurrences of drinking excesses after therapeutic interventions of various nature were observed in 74-91% of patients, even if the periods of abstinence were rather long (from 6 months to 10 years) [1].

At the end of the 20th Century, a concept of alcoholism as a chronic neurological disease was formed. As was believed, negative influences on the toxicant (ethanol) on important neuromediator systems play the role of the main pathogenetic factor of such disease [2]. According to the above concept, neurochemical correction of the respective shifts in the processes of neuromediation is a possible effective approach in stopping craving for alcohol. It was believed that the main potential targets for medicinal agents in the treatment of alcoholism are different links of the transmitter/mediator systems of the brain, namely receptors, systems of transmitter reuptake, and systems of transmitter/mediator synthesis and catabolism [3]. The advances in attempts at neurotransmitter correction of alcoholism remain, however, not very successful (at least, the corresponding techniques did not become routine).

It is natural that changes related to the development of alcoholism affect not only the neurotransmitter cerebral systems but also the main links of the processes of carbohydrate and lipid metabolism in the entire organism and, especially, in the brain. Metabolic disorders in alcoholism are very significant; those are manifested in the development of hypoglycemia, ketoacidosis, adipose degeneration, and other pathological phenomena [4–6]. Disbolism inevitably involves changes in the energy and plastic functions of the cells (affecting,

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first of all, cerebral ones) and their total alteration. It is obvious that abnormal functioning of damaged cells of the cerebral systems inevitably causes the formation of divergent behavior, including pathological craving for one agent or another (in the analyzed case, craving for ethanol).

Since, as was mentioned above, the most important biochemical shifts in alcoholism are hypoglycemia and ketoacidosis, we tested in our recent experiments on the respective experimental model two variants of correction of such metabolic manifestations, corrections of the level of glucose and of that of ketone bodies [7, 8]. The first variant was based on forced long-lasting (30 day long) elevation of the level of glucose in the blood. We assumed that very intense stable hypoglycemia related to chronic alcoholism inevitably exerts destructive influences on the brain. Under conditions of insufficient energy supply, neurons are subjected to significant pathological changes, in particular, to apoptosis [5, 9, 10], which leads to degradation of the cerebral mechanisms [11, 12].

We also tried to correct the state of ketosis using pharmacological neutralization of ketone bodies. It is known that chronic alcoholism is accompanied by stable ketonemia and in a pH drop in the blood (ketoacidosis) [4, 6]. Toxic actions on the brain are exerted by both these factors, acidosis and an enhanced level of ketone bodies (especially of acetone). The ketoacidosis-related symptoms are nausea, emesis, thirst, headache, and loss of consciousness [4, 13–15]; at the high level of the corresponding shifts, even fatal outcome is possible [16, 17]. The above-mentioned manifestations are related to damages of phospholipid cellular membranes, changes in the spatial structure of protein molecules, disorders of the processes of binding of hemoglobin with oxygen, etc.

In our experiments, we found that correction of the level of glycemia in alcoholized animals gives better results than correction of ketosis. The amount of ethanol solution consumed by alcoholized rats under conditions of free choice between this solution and water decreased in a parallel manner with increase in the level of glucose in the blood. Long-lasting (during 30 days) pharmacological suppression of ketosis decreased consumption of ethanol less effectively and even increased this index within the final stages of the test period [8]. Such a result clearly contradicts the recommendations to treat ketoacidosis in clinics in a course mode (e.g., during a one-month-long period using three 7-day-long courses and 3-day-long pauses [16, 18]).

The above-mentioned contradictions showed that a more detailed study of correlation between the level of ketoacidosis and the degree of craving for alcohol is expedient. We believed that, in such a case, monitoring of the level of ketone bodies and of consumption of ethanol by alcoholized rats under conditions of artificial pharmacological modulation of the first of the mentioned indices should be organized with shorter intervals between the measurements (e.g., with 1-hour-long ones). It is obvious that, in the course of performance of such studies, measurements of the level of ketonemia are related to appreciable technical difficulties (considering that the procedure of blood sampling is inevitably traumatic). This is why we believed that the level of ketone bodies can be estimated not in the blood but in the urine (sampling of which is much simpler and less stress-inducing). The above-mentioned indices of ketosis correlate with each other rather closely; changes in the level of ketonuria accompany the respective shifts in ketonemia with rather small time delays (see below).

METHODS

Experiments were carried out on 20 male rats (12 months old, mean body mass about 200 g). Studies were performed within the winter-spring period; the animals were kept under standard vivarium conditions, one rat in the cage, with free access to food (standardized balanced feed for rats). The indoor temperature was maintained at the level of 17–22°C, which ruled out the development of excessive thirst.

The preliminary stage of experiments lasted 90 days. Within this time interval, the animals were subjected to the forced alcoholization, i.e., they should consume a 10% aqueous solution of ethanol (ES) as the liquid for drinking. Every day at 17.00, we measured the ES amount consumed per day and estimated the level of ketone bodies in the urine. To obtain urina samples, each rat was placed in a plastic container with a perforated bottom. In intact rats, the frequency of urination is 1–2 acts per hour [19]. However, in a novel environment (container), the rats, as a rule, urinate with a 2–3-min-long delay. Such approach allowed us to obtain urina samples with minimum stressing of the animal.

The second (main) stage of the experiment lasted three days. The animals were divided into two

groups, experimental and control (n = 10 in each). At 17.00 of every day, we evaluated the amount of ketone bodies in the urine of all rats. Then, the animals of the experimental group were subjected to peroral (with the help of a syringe without a needle) administration of 1.0 ml of 4.2% solution of unithiol, a pharmacological agent that neutralizes ketone bodies. The animals of the control group were treated with 1.0 ml of NaCl physiological solution. Then, the ES-containing drinking liquid was removed from the cage; instead of this, a waterer with pure water was placed. The next day, at 9.00, we initially evaluated the degree of ketonuria in rats and then returned the ES-containing drinking trough in the cage. The waterer with pure water was also left in the cage, i.e., the animals throughout the day could freely choose the liquid for drinking. The degree of ketonuria and the amount of consumed ES were recorded every subsequent hour, from 9.00 to 17.00.

Unithiol was administered in the form of the standardized pharmacological preparation Zoreks (Farma Start, Ukraine). In the molecule of unithiol (sodium dimercaptopropane sulfonate), there are two sulfhydryl (thiol) groups; this agent actively binds with oxygen of keto groups (C=O) of ketone bodies in the peripheral blood and transforms these compounds into nontoxic ones. Unithiol is used as an antidote for suppression of alcoholic (hangover, abstinent) ketoacidosis, removal of nausea, headache, etc. [18]. For rats of the experimental group, we used the dosage of this preparation recommended by the producer for humans (10.5 mg/kg per day). Rats of the control group were kept under identical conditions but did not receive unithiol.

To evaluate the level of ketone bodies in the urine, we used a semiquantitative express estimation with test strips (Citolab, Farmasco, Ukraine). The mentioned strips are soaked with sodium nitroprusside solution; in the presence of ketone bodies, a violet-red color develops. The intensity of labeling depends on the concentration of ketone bodies in the test liquid. According to the scale proposed by the producer (–, ±, +, ++, and +++), the above-mentioned index was estimated using the following rating scale: 0 points, the absence of ketone bodies; 1 point, concentration of ketone bodies at the level of sensitivity of the technique (to 0.5 mM); 2 points, appreciable concentration of ketones (0.6-1.5 mM); 3 points, significant concentration (1.6-4.0 mM), and 4 points, high concentration of ketones (4.1-10.0 mM).

For processing of the numerical data obtained in our experiments, MedStat software was used [20]. Since distributions of the values of the amount of the consumed ES did not fit the normality pattern, we used non-parametric criteria of intergroup comparison (comparison of centers of the linked samplings with the use of the Wilcoxon paired T-test and Kruskal–Wallis one-way analysis of variance). In the case where we found statistically significant intergroup differences, we additionally performed the pairwise comparison using the Dunn criterion. For amounts of the consumed liquids (water and ES), values of the medians of the corresponding distributions and median errors (ml/kg) are shown below; in square brackets, limits of the confidence interval are indicated. For score estimates calculated according to the concentration of ketone bodies in the urine, the arithmetic mean values are indicated.

RESULTS

After the end of the first experimental stage, i.e., after forced alcoholization, each rat consumed, on average, 63 ± 2 ml/kg ES [60–67 ml/kg] per day. After termination of the period of alcoholization, estimates of the degree of ketonuria in four animals corresponded to 4 points, those in 14 rats corresponded to 3 points, while those in two animals corresponded to 2 points. Thus, the mean estimate of the level of ketonuria in the general group of 20 animals was 3.1 ± 0.12 points.

Prior to the second stage, all animals were divided in two maximally equivalent groups, experimental and control. In both these groups (n = 10 in each), there were two rats with the 4-point estimate of the level of ketonuria, seven animals with 3 points, and one rat with 2 points. Naturally, the mean values of the above index in these groups before the second stage of the experiment were identical.

Within the above-mentioned second stage, rats of the experimental group, being under conditions of free choice of pure water or ES for drinking, consumed nearly the same doses of ES as those after completion of the first stage, 61 ± 3 ml/kg per day. The difference between the mean-group values of the ES daily amounts used at the first and second stages was insignificant (P = 0.135). Including consumption of water, the total amount of the liquid used per day of the experimental group was 71 ± 3 ml/kg (64-78 ml/kg) per day.

We found that, throughout the second stage of experiments, animals of the experimental group consumed a rather significant part of the daily ES amount (more than one third of the daily norm, on average, 22 ± 2 ml/kg [21–25 ml/kg]), within the first morning hour of the observation period (from 9.00 to 10.00). We would recall that this was realized during the first hour after a bowl with ES was returned to the cage. In the course of the subsequent seven hours, from 10.00 to 17.00, the amount of ES consumed during 1 h of the observation period varied from 7 ± 1 to 4 ± 1 ml/kg. Thus, the dynamics of amounts of ES consumed by rats of the experimental group during the period of observation (9.00–17.00) were extremely uneven. This feature sharply distinguished animals of the experimental group from control rats that demonstrated insignificant variations of the mean amounts of ES consumed per hour during the observation period, from 8 ± 1 to 9 ± 1 ml/kg. At the same time, total daily consumption of ES by the rats of the experimental and control groups within the above-mentioned period was, in fact, identical (61 ± 3 and 61 ± 1 ml/kg).

The above-described dynamics of the consumption of ES by rats of the experimental group closely correlated with estimates of the level of ketonuria in these animals. As was already mentioned (see Methods), at 17.00 of the previous day, 1.0 ml of 4.2% unithiol solution was perorally introduced into these rats. Due to this, estimates of the level of ketonuria in these animals were minimal at 9.00 of each day throughout the 3-day-long second stage of experiments. In 25 cases of 30 measurements performed in this group within the mentioned three-day-long time interval of the second stage, ketone bodies in the urine were absent completely (0 points), while in five cases the concentration was minimal, below 0.5 mM (1 point). Thus, the mean estimate of the level of ketonuria in rats of the experimental group at the beginning of the observation periods throughout the second stage of experiments was only 0.17 points. Decreases in the degree of ketonuria, as compared with that observed within the first stage in different animals, were dissimilar, most frequently by 3 points (from 3 to 0 points), sometimes by 2 points (from 4 to 2 or from 2 to 0 points), and in one case we observed a drop of this index by 4 points (from 4 to 0 points).

After the access to ES was provided (placement of a drinking bowl with this solution in the cage and the presence of the bowl with pure water), a significant amount of ES was consumed, and the level of ketone bodies in the urine in animals of the experimental group increased. From 9.00 to 10.00, the estimate of the level of ketone bodies in the urine increased by 1 point in 23 cases of measurements of this index during three days of the second experimental stage, while a 2-point increase was found in seven cases. Therefore, the mean increment during the first hour of the observation period was 1.23 points. The increment from 10.00 to 11.00, i.e., after the maximal ethanol consumption, was somewhat greater (by 1 point in 20 measurements and by 2 points in 10 measurements). Thus, the increment within this time interval was the greatest (1.33 points, on average). Then, from 11.00 to 12.00, the increment of the level of ketonuria was rather small, only 0.33 points, on average (in 10 measurements, by 1 point, while in 10 cases no increment was observed). Later on, within the subsequent part of the observation period, the level of ketonuria demonstrated no significant dynamics.

The level of ketonuria in rats of the control group was practically constant and demonstrated no significant variations during the entire period of observation (9.00 to 17.00).

DISCUSSION

There are strong reasons to believe that the amount of ketone bodies in the urine (level of ketonuria) in rats rather closely correlates with the level of these compounds in the blood. The delay between changes in the level of ketonemia and in that of ketonuria in rats is rather short. In these animals, the rate of glomerular filtration in the kidneys is very high (on average, 185.7 ± 4.8 ml/kg per hour; in humans, this index is about two times lower, 94–107 ml/kg per hour [21]). This is why the entire volume of the blood plasma in rats (about 8 ml) should be subjected to filtration in the renal glomeruli within about 13 min. In these animals, the volume of the urinary bladder is relatively small (0.2 ml) [21], i.e., it corresponds to about 1/11 of the diurnal diuresis (2.5 ml [22]). The frequency of urinations in rats is rather high (1–2 per hour).

Therefore, it should be concluded that the level of ketone bodies in the urine changes after a shift of the respective level in the blood with a short delay, about 30 min and not more than 1 h. Ketone bodies circulate in the peripheral blood during several minutes, then these compounds are rapidly absorbed

by tissues, eliminated by the kidneys in the urine, and are not stored in the organism. Therefore, it is clear that dramatic fast oscillations of this index over the circadian period may result exclusively from changes in the rate of their production.

Production of ketone bodies in tissues and organs (first of all, in the liver) is realized via two pathways. One of the latter is incomplete oxidation of fatty acids in the course of energy metabolism. In this case, great amounts of free protons are produced, and ketosis is accompanied by clearly pronounced acidosis (a shift of pH toward lower values). Another pathway corresponds to production of ketone bodies from acetyl-CoA, a co-enzyme extensively distributed in the organism and involved in a number of metabolic reactions. This co-enzyme can be produced in excess amounts and utilized incompletely in the citric acid cycle (Krebs cycle). Both above-mentioned pathways are highly active under conditions of metabolic shifts induced in the organism by ethanol. The functioning of the first pathway is initiated by a deficiency of glucose molecules in the organism, i.e., of the most important energy source mobilized by aerobic oxidation. In the norm, the energy supply of the brain is provided exclusively by metabolization of glucose as a "fuel" substrate. Under conditions of alcoholization and the respective glucose deficiency, ketone bodies also begin to be used as the above substrate; the intensity of such deviation of energy metabolism correlates with the expression of hypoglycemia.

Production of ketone bodies from free fatty acids is a relatively inertial process; utilization of triglycerides from fat depots with their hydrolysis to free fatty acids is initiated by the deficiency of glucose but is realized with a notable delay. Metabolization of ketone bodies is energetically less efficient than that of glucose. After complete oxidation, one molecule of beta-hydroxybutyrate gives 26 ATP molecules, while one molecule of glucose produces 36 ATP molecules.

The second pathway of production of ketone bodies, from acetyl-CoA, is much more labile and depends only on the amount of this co-enzyme. After consumption of ethanol, an excess of acetyl-CoA is created in hepatocytes for two reasons. The ethanol molecule is metabolized *per se*, first to acetaldehyde and then to acetyl-CoA. In addition, alcohol dehydrogenase (the first enzyme responsible for ethanol metabolization) uses NAD as a co-enzyme; this is why the ratio of the oxidized and reduced NAD forms (NAD/NAD-H) is shifted toward NAD-H. The developed NAD deficiency leads to suppression of activity of some enzymes using NAD as the co-enzyme, namely, enzymes of the citric acid cycle. This is why acetyl-CoA cannot be completely utilized in the Krebs cycle and begins to serve as a substrate for construction of ketone bodies.

Taking into account all the above-mentioned data, we believe that the observed rapid dramatic enhancement of the level of ketone bodies in the urine (which clearly reflects a similarly rapid increase in the respective index in the blood) is induced precisely by massive consumption of ES at the beginning of the periods of observation within the second stage of our experiments. Since hypoglycemia in alcoholism is a rather stable phenomenon (as we found in our earlier studies [7, 8]), this biochemical variation cannot be responsible for the observed rapid shifts in the level of ketone bodies.

The strongly decreased level of these bodies in the urine of rats of the experimental group, which is observed at the beginning of the observation period (9.00–10.00), is no doubt due to the action of unithiol administered at 17.00 of the previous day. It is clear that the duration of action of unithiol on the concentration of the above-mentioned bodies is noticeably longer than that claimed by the manufacturer (about 8 h [18]); it lasts not less than 16 h post-injection. A sharp rise in the degree of ketosis became obvious immediately after 9.00, i.e., precisely after consumption of a high dose of ES. It should again be emphasized that such consumption of ES was realized under conditions of free choice between ES and pure water (a source of the latter was present in the cage). Rapid intensification of the production of ketone bodies and consumption of ethanol demonstrate quite comparable dynamics, and these shifts develop against the background of the sharply decreased concentration of the above bodies at the beginning of the period of observation. Thus, depletion of ketone bodies looks like a trigger for intensification of ethanol consumption.

Therefore, there are convincing grounds to believe that there is a cause-and-effect relationship between the two processes (a change in the level of alcoholic ketosis and a behavioral reaction of preferred drinking of ES). In this case, the respective indices are in the inverse relationship; the lower the level of ketosis, the stronger the craving for alcohol.

The facts obtained in our experiments

can be summarized as follows. An artificial pharmacologically induced decrease in the amount of ketone bodies due to the action of administered unithiol evokes, after ES becomes accessible, a sharp increase in consumption of the latter (during the first hour of the observation period, the experimental rats consumed, on average, 22 ml/kg of ES, while the control animals drank only 8 ml/kg). Thus, the experimental animals used more than one-third of the diurnal norm of ethanol against the background of the minimal level of ketosis. Consumption of a high dose of ethanol during the above-mentioned time interval led (with a delay shorter than 1 h) to a significant increase in the level of ketonuria. With increase in the level of ketosis (peaked at about 11.00), consumption of ES decreased. At a relatively stable level of ketonuria, no clearly pronounced dynamics of the alcohol consumption was observed.

It is known that, in chronic alcoholism, the following train of events is realized: the development of chronic clearly pronounced hypoglycemia → energy deficiency ("starvation") of the brain → stimulation of production of ketone bodies → partial transition of the brain from exclusive using of glucose as the energy substrate to partial use of ketone bodies as such substrate. Under such conditions, the brain begins to be dependent, to a significant extent, on the level of these bodies in the blood, i.e., on the degree of ketonemia. With any decrease in this index, the biological requirement to replenish the amount of these compounds develops. Since rapid synthesis of ketone bodies can be readily initiated by introduction of ethanol into the organism, this organism tries to realize the corresponding behavioral reaction, i.e., to consume alcohol.

In other words, energy supply of the alcoholized brain depends, to a considerable extent, on the level of ketone bodies, while energy supply of the healthy brain depends exclusively on the level of glucose in the blood. "Feeding" with ketone bodies becomes a vital necessity. Starvation inducing a drop in the level of glucose in the blood triggers a behavioral reaction directed toward the search for food and controlled by the corresponding cerebral mechanisms. Similarly, the deficiency of energy substrates in the alcoholized brain triggers a comparable vital behavioral reaction directed toward the search and use of alcohol, and consumption of the latter intensifies synthesis of ketone bodies and compensates their deficiency.

Therefore, the most important factor determining alcohol addiction is the level of ketonemia, and the immediate factor that induces situational craving for ethanol in the alcoholized organism is hypoketonemia.

All stages of the study comply with the provisions of the European Convention for the protection of animals used for scientific purposes (86/609 / EEC, 1986, Strasbourg) and with the standards of the Committees on Bioethics in the Bogomolets Kyiv National Medical University and Donetsk National University.

The authors of this study, T. I. Panova and A. K. Bortnikova, confirm that, in the course of performance of the experiments, they had no conflict of interest pertinent to commercial or financial relations and relations with organizations or persons somehow or other related to the study, as well as to relations within the research group.

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