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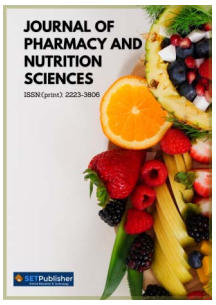

The Effect of Chronic Alcohol Intoxication on the Daily Rhythm of Some Micromorphometric Parameters of Rat Hepatocytes

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Abstract:

The effect of chronic alcohol intoxication on the daily rhythm of micromorphometric parameters characterizing the morphological and functional state of the liver is studied on 80 male Wistar rats of 6 months age, divided into 2 equal groups. The first group served as control; rats of the second group (experiment) were kept under similar conditions but got as a drink a 15% ethanol solution *ad libitum* instead of water. After three weeks of the experiment, animals were euthanized consistently at four-time points during the day. The pathomorphological study of the liver was carried out, the daily dynamics of the nucleus and cell (by area and nuclear-cytoplasmic ratio (NCR)), ploidy of mononuclear hepatocytes, and the proportion of binuclear hepatocytes were measured. The reliability of circadian rhythm (CR) was determined by cosinor analysis. The study indicates complex changes in the organization of rhythmostasis in the experiment. The chrono-destructive effect of experimental alcohol intoxication on the CR of the cell and NCR, as well as the chronomodulating effect to the CR of the nucleus are established. The effect of ethanol on the CR of ploidy and the number of binuclear hepatocytes, as well as on the nature of their variation at the studied time points is established. An increase in the ploidy of hepatocytes and an in the number of binuclear cells is revealed, which indicates the beginning of the deployment of adaptive-compensatory reactions in the organ.

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INTRODUCTION

One of the anthropogenic environmental factors that the human organism has to adapt to is alcohol - the most widely known psychoactive substance in the world, affecting almost all organs and physiological functions.

The development and progression of alcoholic disease largely depends on the level of basal metabolism of ethanol in the liver, which is genetically determined and has an individual nature [1,2]. This is due to the fact that the liver plays a prime role in intersystem cooperation, it is the basic regulator of metabolism in mammals, and this is what determines its importance as the main organ supporting homeostasis, and its morphofunctional state largely conditions the compensatory capabilities of the organism [3,4]. At the same time, the liver is the most vulnerable to alcoholism, and the pathological processes that occur in it significantly change the metabolism of other organs and systems, since even with drunkenness, which is the second stage of alcoholic disease, there is formation of direct and reverse pathological connections between liver, heart, and brain. It is natural that in alcoholic disease a change in the level of a significant number of biological constants occurs, including those characterizing the morphofunctional state of the liver. Among other objects of influence of alcohol, the biological rhythms are found [5-7]

The rhythmicity of functioning is one of the fundamental properties of all living systems of various levels of organization. Of all the biological rhythms, the most significant for mammals are circadian rhythms (CR) [8-11]. The temporal organization of mammalian organism systems, being genetically determined, nevertheless, is modulated quite plastically under the influence of periodic environmental factors - synchronizers, or pacemakers [12,13], the leading role among which belongs to the light regime. The successive cycles of life processes differ in their parameters - amplitude, phase. In those cases when the adaptation processes proceed normally, the degree of influence of stressors on circadian rhythms is insignificant. Otherwise, the rhythmic processes of the organism lose their correctness, regularity, desynchronosis occurs, which can lead to the development of diseases and pathological conditions [14-22].

At present, when considering the effect of alcohol on the mammalian organism, two areas of interest are distinguished. The first one focuses on the chrono-effecter action of alcohol, i.e. on how the effects of alcohol (i.e. its effectiveness) change depending on the time of day at which it is administered, that is, how alcohol interacts with the physiological components of the organism at a certain time of the day. The second area of interest is chronergic, using a wider approach, exploring mainly the effect of alcohol on biorhythms of other parameters of an organism [23].

Clinical and epidemiological observations have shown that alcohol abuse and alcoholism are associated with widespread disturbances in sleep and other circadian biological rhythms. [13].

The effect of alcohol on the circadian rhythms of an organism can be realized in several ways.

The first way consists of indirect influence to the CR-regulating genes. The expression of the main PAS domain containing circadian proteins (CLOCK, BMAL1, PER1, PER2, CRY1, and CRY2) is affected by the presence of alcohol, and the expression of each protein changes in the blood of people with alcoholic disease compared to the control [24-26]. The in vitro study showed that oxidative stress caused by alcohol metabolism leads to an increase in the expression of CLOCK and PER2 circadian proteins, which induces further dysfunction of the ensemble of circadian genes. It was shown that in the presence of alcohol, the circadian rhythm in suprachiasmatic nuclei measured using PER2 was not broken, but in the liver, alcohol caused a significant change in the phase of expression of circadian genes, accompanied by altered lipid metabolism with following the development of hepatic steatosis [27,28].

The second way of action of alcohol on CR is the way it affects extracellular pacemakers - suprachiasmatic nuclei of the hypothalamus, pineal gland. Morphofunctional changes occurring in these organs under the influence of alcohol naturally cause a violation of the circadian rhythms in organs and organ systems, depending on the function of central pacemakers [29].

In any case, alcohol has a pronounced chronic toxic effect, which causes desynchronosis [30-31].

The third way of influence of alcohol on CR includes both the effect on the central mechanisms of maintaining normal rhythm and the effect on the part of the genetic apparatus of the cells responsible for CR [33].

It was shown that alteration in CR, including alcohol-induced, is crucial for increasing the susceptibility of the large intestine and liver to alcohol damage and plays a direct role in the severity of their alcohol-induced pathology. [34-38]. A number of epidemiological and clinical studies show that disturbances in circadian homeostasis make organs such as the liver and intestines more susceptible to alcohol toxicity [39]. In continuation of studies on the development of metabolic disorders in mice, numerous studies in human alcoholics have shown altered expression of circadian genes [40-46]

At the same time, data on the effect of chronic alcohol intoxication on the daily rhythmicity of liver parameters are not numerous.

We found it important to study the diurnal dynamics of some micromorphometric parameters of hepatocytes in Wistar rats at age of 6 months under conditions of a fixed light regime. We studied the dynamics of the cross-sectional area of the nucleus and cells as an indicator of their activity and functional state, the dynamics of the nuclear cytoplasmic ratio (NCR) with the use of cosinor analysis, the dynamics of the number of binuclear hepatocytes, as well as the variation curves of the area and logarithms of the volume of hepatocyte nuclei in each of the studied time points.

MATERIALS AND METHODS

Animals

The study was conducted on 80 male Wistar rats at age of 6 months, weighing 300±20 g. Animals were taken from the Stolbovaya nursery (the "Stolbovaya" affiliate of the FSBIS "Scientific Center for Biomedical Technologies of the Federal Medical and Biological Agency).

Design of Experiment

Rats were divided into 2 equal groups. Animals of the first group served as control. The individuals were housed in plastic cages with free access to water under the conditions of a fixed light regime “light-dark” (10:14 hours) for 3 weeks. The animals of the second group (experiment) were kept under the same conditions, but instead of water, a 15% ethanol ad libitum solution was offered daily as a drink.

The criterion for the selection of rats in the experimental group, along with the absence of visible deviations in the state and behavior, was the initial preference for a 15% solution of ethyl alcohol to tap water. For this, a preliminary experiment was carried out for 3 days in individual cages with free access to both liquids.

Euthanasia was carried out three weeks after the start of the experiment in a carbon dioxide chamber equipped with a device for the upper gas supply (100% CO_2) at 9 AM, 15 PM, 21 PM and 3 AM. The chamber volume was filled with gas at a rate of 20% per minute to avoid dyspnea and pain in animals. After sacrifice, the liver was removed for morphological examination. All animal experiments were performed according to compliance with EC Directive 86/609/EEC and with the Russian law regulating experiments on animals.

Methods of Histological Studies

The liver was fixed in 10% neutral buffered formalin with further passage through alcohols of increasing concentration (50 °, 60 °, 70 °, 80 °, and 96 °) and xylol, followed by pouring into Histomix histological medium (BioVitrum, Russia). When conducting studies of organs embedded in paraffin, serial sections with a thickness of 5-6 μm were prepared. Histological sections were made on the rotor microtome MPS-2 (USSR). Hematoxylin-eosin staining was carried out according to the standard technique. Stained sections were put in a BioMount mounting medium (BioVitrum, Russia).

Microscopy of histological preparations was performed using a Nikon Eclipse 80I digital microscope with the use of a Nikon DI-FI digital camera (Japan). For microscopy, eyepieces ×10, ×15, lenses ×4, ×10, ×20, ×40, ×100 were used. From each studied preparation, 10 digital images of randomly selected visual fields were taken at a magnification of ×400, ×1000, with the use of which karyo- and cytometry were subsequently carried out, the daily dynamics of the nucleus and cells was determined, estimated by their area and nuclear-cytoplasmic ratio. In morphometric studies, the ImageJ program (USA) with the appropriate plug-ins was used to determine the cross-sectional area of hepatocytes and the cross-sectional area of their nuclei [47,48]. The measurements were carried out in micrometers after preliminary geometric calibration on an object-micrometer scale digitized with the same magnification. The nuclear-cytoplasmic ratio in the cells was calculated according to the formula: NCR = Sn/Sc, where: Sn - cell nucleus area; Sc - area of cytoplasm. Then the data array was divided into equal class

intervals in accordance with the rules set out in the guide [49].

Steatosis (percentage of hepatocytes containing lipid droplets) was scored using the non-alcoholic fatty liver disease (NAFLD) activity scoring (NAS) protocol [50,51]. While the NAS protocol is not intended for AD, we applied this system to assign a histopathology score to cases in this experimental animal study. Steatosis was scored as: 0, <5%; 1, 5%-33%; 2, >34%-65%; and 3, >66% of hepatocytes containing lipid droplets.

For ploidometry, paraffin sections were stained with methylene-green - pyronin G, which is then followed by processing of sections with RNA-ase. The hepatocyte ploidy was calculated in units of ploidy relative to the optical density of the staining results of diploid nuclei of small lymphocytes [52,53]

Micromorphometry of only mononuclear interphase hepatocytes without signs of pathological changes was carried out.

To determine the proportion of binuclear hepatocytes, we examined 10 fields of view from each micropreparation with a magnification of the eyepiece ×40. The total number of hepatocytes in the field of view and the number of binuclear cells were determined, and then the percentage of binuclear cells was expressed as a percentage of the total number of hepatocytes.

Methods of Statistical Processing

The obtained data were analyzed using the GraphPad Prism 6.0 program by calculating average values, standard deviation, and arithmetic mean error. The numerical rows characterizing the diurnal fluctuations of the studied physiological rhythms of animals were subjected to mathematical processing, on the basis of which group chronograms were drawn. We studied the form of chronograms and calculated daily average values. Statistical differences in studied parameters were determined using the t-student test. A p-value <0.05 was considered statistically significant.

For the statistical estimation of the amplitude and acrophase of CRs, cosinor analysis was performed, which is an internationally recognized method for the unified study of biological rhythms using the CosinorEllipse2006-1.1 program. The presence of a reliable circadian rhythm was determined, as well as its acrophase and amplitude. Acrophase is a measure of the peak time of the total rhythmic variability over a 24-hour period. The amplitude corresponds to half the total rhythmic variability in the cycle. Acrophase is expressed in hours; amplitude values are expressed in the same units as the studied variables [54,55].

RESULTS

During histological examination, we found that the structure of the liver of rats in the control group corresponds to the norm (Figures **1**,**2**). In the liver of rats of the experimental group (Figures **3**,**4**), the beam structure of the liver was preserved, 12.1±0.57% of hepatocytes became round, with eccentrically located nuclei and vacuoles, indicating the development of steatosis, observed in the cytoplasm. Simultaneously, in the liver of rats of the control group, the proportion of cells in the state of fatty degeneration was 2.40±0.22%. Thus, the steatosis grade was 0 in the control and 1 in the experiment.

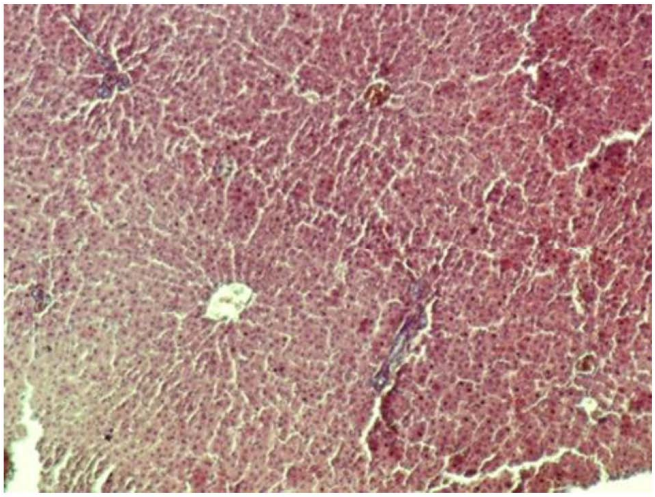

Figure 1: Liver of rat of control group, H&E, ×100.

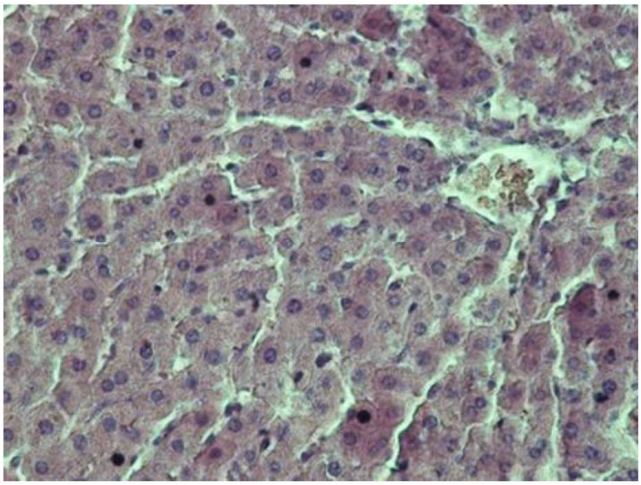

Figure 2: Liver of rat of control group, H&E, ×400.

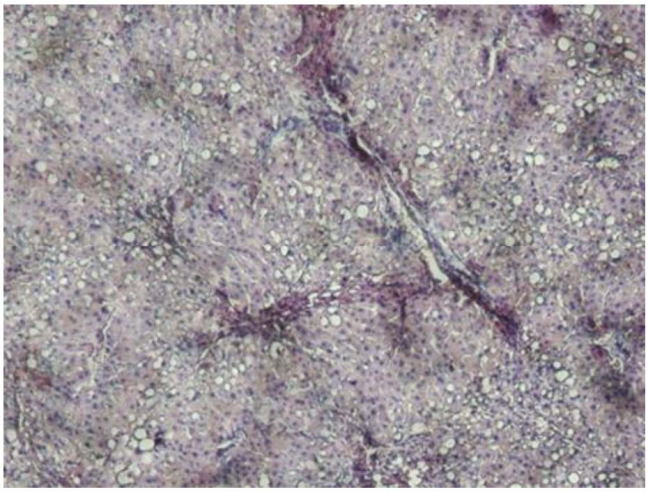

Figure 3: Liver of rat of experimental group, H&E, ×100.

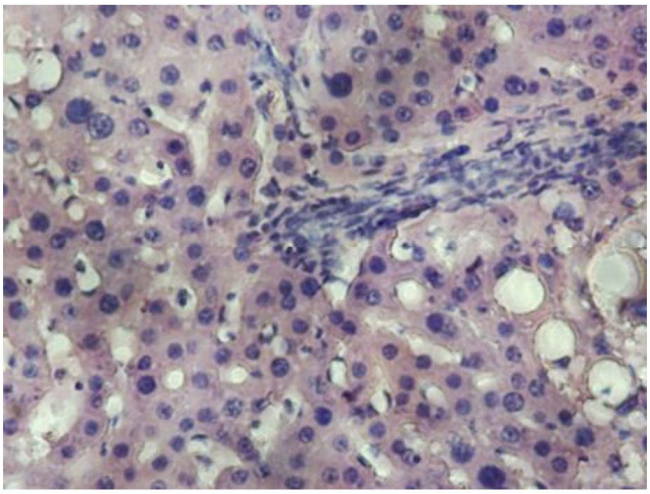

Figure 4: Liver of rat of experimental group, H&E, ×400.

As a result of the study, we found the absence of significant differences in daily average values of the studied micromorphometric parameters (Table **1**).

When considering the daily dynamics of the nucleus in the control, it was found that the maximum cross-sectional area of the hepatocyte nuclei was reached in time point of 15 hours, and then a significant decrease in the value of this parameter to a minimum, which fell in 21 hours, was noted (Figure **5**). In the experimental group, with a maximum remaining at 15 hours, the minimum values were found at 9 hours, but in general, the chronogram was smooth. The results of cosinor analysis show the presence of the reliable circadian rhythm of the cross-sectional area of the hepatocyte nucleus in the control and its change in the liver of rats of the experimental group (Table **2**).

At the consideration of diurnal dynamics of cells, it can be established that there is a presence of reliable circadian rhythm in the control group, but it was

Table 1: The Average Daily Values of the Studied Micromorphometric Parameters

	Area of nucleus of hepatocyte, μm^2	Area of hepatocyte, μm^2	NCR
Control	41.79±8.13	185.80±31.95	0.230±0.056
Experiment	42.65±4.80	190.10±34.03	0.234±0.008

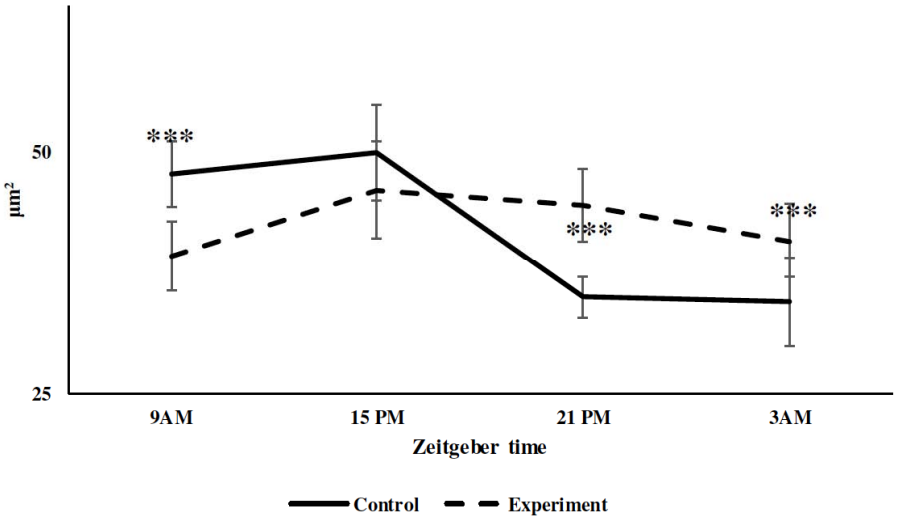

Figure 5: Diurnal dynamics of area of nuclei of hepatocytes.

Hereinafter: *(P≤0,05); **(P≤0,005); ***(P≤0,0005) – statistical significance of differences in comparison with the control group.

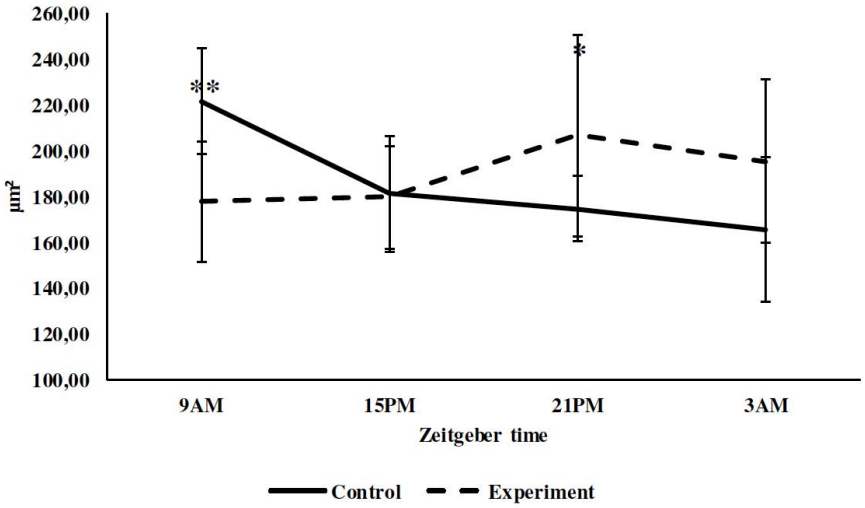

Figure 6: Diurnal dynamics of area of hepatocyte.

destroyed in the experiment. Wherein, the maximum value of the parameter in the liver of control rats was revealed at 9 hours followed by a decrease during the day to the minimum, which was noted at 3 hours (Figure **6**). In the liver of rats of the experimental group, hepatocytes reached their maximum sizes at 21 hours, with a further decrease to the minimum at 9 hours.

Similarly, as with other studied parameters, NCR at the control group had a reliable CR (Table **2**), but it collapsed in the liver of rats of the experimental group. Herewith, the maximum value of NCR was noted at 15 hours, then going down to the minimum at 21 hours (Figure **7**). On the chronogram illustrating the dynamics of the NCR of the rats of the experimental group, there was a slight peak at 15 hours.

When analyzing the graph of the average daily distribution of hepatocyte nuclei by area (Figure **8**), one peak of nuclei (15.3% of all nuclei), whose area lies in the range of 35–40 μm^2, was clearly distinguished in the control. In the experimental group, the karyogram had a two-headed top. The first peak of the nuclei (17.68%) lies in the range of 40-45 μm^2, the second peak with a distribution range from 45 to 50 μm^2 includes 17.88% of the nuclei.

However, when considering the histograms of the distribution of nuclei over the area at the studied time points, the picture differs significantly from the average daily histogram in its group and from the curve of another group.

So, at 9 hours the maximum number of nuclei in the control (20%) had sizes in the range of 50-55 μm^2, but in the experimental group, the maximum number of nuclei (19.7%) lied in the previous range - 45-50 μm^2.

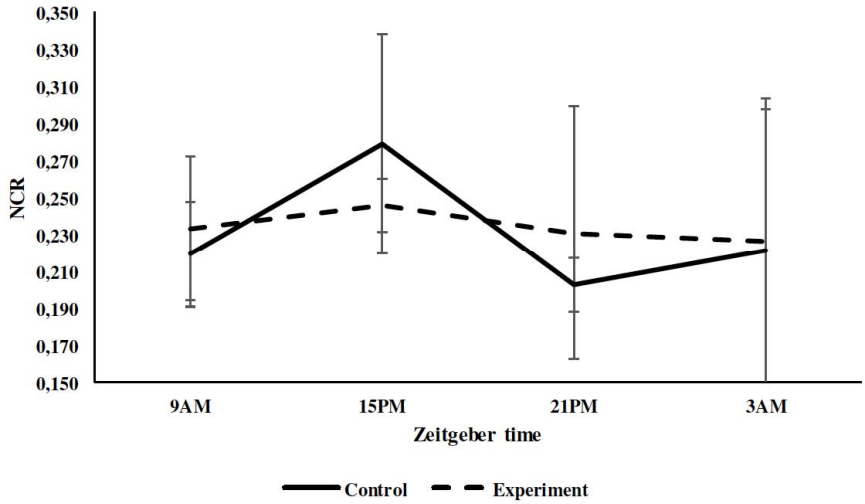

Figure 7: Diurnal dynamics of NCR.

Table 2: Amplitude-Phase Characteristics of Studied Micromorphometric Parameters of Hepatocytes (Based on the Results of Cosinor Analysis)

Parameter	Mesor	Acrophase of rhythm	Amplitude of rhythm
Area of nuclei of hepatocyte, control	41.79 μm^2	12^{21}	10.03 μm^2
Area of nuclei of hepatocyte, experiment	42.63 μm^2	17^{54}	3.73 μm^2
Area of hepatocyte, control	185.84 μm^2	10^{13}	24.84 μm^2
Area of hepatocyte, experiment	No reliable CR		
NCR, control	0.230	13^{56}	0.030
NCR, experiment	No reliable CR		

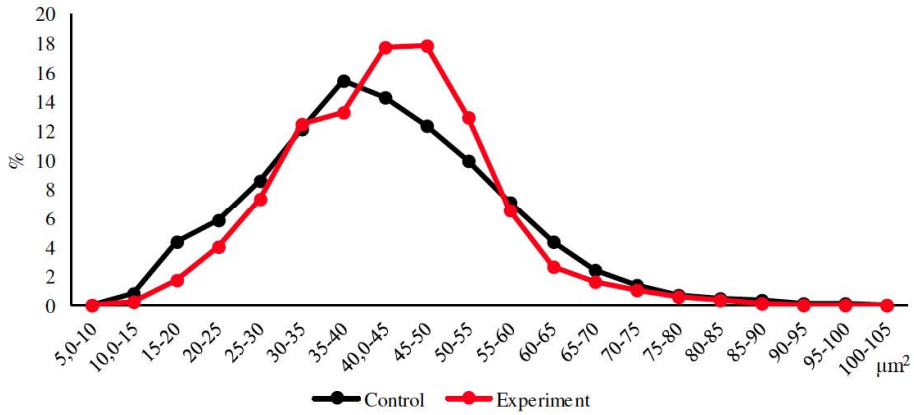

Figure 8: Variation curve of the average daily distribution of hepatocyte nuclei by area.

At 15 hours the curve of the distribution of nuclei by area in the control became more gentle, and the maximum of nuclei with an area in the range of 60-65 μm^2 was observed in 14% of cases. In the experimental group, the distribution curve of hepatocyte nuclei was also slightly shifted to the right, a clear peak was distinguished on it, which amounted to 17.40% of the nuclei lying in the range 55-60 μm^2.

By 21 hours, the curve of the distribution of the area of nuclei in the control shifted significantly to the left, the largest part of nuclei (23.5%) had an area of 35-40 μm^2. By 3 hours the same peak remained, but it makes up 20.6% of the nuclei.

In the experiment at 21 hours, 2 equivalent peaks were detected, which make up 20.20% of the nuclei, lying respectively in the ranges of 40-45 μm^2 and 45-50 μm^2. At 3 hours in this group, the curve had a more flattened shape with a plateau of nuclei in the area range from 30-35 μm^2 to 45-50 μm^2, the maximum number of nuclei fell in the range of 35-40 μm^2 - 17.90%.

When considering the results of ploidometry, we found that the average daily ploidy of the studied hepatocytes in the control was 4.47±2.12n, in the experiment the ploidy was 5.02±2.18n; 3 groups of cells were revealed among the studied hepatocytes - diploid, tetraploid, and octaploid, the percentage of which varies during the day (Figures **9**,**10**).

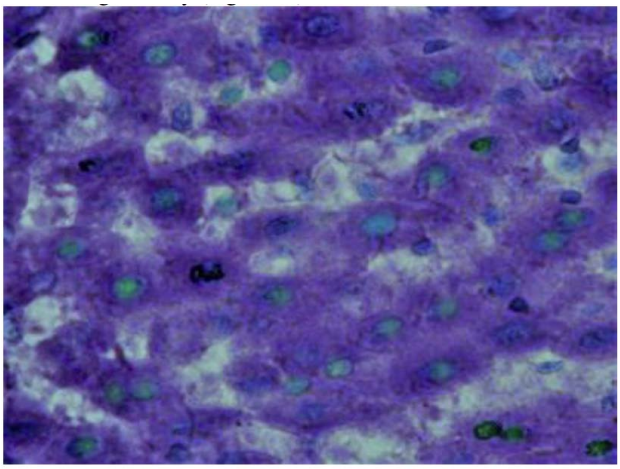

Figure 9: Liver of rat of control group, methylene-green - pyronin G, ×400.

Accordingly, we established the diurnal dynamics of rat hepatocyte ploidy in the studied conditions (Table **3**). In

particular, it was found that the proportion of diploid hepatocytes in the liver of animals of the control group in the morning and afternoon hours is minimal, but it increases significantly in the evening and night hours, and this, apparently, is due to a decrease in the proportion of octaploid nuclei.

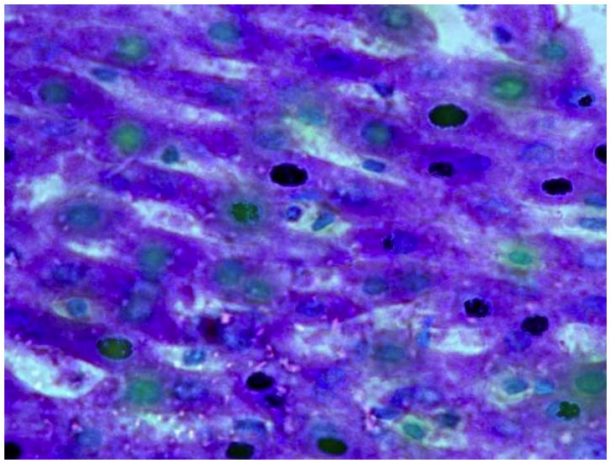

Figure 10: Liver of rat of experimental group, methylene-green - pyronin G, ×400.

In the liver of rats of the experimental group, diurnal oscillations of the nuclei of all ploidy groups were observed, but, unlike the control, the proportion of diploid nuclei was greater in the morning and afternoon. The fraction of tetraploid cells experienced the least diurnal fluctuations, and the minimum percentage of octaploid cells was noted at 9AM. As in the control, tetraploid nuclei are the least variable.
Moreover, in the experimental group there was a decrease in the number of diploid nuclei, but an increase in the proportion of octaploid nuclei.

The study of the nature of the average daily fluctuation of ploidy of the studied hepatocytes showed that the maximum ploidy in the control was observed at 15PM, and the minimum - at 21PM. In the liver of rats of the experimental group, the chronogram was significantly smooth, the maximum ploidy was noted at 15PM, the minimum - at 9AM.

We found that the proportion of binuclear hepatocytes in the liver of rats of the experimental group was 9.08±3.59%, which is higher than the percentage of such cells in the control - 7.44±2.66%.

At the same time, in the control group, the maximum number of binuclear hepatocytes was noted at 21PM, and the minimum - at 9AM, but in the liver of rats of the experimental group the maximum proportion of hepatocytes with two nuclei was found at 9 AM, and it was minimal at 3 AM (Figure **11**).

DISCUSSION

As a result of the study, we found that chronic alcohol intoxication does not cause reliable changes in the average daily values of the nucleus, cell and NCR. At the same time, the change in the diurnal dynamics of these parameters was found, which manifests itself in smoothing of the chronograms of the nucleus and NCR with the disappearance of the expressed extreme points, and in the inversion of the cell chronogram in the experiment relative to the control chronogram. Chronic alcohol intoxication within three weeks causes the development of steatosis in the liver.

According to the cosinor analysis, we found the destruction of the CR of the cell and NCR at revealed

Table 3: Dynamics of Ploidy of Hepatocytes during the Day

Time point	Ploidy of nuclei of hepatocytes		
	2n,%	4n,%	8n,%
9 hours, control	10.4±0.24	51.6±2.67	38.0±1.62
9 hours, experiment	21.4±1.20**	59.5±3.21*	19.1±0.95***
15 hours, control	11.1±0.68	40.2±2.0	48.4±1.90
15 hours, experiment	12.6±0.79	39.1±1.89	48.3±1.95
21 hours, control	35.3±2.11	61.6±2.95	1.8±0.2
21 hours, experiment	9.1±0.50***	54.5±2.54*	36.4±2.55***
3 hours, control	39.1±0.2.68	55.0±2.41	4.7±0.33
3 hours, experiment	13.5±0.72***	60.8±3.25	25.7±1.26***
Average value during the day, control	23.98±1.54	52.1±2.21	23.23±1.20
Average value during the day, experiment	14.15±0.62***	53.47±2.56	32.38±1.88***

Hereinafter: *(P≤0,05); **(P≤0,005); ***(P≤0,0005) – statistical significance of differences in comparison with the control group.

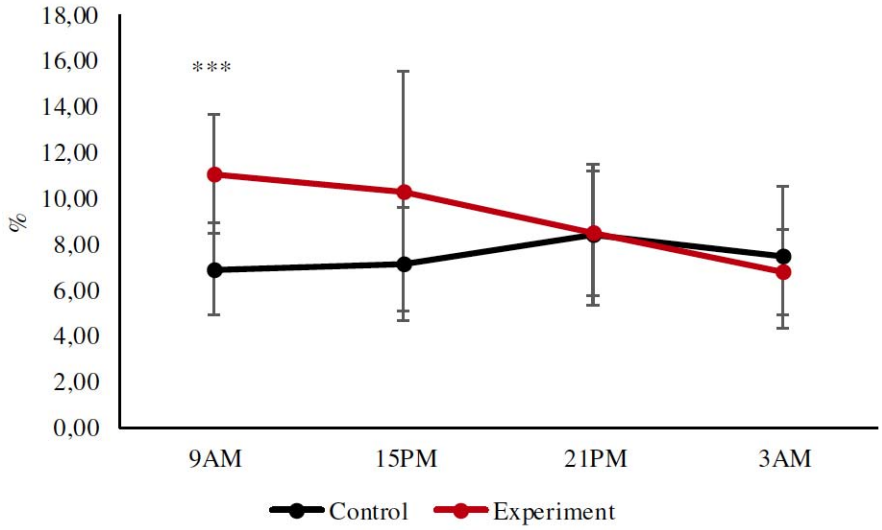

Figure 11: Daily dynamics of the number of binuclear hepatocytes in rat liver.

maintaining of the rhythm of the nucleus. But the rhythm of this parameter in the experiment is characterized by a shift of the acrophase to early evening hours from the late morning hours in the control, as well as a significant decrease in the rhythm amplitude with a practically unchanged mesor.

At the same time, alcohol intoxication caused a shift in the variation curve of the size of the nucleus to the right, which indicates an increase in the proportion of nuclei with a large size. The nature of nuclear fluctuation at each of the studied time points also changes. Also, under the influence of ethanol, an increase in the degree of ploidy of the studied hepatocyte population occurred due to a decrease in the proportion of diploid cells and an increase in the proportion of octaploid cells, but the average daily ploidy fluctuations were less pronounced than in the control.

In addition, under the influence of alcohol intoxication, there was an increase in the number of binuclear hepatocytes relative to control parameters, and the daily dynamics of their content also differed from the control.

Thus, we have established the chrono-destructive effect of experimental alcohol intoxication in relation to the CR of the rhythm of the cell and NCR, as well as the chronomodulating effect in relation to the CR of the nucleus.

The death of liver cells, including that which occurs under the influence of alcohol, stimulates the regeneration of the liver, and its main mechanisms are proliferation, polyploidy and hypertrophy of hepatocytes; polyploidy, and, to a lesser extent, proliferation, lead to an increase in the number of genes in cells [56-60].

The increase in nuclear ploidy of the studied hepatocyte population, as well as an increase in the number of binuclear cells, indicate the beginning of hypertrophic changes in the liver, since it is initially manifested by polyploidization of their nuclei, and the formation of binuclear cells as a result of acitokinetic mitosis is a key step in the process of cell polyploidization [61-64]. The fact that we have not established hypertrophy of the hepatocytes themselves indicates that at this stage the exposure to ethanol does not cause hypertrophic changes at the tissue and cellular level, but they are carried out at the level of the cell nucleus.

In addition, an increase in ploidy is the initial stage of regenerative processes in the liver was shown. According to researches [65-71] with various models of liver damage, it is polyploid hepatocytes that have extensive in situ regenerative ability and regularly undergo mitosis during regenerative reactions. C. Kreutz *et al*., 2017 [72] put forward the hypothesis that ploidy of nuclei is a new factor in the diversity of hepatocytes, and hepatocytes with polyploid nuclei may have other biological functions than diploid ones. This diversity does not depend on the well-known heterogeneity associated with the position of cells along the central axis, which covers the distance between the portal and central veins of the lobule [73-75].

Thus, the increase in the nuclear and cellular ploidy of hepatocytes in the liver of rats under conditions of chronic alcohol intoxication indicates the beginning of the deployment of adaptive-compensatory reactions in the organ.

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CONFLICT OF INTERESTS

The authors declare that there is not any conflict of interest.

COMPLIANCE WITH ETHICS GUIDELINES

All the experimental protocols were performed in accordance with ethical guidelines approved by the Research and Ethics Committee of Scientific Center for Biology of Cells and Applied Biotechnology of the Moscow State Regional University, Moscow, Russian Federation prior to executing the experiments. Experiments were performed as per “Directive 2010/63/EU of the European Parliament for animal use for scientific purpose” and “NIH Guidelines for the Care and Use of Laboratory Animals”.

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