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Circadian rhythm disruption and chronic alcohol intake: effects on oxidative stress levels and cognitive abilities

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Experimental and clinical data demonstrate that circadian misalignment and extreme alcohol consumption often occur together and are associated with enhanced oxidative stress levels. Furthermore, excessive free radical formation is related to numerous adverse health conditions, and learning and memory deficits. The aim of the present study was to investigate the single and combined effects of light-at-night and alcohol abuse on oxidative stress levels and cognitive functions of male rats.

Male Wistar rats were separated in four groups: I group – Control (C); II group - Alcohol (A) (10 % ethanol, ad libitum for a period of six weeks); III group – Circadian Rhythm Disruption (CRD) (constant light for a period of six weeks); IV group – CRD+A.

Our results showed that both models (A) and (CRD) caused increased lipid peroxidation in the plasma of the experimental animals. Also, we observed impaired learning and memory abilities of the rats exposed to disrupted circadian rhythm and chronic alcohol intake. In addition, the cognitive decline corresponded with the activity of xanthine oxidase in the plasma.

The correlation between the learning deficits and the oxidative stress markers indicated an interconnection in the underlying mechanisms of the experimental models which might be an intriguing field for future investigation.

Key words: circadian rhythm disruption, alcohol, cognition, oxidative stress, shuttle-box

INTRODUCTION

Disruptions of natural circadian light rhythms due to shift work, exposures to night-time lighting, sleep disorders, etc. are well-documented challenges in modern life. Recent reports demonstrate that abnormal light-dark cycles promote a range of pathologies and may have multiple deleterious effects on human health [1, 2, 3]. Literature data link circadian misalignment to increased risk of physical (cardiovascular disease, metabolic disorders, cancer, infertility) and neuropsychiatric (depression, schizophrenia, ADHD) conditions [4, 5]. Experimental and clinical evidence indicates that circadian disruption may also impair cognitive abilities and is able to cause learning and memory deficits [3, 6, 7]. On the other hand, research data suggest an interconnection between circadian and redox control systems [8]. It is proposed that altered circadian rhythms may provoke oxidative stress and this could be a pathophysiological basis for various adverse health consequences.

Chronic alcohol intake is known to induce cognitive deficits and memory impairments [9, 10]. The underlying molecular mechanisms often involve neuroinflammation and enhanced levels of oxidative stress [11, 12]. Experimental data suggest that the metabolism of ethanol is associated with excessive formation of reactive oxygen species [13, 14]. In addition, epidemiological and animal studies demonstrate increased oxidative stress in chronic and binge models of alcohol administration [15, 16].

It is well established that oxidative stress causes a range of pathological changes in the body and mediates a myriad of diseases. In the last decades enhanced oxidative stress levels have been increasingly recognized to accompany neurotoxicity, neuronal cell loss, unbalanced neurotransmitter levels and cognitive functions impairment [17, 18, 19]. Also, excessive lipid peroxidation is associated with learning and memory failures [20, 21]. Both animal and human studies have revealed that circadian rhythm disruptions often occur together with substance abuse [22, 23]. Furthermore, research evidence documents bidirectional interactions between circadian dysregulations and excessive alcohol consumption [24]. The basic mechanisms that are involved in their combined effects are an interesting field of research. The aim of the present study was to investigate the effects of circadian rhythm disruption and chronic alcohol intake, alone or in combination, on cognitive functions and plasma oxidative stress levels of rats.

MATERIALS AND METHODS

The experiments were carried out in accordance with the Bulgarian regulations on animal welfare,

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in conformance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and with the approval of Medical University-Sofia ethics committee, Protocol #1156/25.06.2008.

1.Animals

Male Wistar rats (250-300g) were housed 1 per standard polypropylene cage and maintained in a temperature (20±0,5°C) and humidity (65±1%) controlled room for 6 weeks. The animals had free access to food (standard rodent chow) and tap water or 10% ethanol solution. 2-3 days before the experiment the animals were handled and then randomly assigned into four groups (n=5).

Group 1 – ***Control*** – normal light/dark cycle + tap water ad libitum;

Group 2 – ***Alcohol (A)*** - normal light/dark cycle + 10% ethanol solution ad libitum;

Group 3 – ***Circadian rhythm disruption (CRD)*** – exposed to constant light (24/7) + tap water ad libitum;

Group 4 – ***Circadian rhythm disruption + Alcohol (CRD+A)*** - exposed to constant light (24/7) + 10% ethanol solution ad libitum.

2. Chemicals

All chemicals used in the investigation were SIGMA and of finest grade (p.a.). The water was distilled and degassed by sonification, if necessary. The 10% ethanol solution was prepared using 99% ethanol.

3. Blood collection and determination of oxidative stress markers

The blood was collected in EDTA washed test tubes, and the plasma was separated by centrifugation at 2000XG (4°C) for 30 minutes. The protein content of the samples was determined using Biuret method [25]. A 552 UV-VIS spectrophotometer "Perkin-Elmer" with 2ml quartz cuvettes was used for the spectrophotometric measurements.

4.XOA assay

XOA was measured as described by Praja [26]. To determine the Uric Acid (UA) formation the characteristic absorbance at λ=293 nm was monitored for 10 minutes at 298K (the molar extinction coefficient for UA ε= 12400 $m^{-1}cm^{-1}$). One ml of the cuvette contained 0.01 ml supernatant, 0.01 ml 0.3 mM Xanthine (distilled water), and PBS (pH 7.4). One unit (U) of XO activity was defined as the amount of XO producing 1 μmole UA for 1 minute, in 1 ml solution, at 25°C. The XOA was expressed as mU/mg protein.

MDA assay

The malondialdehyde formation was assessed by using the method of Bohnstedt [27]. The characteristic absorbance of MDA at λ=245 nm was monitored for 5 minutes at 25°C, in presence (sample) and in absence (blank) of supernatant. One ml of the cuvette contained 0.01 ml supernatant, 0.01 ml $FeCl_2$/EDTA (3 mM $FeCl_2$ and 0.2 mM EDTA in distilled water) and BPS (pH 7.4). Molar extinction coefficient of 13700 M^{-1} cm^{-1} was used to calculate the MDA and after removing blank from sample measurements, the MDA formation was presented in pmoles/mg protein.

For better understanding, each oxidative stress marker was presented by its Activity Index (AI), as percentage of the corresponding marker for the Control group (e.g., AI = $Marker_{stress}$*100/$Marker_{control}$). The AI for XOA and MDA formation were shown in Figures 3a and 3b.

5. Evaluation of cognitive functions

An automatic reflex conditioner for active avoidance "shuttle box" (Ugo Basile) was used. The apparatus consisted of a standard rectangular shuttle box with Plexiglas walls and stainless steel grid floor. The box was divided into 2 compartments with a plate. There was a hole in the bottom of the plate through which rats could shuttle freely. A learning session of 5 consecutive days was performed. Each day consisted of 25 trials with the following parameters: 4 seconds of light and buzzer (conditioned stimulus) (1000 Hz and 60 dB), 6 seconds of 0.5-mA foot shock (unconditioned stimulus), and 8-second pause. The parameters automatically counted were as follows: (1) latency time (accumulated time before rat's crossing in the other compartment while the stimuli are on) and (2) number of unconditioned stimuli (escapes from the foot shock).

6. Statistical analysis

Each OS marker was determined three times for each animal. Thus, an oxidative marker of a group was estimated using nine parallel measurements. After elimination of the gross errors via Romanowski test [28], the mean values and standard deviations were calculated. The statistical significance of differences among mean data was estimated by INSTAT program package (Bartlett test for significance of differences among the standard deviations followed by ANOVA and Bonferoni post-test).

RESULTS

In the shuttle-box active avoidance test the control rats showed decrease in the latency time in the course of the learning sessions compared to the 1st day (Figure 1). The "alcohol" group demonstrated substantially increased latency time compared to the control group on every day of the learning sessions (p<0,01). "CRD" group had longer latency time on the 1st and on the 2nd day of the learning sessions compared to the control group (p<0,01). "CRD+A" rats demonstrated inconsistent learning skills in the learning sessions but no significant differences with the control group. At the end of the experiment the "CRD+A" group showed considerably shorter latency time than the "A" group and longer latency time than the "CRD" group.

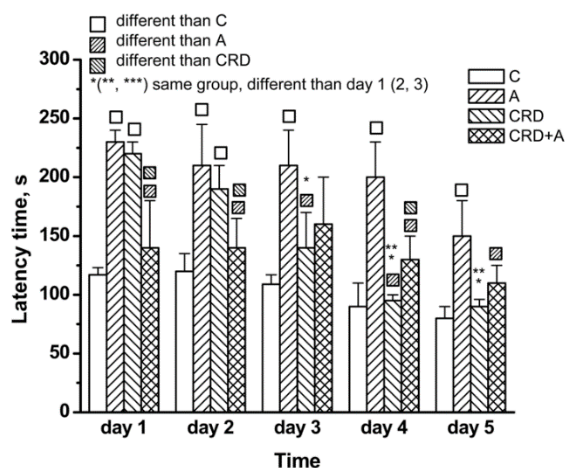

Figure 1. Effects of alcohol and circadian rhythm disruption on the latency time in the shuttle-box test.

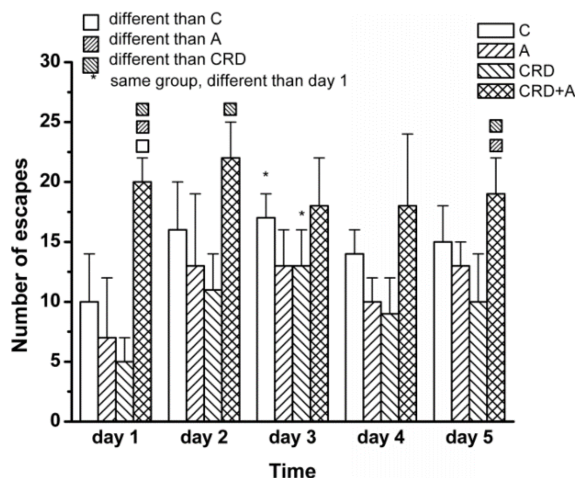

Figure 2. Effect of alcohol and circadian rhythm disruption on the number of escapes (unconditioned stimuli) in the shuttle-box test.

The control group demonstrated increased escape proportions through the end of the experiment (Figure 2). On the third day of the learning sessions the number of escapes was significantly enhanced compared to the 1st day (p<0,01). On the other days no statistically significant differences from the 1st day were registered because of very high standard deviations in the group. In all days of the learning sessions there was observed a considerable decrease in the number of escapes in the "A" and "CRD" groups compared to the same day controls. Again, there was no statistical significance due to high standard deviations in the groups. Interestingly, "CRD+A" rats demonstrated increased number of reactions to unconditioned stimuli compared to the same day "A" and "CRD" rats. However, the escape rate in the "CRD+A" group through the course of the learning sessions was almost the same on each day and the rats did not demonstrate significant improvement of this parameter.

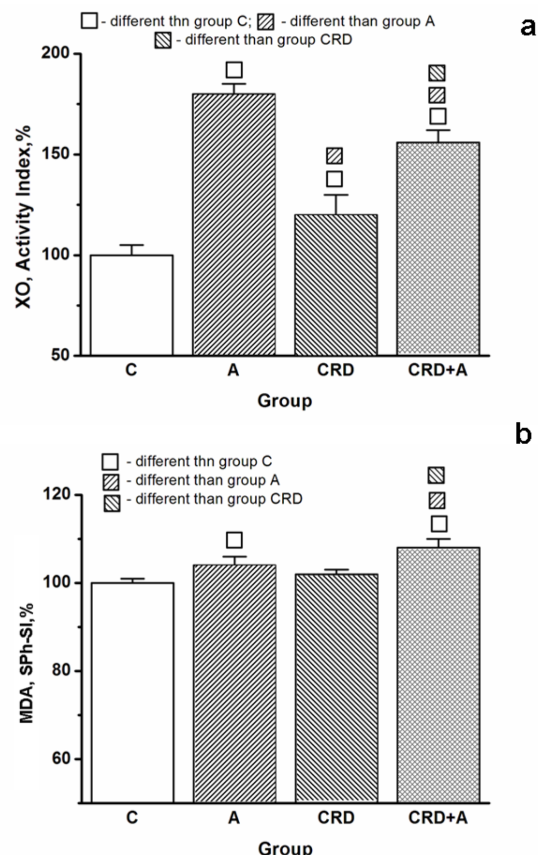

Figure 3. Effects of alcohol and circadian rhythm disruption on activity indices of XOA (3a) and MDA levels (3b) in blood plasma of the experimental animals

Blood plasma MDA levels of the experimental animals decreased in the order (CRD+A)>(A)>(CRD)>(C). The relative increase of MDA was higher in the group exposed to the combination of CRD and alcohol compared to this

in the groups exposed to each of the experimental models alone.

The activity of xanthine oxidase in the blood plasma was higher in the group exposed to alcohol intake, and lower in the control group. The XO activity of the group exposed to the combination of CRD and alcohol was lower than this of the animals exposed only to alcohol, but higher than this of the group exposed only to CRD.

DISCUSSION

The present study provides evidence that circadian rhythm disruption and chronic alcohol intake caused significant learning and memory deficits. The experimental animals from the "CRD" and "A" groups demonstrated increased latency time and decreased escape rate in the shuttle-box test (Figure1 and Figure 2).

Our data also showed that all experimental models produced increased oxidative stress levels in the rats' plasma. Additionally, our results suggest that the combined influence of the experimental models of CRD and chronic alcohol can exacerbate the single adverse effects of each model alone.

The data presented here are consistent with our previous results which have demonstrated that circadian misalignment and chronic alcohol intake induced significant oxidative stress in the brains of the experimental animals [29]. In this study, similar effects were observed in the blood plasma. It may be assumed that the combination of light-at-night and excessive ethanol consumption caused substantial lipid peroxidation and enhanced oxidative stress levels with harmful outcomes for the entire organism of the experimental rats.

A balanced generation of ROS and regulated redox modifications of transcription factors or enzymes play an essential role in the physiological regulatory functions. However, it is well established that excessive levels of ROS and the resulting oxidative stress can cause severe damage to mitochondrial and cellular proteins, lipids, and nucleic acids. Oxidative stress is a common feature and a major mediator of various adverse health conditions. The brain is highly susceptible to oxidative stress due to its high oxygen consumption, high fatty acids levels, and low antioxidant enzyme levels. The hippocampus is a part of the brain which plays an important role in cognition, mood regulation, responses to stress, learning and memory functions, and is particularly vulnerable to oxidative damage [30]. Data from the literature show that excessive free radical levels are associated with learning failures and cognitive decline [31, 32]. Additionally, we have previously shown decreased neuronal density in hippocampal regions associated with impaired cognitive functions of rats with circadian misalignment [33]. This is in consent to a recent finding which has demonstrated that circadian disruptions lead to marked suppression of hippocampal cell proliferation and neurogenesis, associated with deficits in learning and memory [7].

Substantial evidence shows that circadian misalignment and ethanol abuse are frequently associated and the link is bidirectional [34, 35]. Furthermore, experimental data demonstrate enhanced oxidative stress levels in animal models of alcohol intake [13, 15] and circadian disruption [36]. It has been shown that cognitive processes are exquisitely sensitive to changes in the redox balance [37]. Clinical and experimental evidence demonstrate that alcohol consumption is related to learning and memory deficits and these are often associated with activation of oxidative-inflammatory cascade in different brain regions [38, 39].

Our current results showed that alcohol intake induced cognitive impairment in the experimental rats. In the shuttle-box test the animals treated with ethanol solution demonstrated substantially increased latency time and decreased escape rates in comparison with the control animals. Furthermore, the MDA levels were significantly increased in all experimental groups and the highest relative increase was registered in the animals with disrupted circadian rhythm and simultaneously treated with alcohol. In the same time the XO activity index was remarkably higher than this of the control group. This might be related to the fact that acetaldehyde which is a metabolic product of ethanol is also a substrate for xanthine oxidase. The increased XO activity index in the CRD group may be associated with adaptive overreaction to the exposure to constant light. The collective effect of both models (CRD+A) was lower compared with group A, but higher than this of group CRD. These relative changes might be explained with the adjustment of the animals to the more demanding living conditions, in which the effect of CRD dominated this of alcohol.

The data in Figures 3a and 3b suggest a possible activation of XO by acetaldehyde. The activity of XO did not correspond to the levels of MDA. This might happen only if XO is being involved in transformation of acetaldehyde instead of purine metabolism. Nevertheless, in the group CRD+A the lipid peroxidation was significantly higher ($P<0.001$) than in all other samples. The results in Figures 3a and 3b suggest that XO was involved

both in the purine metabolism (group CRD) and the acetaldehyde transformation (A, CRD+A). Our data also propose that in the collective model (CRD+A) the CRD effect is prevailing the effect of ethanol.

In agreement with the abovementioned reports and our previous results our current data showed that light at night and chronic alcohol consumption induced significant oxidative stress and impaired learning and memory in the experimental animals. Moreover, our findings suggest that the combination of circadian misalignment and excessive alcohol intake may lead to significantly higher levels of MDA and oxidative damage in the rat plasma, than each of them alone. These facts propose shared mechanisms between circadian rhythm disruptions and abusive alcohol intake which overlap with an imbalanced oxidative status. Further investigations in this field are needed for a detailed understanding of these observations. An improved comprehension of the underlying links between circadian misalignment and alcohol abuse may be of significant clinical value and may contribute to the development of more advanced therapeutic approaches for individuals at risk.

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