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Circadian misalignment and alcohol intake change the oxidative status of rat blood plasma

M. G. Varadinova[1,] *, M. L. Valcheva-Traykova[2]

[1] *Department of Pharmacology and Toxicology, Medical Faculty, Medical University - Sofia, Bulgaria*
[2] *Department of Medical Physics and Biophysics, Medical Faculty, Medical University - Sofia, Bulgaria*

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Circadian misalignment and chronic alcohol intake often occur together and are known to result in decreased endogenous antioxidant resistance and oxidative stress in human and animal models. This is associated with a myriad of chronic health consequences and thus may have a large-scale financial burden on society and healthcare system. There is evidence for the antioxidant activity of ethanol in model solutions. However, some data suggest that ethanol can display pro-oxidative properties *in vivo*.

The aim of this study was to investigate the effect of *in vitro* ethanol supplementation on the oxidative status of rat blood plasma in models of circadian rhythm disruption (CRD) and chronic alcohol consumption (A). Our results demonstrated that ethanol exhibited pro-oxidative activity in blood plasma. The plasma oxidant status was impaired in both models. Moreover, the combination of CRD and A increased malondialdehyde (MDA) levels nearly twice.

Our data suggest that the combined influence of CRD and A can exacerbate the single adverse effects of each factor on the plasma oxidative status. The explanation of these observations needs further investigation on mechanisms of association between circadian misalignment and chronic alcohol consumption.

Key words: chronic alcohol intake; circadian rhythm disruption; malondialdehyde; oxidative stress.

INTRODUCTION

Epidemiological data reveal that disruption of circadian rhythm due to shift work, jet-lag, sleep disorders, and other modern life style choices and work practices is now very common in our society. Circadian misalignment is associated with a wide variety of adverse health consequences including cancer, metabolic disorders, cardiovascular dysfunction, immune dysregulation, impaired reproduction and neuropsychiatric conditions [1, 2, 3].

Recent studies have reported that disrupted circadian rhythms and increased alcohol consumption are often related [4, 5, 6]. Moreover, it has been suggested that the link is bidirectional [7]. Circadian misalignment and excessive alcohol intake may have considerable harmful effects on molecular and organismal levels [7, 8]. It has been proposed that circadian and redox regulatory systems are tightly interconnected [9]. Experimental evidence demonstrates that circadian misalignment may cause substantial alterations on the redox balance and may enhance susceptibility to lipid peroxidation [10, 11, 12]. In addition, chronic and acute models of alcohol exposure are reported to produce increased oxidative stress [8, 13, 14].

In the literature, there is a controversy about the pro-oxidative/antioxidative properties of alcohol *in vitro* [15, 16, 17]. Interestingly, the effect of the combination of circadian misalignment and chronic alcohol intake on plasma antioxidant resistance still remains unexplored.

The aim of this study was to investigate the effect of *in vitro* ethanol supplementation on the oxidative status of rat blood plasma in models of circadian rhythm disruption and chronic alcohol consumption. Endogenous lipid peroxidation was assessed by measuring the levels of malondialdehyde (MDA) in blood plasma.

MATERIALS AND METHODS

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.

Animals

Male Wistar rats (220-240) 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

* To whom all correspondence should be sent:
E-mail: miria@abv.bg

or 10% ethanol solution. 2-3 days before the experiment the animals were handled and then randomly assigned to four groups (n=5).

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

Group 2 – Circadian rhythm disruption (CRD) – exposed to light-at-night + tap water *ad libitum*;

Group 3 – Alcohol (A) - normal light/dark cycle + 10% ethanol solution *ad libitum*;

Group 4 – Circadian rhythm disruption + alcohol (CRD+A) - exposed to light-at-night + 10% ethanol solution *ad libitum*.

The experiments were carried out in accordance with the Bulgarian regulations on animal welfare and in conformance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

The blood was collected in EDTA washed test tubes, and the plasma was separated by centrifugation at 2000XG (4°C) for 30 min. The protein content of the samples was determined using the Biuret method [18]. A 552 UV-VIS spectrophotometer "Perkin-Elmer" with 2 ml quartz cuvettes was used for the spectrophotometric measurements. The *in vitro* ethanol treatment was performed at the following conditions: 50 µl of blood plasma, 300 µl ethanol (10^{-3} M) and 1600 µl PBS were incubated at 37°C for 15 min. The same amount of blood plasma was incubated at the same temperature in 1900 µl PBS. Then the MDA was measured in all samples.

MDA assay

The MDA formation was assessed as described in [19]. 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), BPS (pH 7.4) and 0.01 ml 0.003M H_2O_2. 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.

- MDA levels determination in the absence of alcohol in the sample: 50 µl of blood plasma and 1900 µl PBS were incubated at 20°C for 15 min. 50 µl $FeCl_2$ and 10 µl H_2O_2 were added.
- MDA levels determination in the presence of alcohol in the sample: 50 µl of blood plasma, 300 µl ethanol (10^{-3} M) and 1600 µl PBS were incubated at 20°C for 15 min. 50 µl $FeCl_2$ and 10 µl H_2O_2 were added. For the blank measurement only $FeCl_2$ and H_2O_2 were added to PBS.

Data presentation

For a better understanding, MDA was presented by its Activity Index (AI), as a percentage of the corresponding marker for the Control group (e.g., $AI=Marker_{stress}*100/Marker_{control}$). The AI for MDA formation are shown in Figures 1 and 2.

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* the Romanowski test [20], the mean values and standard deviations were calculated. The statistical significance of the differences between the mean data was estimated by the INSTAT program package (Bartlett test for significance of differences among the standard deviations followed by ANOVA and Bonferoni post-test).

RESULTS

There were no statistically significant differences between the MDA levels in the blood samples of all animals, exposed to light at night or receiving ethanol, or both, compared to the control group (Fig.1). The absolute values of our data were in agreement with these described in the literature for a different model of ethanol intake [21]. Figure 2 illustrates that elevated MDA levels were registered in all experimental groups compared to the same groups in the absence of alcohol. The combined effects of circadian disruption and alcohol caused the highest relative increase of MDA in the "CRD+A" group.

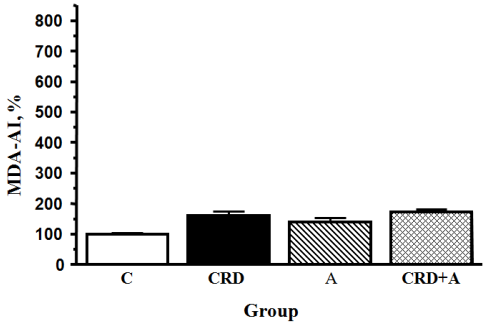

Fig. 1. MDA levels in the blood plasma without alcohol in the sample.

DISCUSSION

The present results demonstrated that, within our model of systemic ethanol intake, there were no significant differences in MDA levels (Fig. 1). The data in Figure 2 show that ethanol *in vitro* supplementation caused increased levels of MDA in all experimental groups compared to the same groups in the absence of alcohol. The increased MDA level indicated more profound lipid

peroxidation due to inefficient antioxidant defense. The lowest relative increase of the MDA level was in the Control group. In agreement with [8] this may be related with the metabolic transformation of ethyl alcohol in the blood plasma of the control animals in conditions of undisturbed antioxidant defense. Disrupted circadian rhythm and chronic ethanol intake, alone and in combination, resulted in a significantly increased level of MDA in rat blood plasma. This may be because of increased free radicals formation, and/or diminished antioxidant defense. Our data also suggest that the combined influence of circadian rhythm disruption and chronic alcohol can exacerbate the single adverse effects of each factor on the plasma oxidative status.

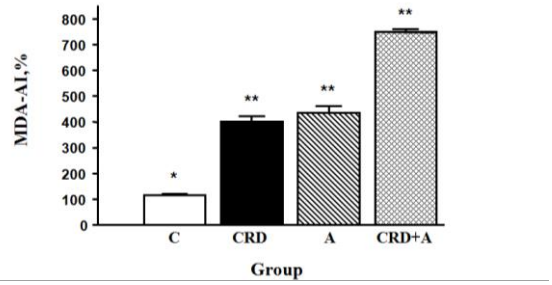

Fig. 2. MDA levels in the blood plasma in the presence of alcohol in the sample.
*P=0.0063; **P<0.0001 *vs* the same group in the absence of alcohol.

Our previous results showed that light at night and chronic alcohol consumption induced significant oxidative stress in the brains of the experimental animals. Furthermore, our data proposed that the combination of circadian misalignment and chronic alcohol might result in significantly higher MDA levels in the rat brain, than any of these models alone [22]. In this investigation, similar effects in rat blood plasma were observed. It may be assumed that the combination of circadian misalignment and chronic ethanol intake compromise the antioxidant defense and result in a massive lipid peroxidation within the entire organism of the model animals.

It is believed that oxidative stress plays a major role in the pathogenesis of a variety of adverse health conditions. In addition, lots of disorders have been found to have strong oxidative stress and circadian rhythm connections, including physical and psychiatric dysfunctions. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are important regulators of cellular metabolism, signal transduction, and gene expression and they are implicated in the regulation of physiological processes [9]. The exposure to light at night increases the lipid peroxidation in tissues and decreases both the total antioxidant activity and superoxide dismutase activity [12]. It is proposed that impairment of redox regulation and circadian rhythms may lead to a number of adverse effects on human health [23].

One causative pathway between excessive alcohol consumption and disease may be the circadian misalignment because they are often related [5, 24]. Furthermore, high ethanol consumption significantly enhances endogenous lipid peroxidation resulting in significantly elevated MDA and increased oxidative stress [10, 25]. In addition, exposure of cells to excessive ethanol result in a significant increase in the ROS production [16].

On the other hand, there is evidence for the antioxidant activity of ethanol in experimental models. For instance, Trevithick *et al*. [26] demonstrated antioxidant properties of ethanol *in vivo*, associated with anti-atherosclerotic effects. Also, ethanol protected LDL from oxidation initiated by superoxide and hydroxyl radicals *in vitro* [15]. Additionally, Tyulina *et al.* [17] reported that ethanol caused a decrease in erythrocyte reactive oxygen species levels and displayed protective activity on erythrocytes *in vitro*.

These controversial data gave us a reason to investigate the effects of supplementation of ethanol to plasma in our experimental models. It is known that in pathological conditions the antioxidant system may be overwhelmed. Oxidative stress occurs when ROS outweigh the antioxidant defense. In our experiment ethanol exhibited pro-oxidative activity in blood plasma *in vitro*. In agreement with the abovementioned data and our results, we can speculate that in our models there was increased lipid peroxidation and decreased antioxidant resistance in the blood plasma.

Compelling evidence from experimental and clinical studies links circadian misalignment and chronic alcohol abuse to disruptions in the neuroendocrine, immune and oxidative stress systems. Assessment of various related mechanisms is still a limited and novel field, but may be of considerable clinical relevance, having in mind the increasing number of affected individuals. Our results suggest an interconnection between circadian disruption, alcohol intake and imbalanced oxidative status, due to enhanced lipid peroxidation and/or reduced antioxidant resistance. The explanation of these observations needs further investigation and may contribute to the development of more efficacious preventive and therapeutic approaches for endangered patients.

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НАРУШЕНИЯТ ЦИРКАДЕН РИТЪМ И АЛКОХОЛНИЯТ ПРИЕМ ПРОМЕНЯТ ОКСИДАТИВНИЯ СТАТУС НА КРЪВНА ПЛАЗМА НА ПЛЪХ

М. Г. Варадинова[1], М. Л. Вълчева-Трайкова

[1] Катедра по фармакология и токсикология, Медицински факултет, Медицински Университет – София, България

[2] Катедра по медицинскафизика и биофизика, Медицински факултет, Медицински Университет – София, България

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(Резюме)

Нарушеният циркаден ритъм и хроничният алкохолен прием често се съчетават и водят до понижена антиоксидантна защита и оксидативен стрес при човешки и животински опитни постановки. Това се асоциира с редица хронични здравни проблеми, които от своя страна имат сериозни финансови последствия върху обществото и системата на здравеопазване.

Съществуват доказателства за антиоксидантна роля на етанол в експериментални модели. Същевременно, редица данни демонстрират про-оксидативни свойства на алкохол в in vivo условия.

Цел на настоящето изследване бе да проучи ефекта на етанол in vitro върху оксидативния статус на кръвна плазма от плъх при модели на нарушен циркаден ритъм (НЦР) и хроничен алкохолен прием (А). Получените резултати показаха про-оксидативна активност на етанол в кръвна плазма. Оксидативният статус на плазмата бе нарушен и при двата модела. В допълнение, при комбинацията от НЦР и А нивата на малондиалдехид бяха увеличени близо два пъти.

Нашите данни демонстрират, че съчетанието от нарушен циркаден ритъм и хроничен алкохолен прием могат да отежнят самостоятелния отрицателен ефект на всеки от факторите върху оксидативния баланс на кръвна плазма. Обяснението на тези наблюдения изисква по-нататъшни изследвания на механизмите на взаимовръзка между НЦР и продължителен алкохолен прием.