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MELATONIN PREVENTS OXIDATIVE STRESS IN RATS EXPOSED TO ETHANOL INTOXICATION, ITS COMBINATION WITH CAFFEINE AND LIGHT EXPOSURE

N. V. Davydova, N. P. Hryhorieva

Bukovinian State Medical University, Chernivtsi, Ukraine

The aim of the research *– to investigate the effects of melatonin on oxidative stress biomarkers malonic dialdehyde (MDA) and oxidatively modified proteins (OMP) in blood, liver and kidneys of rats exposed to subacute alcohol intoxication, its combination with constant light exposure, and caffeine intake.*

Materials and methods. *Experiments were performed on 42 male rats weighing 180-200 g kept under standard conditions of vivarium and artificial equinox. Subacute alcohol intoxication was induced by intragastric administration of 40 % ethanol at a dose of 7 ml/kg of body weight for 7 days. Caffeine was administered by gavage at a dose of 30 mg/kg of body weight.*

Results. *Ethanol poisoning results in increase in MDA and OMB, and liver was the most affected (90 % and 42 % higher than control). The combination of ethanol with caffeine reduced the intensity of oxidative stress in the blood but increased the toxic effects of ethanol on the liver (MDA and OMP were 116 % and 60 % above control range). The most significant increase in MDA was in the liver and kidneys under combination of alcohol intoxication with exposure to constant light (139 % and 33 % respectively). There was a decrease in OMB level along with elevation of MDA level in kidneys of all groups of alcoholized animals.*

Administration of 5 mg/kg melatonin for 7 days limited the rise in MDA in the blood and liver of animals and changes in OMB content in the blood liver and kidneys of animals exposed to ethanol and constant lighting. Melatonin showed to be less effective in the liver and kidneys of rats treated with a combination of ethanol and caffeine.

Conclusion. *The data obtained are evidence of the pronounced antioxidant melatonin action and its capacity to prevent toxic effects of ethanol and its combination with constant lighting and caffeine introduction into organism.*

Key words:
ethanol, caffeine, light exposure, melatonin, rats.

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E-mail:
davydova.nataliia@bsmu.edu.ua

МЕЛАТОНІН ЗАПОБІГАЄ ОКСИДАТИВНОМУ СТРЕСУ У ЩУРІВ ЗА УМОВ АЛКОГОЛЬНОЇ ІНТОКСИКАЦІЇ, ЇЇ ПОЄДНАННЯ ІЗ ВВЕДЕННЯМ КОФЕЇНУ ТА ПОСТІЙНИМ ОСВІТЛЕННЯМ

Н.В. Давидова, Н.П. Григор'єва

Буковинський державний медичний університет, м. Чернівці, Україна

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Мета роботи – *дослідити вплив мелатоніну на біомаркери окисного стресу: малоновий діальдегід (МДА) та окисно модифіковані білки (ОМБ), у крові, печінці та нирках щурів за умов алкогольної інтоксикації, її поєднання з постійним освітленням та введенням кофеїну.*

Матеріали та методи. *Експерименти проводили на 42 самцях щурів масою 180-200 г, яких утримували за стандартних умов віварію і штучного рівнодення. Алкогольну інтоксикацію викликали внутрішньошлунковим введенням 40 % етанолу в дозі 7 мл/кг маси тіла впродовж 7 діб. Кофеїн вводили внутрішньошлунково в дозі 30 мг/кг маси тіла.*

Результати. *Отруєння етанолом призводило до збільшення рівня МДА та ОМБ, найбільш вираженого в печінці щурів (на 90 % та 42 % вище контролю). Поєднання введення етанолу з кофеїном зменшувало інтенсивність окиснювального стресу в крові, проте посилювало токсичний вплив етанолу на печінку (вміст МДА та ОМБ були на 116 % та 60 % вище контролю). Найбільш інтенсивне зростання МДА спостерігали в печінці та нирках щурів за умов поєднаного впливу етанолу та постійного освітлення (на 139 % та 33 % вище контролю відповідно). У нирках алкоголізованих тварин усіх груп на фоні зростання рівня МДА спостерігали зниження вмісту ОМБ.*

Введення мелатоніну в дозі 5 мг/кг маси тіла впродовж 7 діб запобігало вірогідному зростанню вмісту МДА у крові та печінці тварин та змінам вмісту ОМБ у крові, печінці та нирках тварин, які зазнали впливу етанолу та постійного освітлення. Вплив мелатоніну був менш ефективним у печінці та нирках щурів, які отримували комбінацію етанолу та кофеїну.

Висновок. *Отримані дані засвідчують про виражену антиоксидантну дію мелатоніну та здатність його запобігати токсичним ефектам етанолу та його поєднання з постійним освітленням і введенням кофеїну в організм.*

Introduction

Although the negative effects of excessive alcohol consumption are generally known in the human population, the drinking of alcoholic beverages is prevalent in society. According to WHO, alcohol abuse contributes to three million deaths per year globally and millions of people's disabilities and organ damage. Overall, alcohol causes more than 5 % of the global disease burden worldwide [4].

Multiple laboratory and clinical studies have shown that generation of reactive oxygen species (ROS) and activation of free radical oxidation of biomolecules is the key for ethanol toxic effects [9]. Ethanol detoxification occurs mainly in the liver by alcohol dehydrogenase, catalase, and microsomal monooxygenase system yielding acetaldehyde. Acetaldehyde mediates an increase in nitrogen and oxygen reactive species, activation of free radical oxidation of unsaturated fatty acids, proteins, nucleic acids, depletion of antioxidants, which defines the concept of oxidative stress and leads to irreversible macromolecular changes in cells [1]. Another important source of ROS is cytochrome P450 2E1 (CYP2E1) which expression is induced by ethanol intake. CYP2E1 possesses a remarkably high NADPH oxidase activity, resulting in electron leakage from the mitochondrial respiratory chain and oxidative stress progress [9].

In recent years, the consumption of energy drinks has considerably increased worldwide through tactical marketing and advertising strategies. It is the rapidly rising popularity of highly caffeinated energy drinks, and their use with alcohol, that has caused concern [15]. Caffeine interferes with sleep homeostasis by antagonizing the A1 and A2A adenosine receptors as mirrored in reduced sleepiness, improved behavioral performance [2]. Caffeine appears to increase the subjective pleasurable effects of alcohol, selectively increases positive reinforcing effects of self-administered alcohol. Combined ethanol and caffeine intake causes deeper changes in behavioral responses and worsens the withdrawal symptoms [10].

Numerous experimental studies evidence the antioxidant effect of caffeine [11, 13]. However, a number of researchers point to its ability to stimulate apoptosis, mutagenic action, and prooxidant effect at certain concentrations and in the presence of Cu (II) ions [8, 13]. Furthermore, the state of the pro- and antioxidant system under combined ethanol and caffeine effects has not been studied.

Negative effects resulted from ethanol exposure can be prevented by therapeutic agents with potentially antioxidative capabilities.

Melatonin, chemically N-acetyl-5-methoxytryptamine, is a pineal gland hormone which has an extremely wide range of physiological functions. Melatonin regulates circadian rhythm, thermoregulation, reproductive cycles, and has immunomodulatory, antitumor, antiischemic, antihypertensive, and antiaging effects [3]. In addition, melatonin is one of the most potent endogenous antioxidants, as evidenced by numerous in vitro and in vivo studies [7].

Melatonin secretion by the pineal gland is controlled by an endogenous circadian rhythm and plays an important role in the regulation of this rhythm, rising at night and suppressed by light [3].

Furthermore, evidence accumulates that caffeine also impacts on human circadian rhythms, as indexed by changes in salivary melatonin levels [16]. Acute caffeine intake in the evening and at night has been shown to delay the onset of melatonin secretion and decrease nighttime melatonin levels [2]. Besides this, alcohol intake is usually combined with circadian disruption.

The aim of the research

To investigate the effects of melatonin on oxidative stress biomarkers (malonic dialdehyde and oxidatively modified proteins) in blood plasma, liver and kidneys of rats exposed to subacute alcohol intoxication, its combination with constant light exposure, and caffeine intake.

Materials and methods

The study was performed in compliance with the Rules of the work using experimental animals (1977) and the Council of Europe Convention on the Protection of Vertebrate Animals used in experiments and other scientific purposes (Strasbourg, 1986). It has been performed according to the directions of International Committee of Medical Journals Editors (ICMJE), as well as «Bioethical expertise of preclinical and other scientific research performed on animals» (Kyiv, 2006).

Experiments were performed on 42 male Wistar rats weighing 180-200 g which were randomly grouped and kept in polycarbonate cages (3-4 rats per cage) in a room under controlled environmental condition (temperature 21±1 °C and 12:12 h light/dark cycle, with lights on 8:00 a.m.). Animals received food and water ad libitum.

Subacute alcohol intoxication was induced by intragastric administration of 40 % ethanol at a dose of 7 ml/kg of body weight for 7 days. Caffeine was administered intragastrically by gavage at a dose of 30 mg/kg of body weight which is equivalent to a dose of 6 mg/kg in humans and corresponds to moderate coffee consumption (5 cups of coffee or about 400 mg of caffeine). The light exposure was caused by a constant fluorescent light of 1500 lux intensity for 24 hours a day. Melatonin («Vita-melatonin», JSC «Kyiv Vitamin Plant») was given by gavage at dose of 5 mg/kg of body weight at 20^0 for 7 days along with alcohol intoxication. The control group of animals received equivolume amount of water.

Rats were randomly assigned into 7 groups: group 1 – untreated control; group 2 – induced subacute alcohol intoxication; group 3 – alcohol intoxication + melatonin administration; group 4 – alcohol intoxication + caffeine administration; group 5 – alcohol intoxication + caffeine + melatonin; group 6 – alcohol intoxication + constant

light exposure; group 7 – alcohol intoxication + constant light exposure + melatonin.

Animals were decapitated under light ether anesthesia on the 7th day after beginning of the experiment. Blood samples were collected in the presence of anticoagulant EDTA (1 mg/ml of blood). Erythrocytes were washed three times with five volumes of saline solution and centrifuged at 3000 rpm for 10 min and used for the determination of 2-thiobarbituric acid reactive substances. Blood plasma was used to measure oxidative protein modification.

The tissues samples (liver and kidneys) were excised, minced, rinsed with cold 50 mm Tris-HCl buffer (pH=7.4) to remove blood and homogenized in a glass homogenizer with a motor-driven teflon pestle on ice to prepare 5 % homogenates. The homogenates were centrifuged for 10 min at 900g.

Malonic dialdehyde (MDA) content was assayed in erythrocytes, liver and kidneys homogenates by the method of Kamyshnikov (2004) based on spectrophotometric measurement of trimethine colored complex formed in reaction with thiobarbituric acid at high temperature and acidic pH [12,14].

The content of oxidatively modified proteins (OMP) in blood plasma, liver and kidneys homogenates was assayed by reaction of the resultant carbonyl derivatives of amino acids with 2.4-dinitrophenyl hydrazine which results in formation of hydrazones having specific absorption spectrum as described by Dubinina et al. (1995) [5,14]. The value of OMP is quantified by the number of aldehyde and ketone groups formed. Aldehydes derivates were determined at 370 nm.

Total protein content was assayed by Lowry using standard reagent kit for clinical diagnostics («Filisit-Diagnostics» Co., Ltd.).

The results were statistically processed using the STATISTICA 10 software (StatSoft Inc.). A Shapiro-Wilk test was performed to verify normality of data distribution and then Mann-Whitney test was used to consider sufficient for valid conclusions to be made. Data are illustrated as mean±SEM (n=6 animals per group). $P<0.05$ was considered as statistically significant differences.

Results and their discussion

In this study, we demonstrated that the model of subacute ethanol stress caused an increase in malonic dialdehyde content in rats' blood and kidneys 38 % and 28 % (table 1). The value of MDA in the liver was 90 % higher vs. control group, indicating much greater oxidative stress in hepatocytes, which are directly involved in ethanol biotransformation.

Along with the increase in lipid peroxidation, there was an intensification of free radical oxidative modification of proteins evidenced by increase in the content of OMP aldehyde derivates in rats' blood plasma and liver in terms of alcohol intoxication 40 % and 42 % vs control group, respectively.

Table 1

The content of MDA and OMP in rats in terms of alcohol intoxication combined with light exposure, caffeine, and melatonin intake (M±m)

	Malonic dialdehyde			Oxidatively modified proteins (370 nm)		
Groups/ Indices	RBCs, nMol/ml	Liver, µMol/g tissue	Kidneys, µMol/g tissue	Blood plasma, mMol/g protein	Liver, mMol/g protein	Kidneys, mMol/g protein
Control	11.6±1.2	28.0±2.5	50.3±2.9	0.76±0.06	2.16±0.11	2.06±0.12
Ethanol	16.0±1.6*	53.3±4.9*	64.4±1.2*	1.07±0.08*	3.07±0.23*	1.38±0.17*
Ethanol + melatonin	13.2±1.1*	34.5±3.3*	63.8±1.6*	0.87±0.10	2.31±0.29	1.51±0.16*
Ethanol + caffeine	14.3±0.9*#	60.6±2.9*#	70.1±6.1*#	1.02±0.09*	3.45±0.20*	1.40±0.15*
Ethanol + caffeine + melatonin	10.4±1.1	40.4±4.0*	69.4±3.3*	0.91±0.15	2.86±0.31*	1.45±0.31*
Ethanol + light	18.1±2.2*	66.9±5.5*#	67.1±1.5*#	1.31±0.22*#	4.07±0.48*#	1.55±0.36*
Ethanol + light + melatonin	14.1±2.2*	35.9±4.2*	76.9±6.4*	0.94±0.12*	2.59±0.16*	2.28±0.21

*Note: * – statistically significant difference compared to the control group (p≤0,05);*
 # – statistically significant difference compared to ethanol treated group (p≤0,05).

Combined ethanol and caffeine intake for 7 days resulted in increase in MDA in erythrocytes and OMP in blood plasma of animals 23 % and 34 % level vs control group. These values appeared to be statistically lower than in animals treated with ethanol only, which may confirm the data on the caffeine antioxidant effect. However, the levels of malonic dialdehyde and OMP in the liver of such rats were significantly higher than in animals treated with ethanol only and exceeded the control level 116 %, and 60 %, respectively. This may be due to the significant activation of the microsomal oxidative system in hepatocytes, which are directly involved in both ethanol neutralization and caffeine metabolism when combined in high concentrations.

Caffeine administration caused a greater increase in MDA levels (39 % above the control) in the kidneys

of alcoholized animals compared to animals treated with ethanol alone. However, the content of OMB in the kidneys of rats treated with ethanol or combination of ethanol and caffeine was below the control group 33 % and 32 % respectively.

We revealed that ethanol stress along with constant light caused a significant increase in lipid peroxidation and OMP, as evidenced by an increase in malonic dialdehyde in blood, liver, and kidneys of rats (56 %, 139 %, and 33 % above the control) and increase in oxidatively modified proteins in blood plasma and liver of animals (72 % and 88 % above control, respectively). These values were significantly higher than in alcohol-treated rats under normal light conditions. The more significant free radical damage of biomolecules under the combination of alcohol intoxication with exposure to constant light is probably due to inhibition of melatonin synthesis by constant light. In kidneys combination of ethanol poisoning with constant light resulted in OMP decrease 25 %. Decrease in OMP in kidneys observed in all groups of ethanol-stressed rats may be due to their enhanced breakdown by multicatalytic proteases such as trypsin, pepsin, subtilisin, calpain, and cathepsin D. Initial proteins oxidative modification result in their denaturation and increased hydrophobicity which are considered as a trigger for intracellular proteolysis [6]. A pronounced oxidative damage of proteins leads to a decrease in proteolytic susceptibility due to progressive covalent cross-linking, intramolecular interactions, aggregation, and decreased solubility [6]. It results in accumulation of OMP as we have observed in the blood and liver of all groups of ethanol-stressed rats.

In the study, we demonstrated (table 1) that administration of 5 mg/kg melatonin during subacute ethanol stress and its combination with caffeine intake or constant light for 7 days limited the rise in lipid peroxidation products and oxidatively modified proteins. Thus, the content of MDA exceeded the level of control in the blood plasma and liver of alcohol-exposed animals 15 % and 23 % only. The melatonin administration prevented statistically changes in OMP content in the blood plasma of animals treated with ethanol, as well as its combination with caffeine intake.

The impact of melatonin was less effective in the liver of rats exposed to combination of ethanol and caffeine. The content of MDA and OMP in the liver of this group of animals, although it tended to normalize, but remained above the control level 44 % and 32 %, respectively.

There was no apparent influence of melatonin on MDA and OMP in kidneys of alcoholized rats and rats which underwent combination of ethanol and caffeine.

Administration of melatonin to ethanol-exposed rats kept under constant light proved to be quite effective in normalizing the processes of biomolecules free radical oxidation. The content of MDA and OMP in the blood and liver of such animals was significantly lower than in untreated but remained higher than in control group (21 % and 23 % in the blood and 28 % and 20 % in the liver). But in kidneys of such rats the MDA level exceeded control level 53 % that was stigmatically higher than in animals non-treated with melatonin. Wherein melatonin results no statistically changes in OMP in kidneys compared to control group.

Our results evidence the potent antioxidant effect of melatonin and its ability to counteract macromolecules free radical oxidation. The effect of melatonin may be related to its ability for scavenging a variety of free radicals including $^{\cdot}OH$, $^{\cdot}OOH$, $^{\cdot}N_3$, $^{\cdot}NO$, $^{\cdot}NO_2$, $^{\cdot}OOCCl_3$, $ONOO^-$, quenching singlet oxygen ($^1O2$) at fast rates thus counteracting the cytotoxic action this oxidant. It can also act as OH-inactivating ligand by chelating transition metal ions, increase the antioxidant effects of glutathione, ascorbic acid which is attributed to the capability of melatonin to regenerate these compounds through electron transfer processes [7]. There is evidence supporting its role in the activation of antioxidant enzymes, the inhibition of pro-oxidative enzymes seems evident that melatonin can be classified as a multipurpose antioxidant [3].

Conclusions

1. Thus, ethanol poisoning and its combination with caffeine intake or light exposure results in a significant increase in free radical oxidation of biomolecules, which evidences the oxidative stress progress. The most pronounced changes were observed in the liver, which is probably due to its direct involvement in ethanol detoxification.
2. The combination of ethanol with caffeine or constant light potentiated the toxic effects of ethanol in the liver and kidneys, resulting in a greater increase in malonic dialdehyde and OMP compared to animals treated with ethanol alone but reduces oxidative stress in the blood.
3. According to our data, the most intensive rise in free radical oxidation of lipids and proteins was in the liver of rats exposed to alcohol poisoning under constant light exposure. Therefore, the violation of the light regime significantly reduces the body's resistance to the toxic effects of ethanol intake.
4. Melatonin administration at a dose of 5 mg/kg body weight prevents ethanol-induced toxicity and oxidative stress in rats exposed to ethanol and its combination with caffeine or constant light for 7 days by significantly reducing the level of malonic dialdehyde and aldehyde protein derivatives in blood, liver, and kidneys as biomarkers of macromolecules destruction processes in tissue damage in most of animal groups.

Prospects for further research

To study the state of antioxidant system in blood plasma, liver and kidneys of rats exposed to subacute alcohol intoxication, its combination with constant light exposure, and caffeine intake.

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Information about the authors:

Davydova N.V. – Ph.D., Associate Professor of Bioorganic and Biological Chemistry and Clinical Biochemistry Department, Bukovinian State Medical University, Chernivtsi, Ukraine.
E-mail: davydova.nataliia@bsmu.edu.ua
ORCID ID: https://orcid.org/0000-0003-4405-8696
Grigorieva N.P. – Ph.D., Associate Professor, Head of the Bioorganic and Biological Chemistry and Clinical Biochemistry Department, Bukovinian State Medical University, Chernivtsi, Ukraine.
E-mail: hryhorieva.nadiia@bsmu.edu.ua
ORCID ID: https://orcid.org/0000-0002-1422-0798

Відомості про авторів:

Давидова Н.В. – к.м.н., доцент кафедри біоорганічної і біологічної хімії та клінічної біохімії Буковинського державного медичного університету, м. Чернівці, Україна.
E-mail: davydova.nataliia@bsmu.edu.ua
ORCID ID: https://orcid.org/0000-0003-4405-8696
Григор’єва Н.П. – к.б.н., доцент, завідувач кафедри біоорганічної і біологічної хімії та клінічної біохімії Буковинського державного медичного університету, м. Чернівці, Україна.
E-mail: hryhorieva.nadiia@bsmu.edu.ua
ORCID ID: https://orcid.org/0000-0002-1422-0798

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