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Melatonin Restores White Blood Cell Count, Diminishes Glycated Haemoglobin Level and Prevents Liver, Kidney and Muscle Oxidative Stress in Mice Exposed to Acute Ethanol Intoxication

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Abstract

Aims: The aim of the study was to examine the effects of melatonin impact on changes in haematological profile, biomarkers of oxidative stress (dienes conjugates, malondialdehyde (MDA), oxidatively modified protein levels, total antioxidant capacity and antioxidant enzyme activity) in liver, muscle, kidney and erythrocytes, and glycated haemoglobin (HBA1c) in mice during acute ethanol stress.
Methods: Assays were carried out in quadruplicate: control, melatonin (10 mg/kg, 10 days), acute ethanol stress (0.75 g/kg/day, 10 days) and acute ethanol stress plus melatonin groups.
Results: Acute ethanol stress caused a significant increase in the total number of white blood cells (WBC), especially neutrophils in the blood, and HBA1c levels vs. control mice. The correlation between lipid peroxidation and the glycated haemoglobin level was shown ($r = 0.93$, $P = 0.007$). Ethanol reduced the antioxidant capacity by increasing reactive oxygen species (ROS) production and the level of oxidatively modified protein content, diene conjugates and MDA. Melatonin administration in animals during acute ethanol stress reduced antioxidant stress biomarkers, WBC, HBA1c levels and ROS production.
Conclusions: Melatonin had protective effects on liver, kidney and muscle tissues by preventing the intensive lipid peroxidation processes in initial (diene conjugation production) and late stages (MDA level), and significantly reduced the level of aldehyde and ketone protein derivatives. Furthermore, melatonin restored elevated WBC count and HBA1c level and diminished ROS production.
Short Summary: Ethanol reduces antioxidant capacity and leads to exaggerated reactive oxygen species production and consequent increases in oxidatively modified proteins. Melatonin exerts protective effects by preventing the intensive lipid peroxidation processes. Melatonin significantly reduces the level of aldehyde and ketone protein derivatives, restores glycated haemoglobin level and white blood cell count.

INTRODUCTION

Liver is the main organ to metabolize ethanol. Major ethanol metabolic pathways in the liver include ethanol dehydrogenase in the cytosol, microsomal ethanol oxidizing system in the endoplasmic reticulum, aldehyde oxidase in the mitochondria and catalase (CAT) (Correa *et al.*, 2004). All reactions of microsomal and mitochondrial oxidation resulting from incomplete reduction of oxygen to water may result in formation of reactive oxygen species (ROS), such as singlet oxygen ($_1O^2$), superoxide anion radical ($O_2^{\bullet}$), hydroxyl radical (HO), hydrogen peroxide (H_2O_2). ROS reactions with unsaturated fatty acids, in the presence of variable valence metal ions, form peroxide compounds (Das *et al.*, 2009). All of these processes may result in pathological developments (Patere *et al.*, 2011). The antioxidant enzymes—superoxide dismutase (SOD), CAT, glutathione-peroxidase (GPx), glutathionreductase (GR) and ceruloplasmin—metabolize oxidative toxic intermediates (Mansouri *et al.*, 2001).

The glycated haemoglobin (HbA1c) level is used in medical practice for long-range glycaemic control in patients with alcoholic liver diseases and non-alcoholic fatty liver pathologies (Nedelson *et al.*, 2016). Alcohol liver disease is often associated with glucose intolerance (Isida *et al.*, 2017) and diminished erythrocyte survival ultimately resulting in anaemia (Maruyama *et al.*, 2001). Therefore, HbA1c can be used not only as a marker of glycaemic control but also as an effective parameter to monitor liver injury. The results of biochemical tests together with histological observations suggest that ethanol leads to serious changes in the histology of kidney by increased formation of lipid peroxides and associated ROS with next membrane integrity (Sönmez *et al.*, 2012).

Oxidative stress resulting from ethanol exposure affects different organs and systems (Mansouri *et al.*, 2001). Negative effects of ethanol can be prevented by therapeutic agents with potentially antioxidative capabilities. Melatonin is one of such substances that induce cellular protective responses within the nervous, immune and endocrine systems (Petrosillo *et al.*, 2008). Melatonin, chemically *N*-acetyl-5-methoxytryptamine, is an important hormone of the pineal gland. Melatonin has an extremely wide range of physiological functions. It regulates circadian rhythm including sleep induction, reproductive cycles, thermoregulation and has potent antioxidant immunomodulatory and anti-stress effects (Bonnefont-Rousselot and Collin, 2010). Melatonin stabilizes the mitochondrial bioenergetic function, possibly declining the pace of aging and supporting treatment of several diseases (Carrasco *et al.*, 2015). Melatonin possesses antitumor activities and reduces viability in several cancer cell lines (Kim *et al.*, 2005). In patients using alcohol, oxidative stress induced by the presence of excessive ROS is closely associated with chronic inflammation, leading to potential tissue damage. As oxidative stress is the principal reason for diabetic complications, the use of antioxidants appears to be one of the most rational restorative approaches.

Use of therapeutic agents with potentially antioxidative capabilities may appear protective from ethanol poisoning. Therefore, the aim of the study was to investigate the effect of melatonin, on the haematological profile, glycate haemoglobin, lipid peroxidation processes, oxidized modified proteins level and antioxidant enzyme activities in selected tissues in mice exposed to acute ethanol stress.

MATERIALS AND METHODS

Animals and experimental design

The experiments were conducted in accordance with the Guidelines of the European Union Council and the current laws in Ukraine and Poland, and approved by the Ethical Commission of National Pedagogical University in Chernihiv (2612/2016). To eliminate circadian rhythm changes, all examinations started at 10.00 am and ended at 12.00 am. The animals used in this experiment were male white mice (*Mus musculus*), 2–3 months of age. In total, 24 healthy adult male mice, weighing ~20–30 g, were divided into four groups. The mice were housed at a constant temperature of 20 ± 2°C. The animals had free access to food and water throughout the experiments.

Drugs and solutions

EDTA, HEPES, KCl, K_2CO_3, KH_2PO_4, EDTA and 2-tiobarbituric acid were purchased from Sigma-Aldrich (Sigma-Aldrich Sp. z.o.o, Poznan, Poland). All drugs were freshly prepared. All other reagents used were of analytical reagent grade.

Sampling

Three millilitre blood samples were collected in tubes with K-EDTA. After centrifugation, plasma samples were removed and frozen at −20°C and stored until analysis. Erythrocytes were washed three times with five volumes of saline solution and centrifuged at 3000 *g* for 10 min. Haemolysed erythrocytes were used for the determination of 2-thiobarbituric acid reactive substances.

Tissue isolation

The tissues were removed from the mice after decapitation. Briefly, the tissues were excised, weighed, washed in ice-cold buffer and minced. The minced tissue was rinsed with cold isolation buffer to remove blood and homogenized in a glass Potter-Elvehjem homogenizer with a motor-driven Teflon pestle on ice. The isolation buffer consisted of 120 mM KCl, 2 mM K_2CO_3, 10 mM HEPES and 1 mM EDTA; the pH was adjusted to 7.2 with KOH.

Experimental groups

Mice were randomly assigned into four groups: untreated control (six animals), melatonin administration (six animals), acute ethanol stress (six animals) and acute ethanol stress + melatonin administration (six animals).

Melatonin was given in daily intraperitoneal injections with 10 mg/kg of melatonin for 10 days. Melatonin was dissolved in a minimum volume of ethanol and diluted in 0.9% NaCl to yield a dose of 10 mg/kg body weight, as described in previous studies (Bonnefont-Rousselot and Collin, 2010) and Shin *et al.* (2015).

Acute ethanol stress was induced by intraperitoneal injection of 0.75 g/kg/day ethanol dose. Ethanol was diluted from a 95% (v/v) solution to a concentration of 20% (v/v) with physiological saline (0.9%) and was administered as intraperitoneal injections at doses of 0.75 g/kg of body weight in an injection volume of 4.73 ml/kg for 10 days of the experiment, as described by Powers and Chester (2014).

Control mice were injected with 0.9% NaCl.

Haematological profile

K_2EDTA blood was collected and analysed in an automated manner (Abacus Junior Vet, Diatron MI Zrt., Budapest, Hungary) considering the following parameters: red blood cell (RBC) count (10^6/µl), white blood cell (WBC) count (10^3/µl), lymphocyte (10^3/µl), monocyte (10^3/µl), neutrophil (10^3/µl), lymphocyte (%), monocyte (%) and neutrophil (%), haemoglobin (Hb, dl/g), packed cell volume (PCV), haematocrit HCT (%), mean corpuscular volume (MCV, fl), mean

corpuscular haemoglobin (MCH, pg), mean corpuscular haemoglobin concentration (MCHC, g/dl), RBC distribution width (RDWc, %), platelet count (PLT, 10^3/μl), packed cell volume (PCV, %), platelet volume (MPV, fl), platelet distribution width (PDWc, %).

The HBA1c level in the blood of mice was estimated by the HemoCue HbA1c 501 (HemoCue AB, Angelholm, Sweden) system and was expressed as a percentage.

The enzymatic reactions were started by the tissue supernatant addition. The specific assay conditions were as follows.

Biochemical assays

Conjugated dienes assay

The level of conjugated dienes was determined according to the Kamyshnikov method (Kamyshnikov, 2004). Conjugated dienes are formed during lipid peroxidation processes as a result of reconfiguration of double bonds after detachment of hydrogen forms the rest of the polyunsatured fatty acid. Conjugated diene groups were determined by the absorption peak at the wavelength of 233 nm and expressed in nmol per gram of protein.

Thiobarbituric acid reactive substances assay

Thiobarbituric acid reactive substances (TBARS) were measured by the method of Kamyshnikov (2004). TBARS level was expressed in nmol of malondialdehyde (MDA) per litre of blood or nmol of MDA per mg protein.

Protein carbonyl derivatives assay

The oxidatively modified protein (OMP) rate was estimated by the reaction of the resultant carbonyl derivatives of amino acids with 2.4-dinitrophenyl hydrazine (DNFH) as described by Levine *et al.* (1990) in modification by Dubinina *et al.* (1995). The final solution was centrifuged to remove any insoluble material. The carbonyl content was calculated from the absorbance measurement at 370 and 430 nm and an absorption coefficient of 22,000 M^{-1} cm^{-1}. Carbonyl groups were determined spectrophotometrically at 370 nm (aldehyde derivates (AD), OMP_{370}) and 430 nm (ketonic derivates (KD), OMP_{430}), and expressed in nmol per mg of protein.

SOD activity assay

SOD activity in the supernatant was determined according to Kostiuk *et al.* (1990). SOD activity was assessed by its ability to dismutate superoxide produced during auto-oxidation of quercetin in an alkaline medium (pH 10.0). Absorbance at 406 nm was measured immediately and after 20 min. Activity is expressed in units of SOD per mg of protein.

Catalase activity assay

CAT activity was determined by measuring the decrease of H_2O_2 in the reaction mixture by the method of Koroliuk *et al.* (1988). One unit of CAT activity is defined as the amount of enzyme required for decomposition of 1 μmol H_2O_2 per min per mg of protein.

Glutathione reductase activity assay

Glutathione reductase (GR) activity in the blood and tissues was measured according to the method of Glatzle *et al.* (1974). The enzymatic activity was assayed spectrophotometrically by measuring nicotinamide adenine dinucleotide phosphate (NADPH) consumption. A blank sample without NADPH was used. The GR activity was expressed as nmol NADPH per mg protein.

Glutathione peroxidase activity assay

Glutathione peroxidase (GPx) activity was determined by the detection of reduced glutathione (GSH) utilization as the reacting substrate at 412 nm. Assays were carried out after incubation with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) according to the method of Moin (1986). GPx activity was expressed as nmol GSH per mg protein.

Total antioxidant capacity assay

Total antioxidant capacity (TAC) levels in the liver, muscle and kidney tissues were estimated spectrophotometrically using the TBARS level and following the method with Tween 80 oxidation described by the authors (Galaktionova *et al.*, 1998). The level of TAC in the sample (%) was calculated according to the absorbance of a blank.

For the quantification of proteins, the Bradford method (Bradford, 1976) with bovine serum albumin as a standard was used. Absorbance was recorded at 595 nm. All enzymatic assays were carried out at 22 ± 0.5°C using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany) in duplicate.

Statistical analysis

Results were expressed as mean ± SEM. All variables were tested for normal distribution using the Kolmogorov–Smirnov test ($P > 0.05$) and homogeneity of variance by using the Levene's test. The significance of differences in the level of lipid peroxidation, amino acids carbonyl derivatives, and antioxidant enzyme activities between control and examined groups were examined using one-way analysis of variance (ANOVA). We used Bonferonni's post-test. Differences were considered significant at $P < 0.05$. In addition, the associations between data of all individuals were evaluated using Pearson's correlation analysis. All statistical calculations were performed on separate data from each individual with Statistica 8.0 software (StatSoft Inc., Poland).

RESULTS

Haematological parameters are presented in Table 1. Melatonin did not cause statistically significant changes in the blood cell counts in mice. The model of acute ethanol stress (0.75 g/kg/day, 10 days) caused a significant increase of WBC count, especially neutrophils. Treatment with melatonin significantly decreased WBC, lymphocyte and neutrophil count compared to ethanol intoxication. In the ethanol and melatonin group MCV and RDWc values increased.

In most cases, alcohol dependence is associated with impaired transport and metabolism of glucose in the blood and tissues. Therefore, the next step of our investigation was devoted to HBA1c level during acute ethanol stress and melatonin administration (Table 1). Melatonin in our investigation did not change HBA1c levels vs. control mice while ethanol statistically increased HBA1c levels vs. control mice. Administration of melatonin to ethanol-exposed mice decreased HBA1c compared to the ethanol-treated group.

Diene conjugation in the fatty acid molecules and their hydroperoxides appears at the initial stages of free radical oxidation of lipids. Therefore, they are considered to be the primary product of the free radical oxidation of lipids as the diene conjugates and ketodienes. For this purpose, the determination of the intensity of lipid peroxidation processes, we carried out the evaluation of initial substrate accumulation in this stage of free radical oxidation (Table 2). Melatonin administration did not exert any statistically significant effect compared to control mice. After ethanol treatment in analysed tissues such as liver ($F = 10.09$, $P = 0.000$), muscle ($F = 6.18$, $P = 0.000$) and kidney ($F = 11.82$, $P = 0.000$), the level of conjugated dienes was significantly higher

Table 1. Effects of melatonin on the morphological blood parameter and glycated haemoglobin level changes during acute ethanol stress in mice

Parameters	Control	Mel	AES	AES + Mel
RBC, 10^6/µl	7.91 ± 0.29	7.18 ± 0.44	7.96 ± 0.17	6.97 ± 0.44
WBC, 10^3/µl	4.79 ± 0.36	5.93 ± 0.83	8.52 ± 1.36[a] ($P = 0.031$)	4.61 ± 0.45[aa] ($P = 0.001$)
LYM, 10^3/µl	3.60 ± 0.23	3.94 ± 0.08	5.73 ± 0.86	3.35 ± 0.28[aa] ($P = 0.035$)
MON, 10^3/µl	0.18 ± 0.04	0.25 ± 0.52	0.28 ± 0.06	0.22 ± 0.07
NEU, 10^3/µl	1.01 ± 2.26	1.74 ± 0.46	2.52 ± 0.56[a] ($P = 0.037$)	1.04 ± 0.17[aa] ($P = 0.026$)
LYM, %	75.71 ± 2.63	66.38 ± 4.86	67.78 ± 1.18	73.30 ± 2,11
MON, %	3.70 ± 0.61	4.38 ± 0.52	4.17 ± 1.15	4.63 ± 1.43
NEU, %	20.57 ± 2.26	29.22 ± 5.27	28.08 ± 2.02	22.07 ± 1.46
Hb, dl/g	13.54 ± 0.59	12.45 ± 0.55	13.67 ± 0.35	12.63 ± 0.87
HCT, %	42.70 ± 1.86	39.31 ± 2.46	42.61 ± 0.81	40.09 ± 2.59
MCV, fl	53.71 ± 0.78	54.83 ± 0.65	53.33 ± 0.21	57.33 ± 0.49[aa] ($P = 0.001$)
MCH, pg	17.07 ± 0.20	17.47 ± 0.37	17.15 ± 0.18	18.07 ± 0.27
MCHC, g/dl	31.74 ± 0.36	31.93 ± 0.68	32.02 ± 0.37	31.43 ± 0.29
RDWc, %	18.13 ± 0.30	18.0 ± 0.17	17.32 ± 0.10	18.38 ± 0.47[aa] ($P = 0.025$)
PLT, 10^3/µl	598.43 ± 39.53	634.33 ± 146.35	474.0 ± 44.75	493.66 ± 91.81
PCT, %	0.47 ± 0.03	0.49 ± 0.11	0.38 ± 0.04	0.39 ± 0.07
MPV, fl	7.81 ± 0.05	7.83 ± 0.11	7.88 ± 0.1	7.80 ± 0.68
PDWc, %	30.91 ± 0.15	30.62 ± 0.20	30.80 ± 0.32	31.01 ± 0.32
Glycated haemoglobin (Hb)	5.30 ± 0.28	4.89 ± 0.42	8.57 ± 0.64[a]	6.54 ± 0.46[aa]

Control—control animals.
Mel—melatonin administration.
AES—acute ethanol stress model.
AES + Mel—acute ethanol stress model and melatonin administration.
Significant differences between groups are designated as follows:
[a]AES group vs. control group.
[aa]AES + Mel group vs. AES group.

Table 2. Effects of melatonin on conjugated dienes and protein carbonyl derivatives contents in selected tissues during acute ethanol stress in mice

Parameters	Control	Mel	AAS	AAS + Mel
Conjugated dienes, E233 mg protein				
Liver	2.13 ± 0.11	1.87 ± 0.12	5.89 ± 0.24[a]	4.48 ± 0.36[aa]
Muscle	2.46 ± 0.32	3.04 ± 0.29	7.12 ± 0.37[a]	5.69 ± 0.54[aa]
Kidney	7.08 ± 0.69	6.33 ± 0.26	13.10 ± 0.84[a]	9.86 ± 0.44[aa]
Protein carbonyl derivatives, AD, E370 mg protein				
Liver	6.60 ± 0.54	6.59 ± 0.51	16.81 ± 0.69[a]	11.79 ± 0.85[aa]
Muscle	9.88 ± 0.47	9.46 ± 1.41	18.07 ± 1.61[a]	26.62 ± 11.71
Kidney	16.57 ± 1.77	15.18 ± 0.71	21.98 ± 2.49	14.51 ± 0.86[aa]
Protein carbonyl derivatives, KD, E420 mg protein				
Liver	8.76 ± 0.69	8.36 ± 0.49	19.21 ± 0.87[a]	14.65 ± 0.80[aa]
Muscle	12.00 ± 1.24	11.17 ± 0.83	24.07 ± 1.55[a]	17.99 ± 0.82[aa]
Kidney	15.20 ± 1.25	11.21 ± 1.09	20.86 ± 1.27[a]	16.35 ± 0.67[aa]

Control—control animals.
Mel—melatonin administration.
AES—acute ethanol stress model.
AES + Mel—acute ethanol stress model and melatonin administration.
AD—aldehyde derivates, KD—kenotic derivates.
Significant differences between groups are designated as follows:
[a]AES group vs. control group.
[aa]AES + Mel group vs. AES group.

compared to those observed in the control group. Melatonin administration to ethanol-treated mice statistically decreased the level of conjugated dienes in all investigated tissues compared to the ethanol group.

Primary products of free radical oxidation have a high reactivity and damage various biomolecules, especially proteins. Therefore, we determined the level of OMP—AD and KD derivatives in the liver ($F = 27.27$, $P = 0.000$ and $F = 20.44$, $P = 0.000$ for AD and KD, respectively), muscle ($F = 50.07$, $P = 0.000$ and $F = 4.33$, $P = 0.017$ for AD and KD, respectively) and kidney ($F = 13.07$, $P = 0.000$ for KD) tissues under acute ethanol stress and melatonin treatment (Table 2). Our results did not show any significant effect of melatonin, whereas ethanol intoxication resulted in increase in liver and

muscle OMP AD levels, and the OMP KD levels in three analysed tissues simultaneously. In large part, melatonin limited the intensification of free radical processes in studied tissues.

Our results indicate that in ethanol-intoxicated mice vs. control group MDA level changed significantly in liver and muscle, while in kidney and erythrocytes we did not observe statistically important differences (Fig. 1). Melatonin alone did not exert any effect compared to control group. However, we showed a statistically significant decrease in MDA in liver and kidney tissue levels after melatonin treatment in ethanol-intoxicated group of mice.

TAC level was decreased statistically (Fig. 2) after ethanol treatment in liver ($F = 10.09$, $P = 0.000$) and kidney ($F = 11.82$, $P = 0.000$) compared to the control. Melatonin statistically increased the TAC value in liver tissue of control mice and those exposed to ethanol. These observations correlated with the data of antioxidant enzyme activity upon treatment with melatonin and ethanol.

Antioxidant enzyme activity in the liver, muscle and kidney in response to ethanol and melatonin was very tissue specific (Table 3). Melatonin increased GPx in liver and muscle, and augmented $NADPH_2$ in kidney. In liver and kidney, ethanol increased SOD and GR, but decreased CAT and GPx. Melatonin reversed the effect of ethanol on CAT and GPx, only very slightly modified GR increases induced by ethanol, and actually augmented (liver) or did not change (kidney) the ethanol influence on SOD. In muscle, melatonin did not reverse ethanol evoked changes in antioxidant enzyme activities, and in the case of CAT it showed a synergistic effect.

In the present study we have observed dependences between investigated oxidative stress parameters and activity of antioxidant enzymes upon ethanol treatment, and melatonin administration. Correlation and regression analysis unveiled interactions between lipid peroxidation and HbA1c concentrations in erythrocytes during acute ethanol stress ($r = 0.93$, $P = 0.007$). Melatonin during acute ethanol stress conditions induced the following interdependences in tissues: in liver, a significant relationship between the conjugated dienes and GR ($r = 0.92$, $P = 0.040$) and TAC-OMP KD ($r = 0.85$, $P = 0.034$) was induced; in muscle, interrelations between the conjugated dienes and OMP KD ($r = -0.92$, $P = 0.010$) and SOD – TAC ($r = 0.90$, $p = 0.014$) were induced. Positive correlations were also found between the conjugated dienes and CAT ($r = 0.84$, $P = 0.035$) and negative correlations were found between OMP AD and TAC ($r = -0.88$, $P = 0.019$) in kidney. The results obtained demonstrated that the effect of melatonin during acute ethanol stress mediated different metabolic redox pathways of antioxidant defence.

DISCUSSION

The present research reports the effects of melatonin on the oxidative damage induced by ethanol treatment in mice and its possible role in ameliorating significant changes in WBC count, antioxidant enzyme functioning in blood, liver, kidney and muscle tissues and the development of diabetes predispositions. The main finding of this study is that melatonin prevents ethanol-induced toxicity and oxidative stress

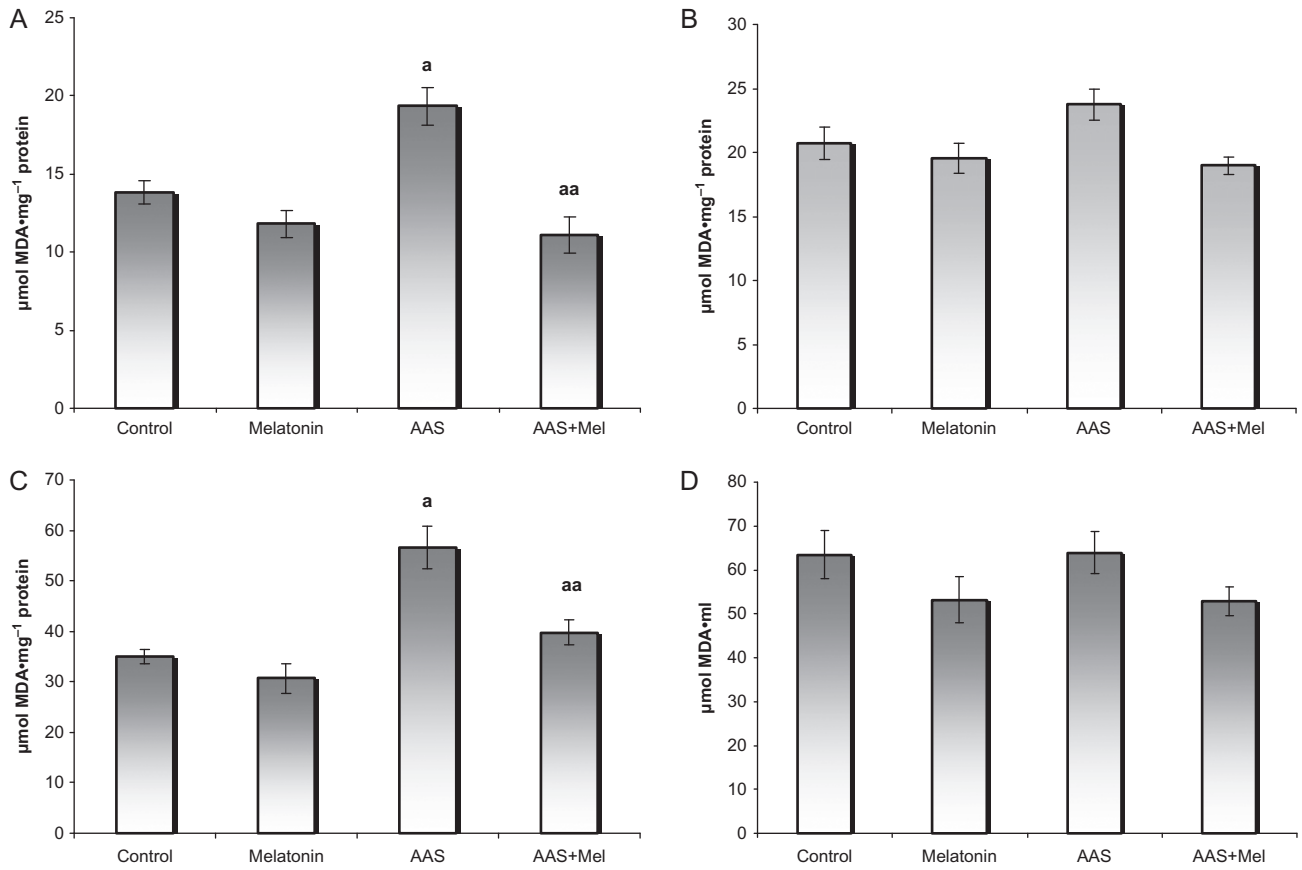

Fig. 1. Effect of melatonin (Mel) on ethanol-induced (AES) lipid peroxide levels (µmol MDA mg^{-1} protein or µmol MDA·ml) in the liver (**A**), muscle (**B**), kidney (**C**) and erythrocytes (**D**) of mice. Significant differences between groups are designated as follows: [a]AES group vs. control group; [aa]AES + Mel group vs. AES group.

in mice by significantly reducing the level of aldehyde and ketone protein derivatives as biomarkers of protein destruction processes in tissue damage. In alcoholic patients, oxidative stress induced by the presence of excessive ROS is closely associated with chronic inflammation, leading to potential tissue damage. The most important results of the present study were the demonstration of decreased oxidative stress parameters, leading to decreased haemoglobin glycation levels, in the development of inflammatory conditions caused by a significant release of WBC and an increase of TAC functional body systems by melatonin administration to ethanol-induced mice over 10 days. The protective effects of melatonin on antioxidant enzyme activity are tissue dependent and most evident in organs of high metabolic activity like liver and kidney.

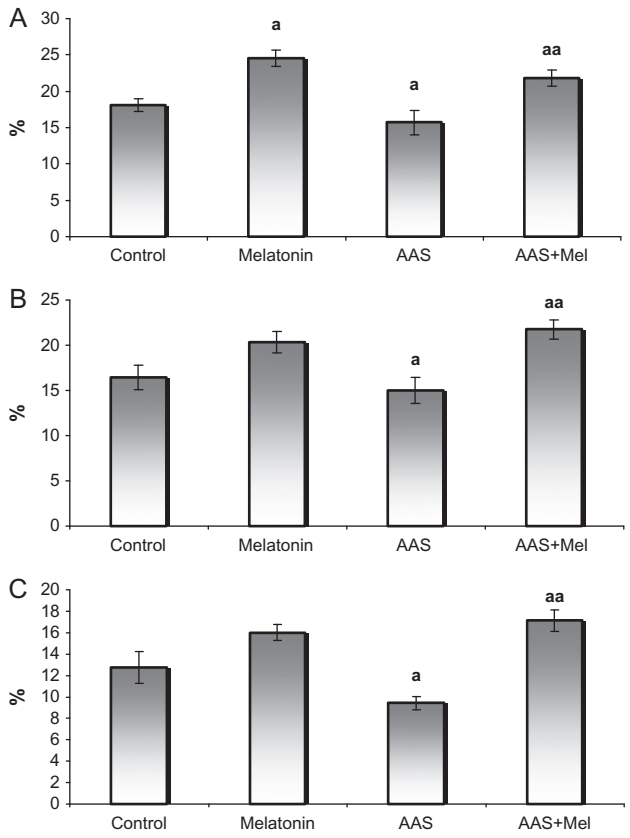

Fig. 2. Effect of melatonin (Mel) on Total Antioxidant Capacity level (%) in the liver (**A**), muscle (**B**), and kidney (**C**) during acute ethanol stress (AES) in mice. Significant differences between groups are designated as follows: [a]AES group vs. control group; [aa]AES + Mel group vs. AES group.

In this study, oxidative stress levels were evaluated based on the mouse blood, liver, muscle and kidney tissues, the total antioxidant capacities of these tissues, SOD, CAT, GR and GPx activities, and the initial (diene conjugation production) and terminal stage (MDA concentration) lipid peroxidation levels. MDA was accepted as an end marker of lipid oxidation processes.

Experimental data showed dependencies between the duration of ethanol administration (e.g. single or chronic) on morphological blood cell content (Maruyama *et al.*, 2001). In our model of acute ethanol stress (0.75 g/kg/day, 10 days) we observed a significant increase to the total number of WBC, especially neutrophils. In a paper by Kawashima *et al.* (2011) single administration effects of ethanol in doses of 2.0 g/kg body weight increased neutrophil count, monocyte, basophil and total WBCs. In contrast to these results, Jabri *et al.* (2016) reported that acute oral ethanol administration (4.0 g/kg body weight) decreased the WBC and PLT count. An anti-migratory effect of melatonin on neutrophils has been recently described (Ren *et al.*, 2015). Nevertheless, to the best of our knowledge we are the first to demonstrate that melatonin attenuates ethanol-induced increases of WBC count, both in neutrophils and lymphocytes.

During liver disease, ethanol promotes glucose intolerance and diabetes through various mechanisms including insulin resistance and impaired insulin secretion, as shown by Nedelson *et al.* (2016). A large number of patients with liver disease have glucose intolerance and the possibility of developing overt diabetes. In patients with alcoholic liver disease, direct toxic effect of ethanol on bone marrow with anaemia's effects is observed (Moreno Otero and Cortés, 2008). HBA1c is

Table 3. Antioxidant enzyme activity in the liver, muscle and kidney tissues during acute ethanol stress (AES) and melatonin (Mel) administration in mice

Parameters	Control	Mel	AES	AES + Mel
Liver				
SOD, U mg^{-1} protein	360.84 ± 16.19	435.65 ± 43.37	653.49 ± 43.12[a]	668.78 ± 55.99[ab]
CAT, μmol min^{-1} mg^{-1} protein	10.43 ± 0.40	9.42 ± 0.39	7.19 ± 0.92[a]	10.51 ± 1.06[ab]
GR, nmol $NADPH_2$ min^{-1} mg^{-1} protein	128.20 ± 6.80	143.61 ± 7.06	178.26 ± 5.69[a]	155.94 ± 9.26
GPx, nmol GSH min^{-1} mg^{-1} protein	43.17 ± 1.31	58.94 ± 1.83[a]	28.14 ± 2.05[a]	40.51 ± 5.04[aa, ab]
Muscle				
SOD, U mg^{-1} protein	443.87 ± 37.76	339.87 ± 16.16	558.92 ± 35.66	436.09 ± 40.80
CAT, μmol min^{-1} mg^{-1} protein	11.19 ± 0.75	9.28 ± 0.77	12.04 ± 0.62	13.24 ± 0.95[ab]
GR, nmol $NADPH_2$ min^{-1} mg^{-1} protein	76.70 ± 6.85	99.92 ± 2.26	123.63 ± 7.01[aa]	130.01 ± 10.34
GPx, nmol GSH min^{-1} mg^{-1} protein	27.85 ± 2.19	51.22 ± 2.95[a]	59.36 ± 9.29[aa]	58.91 ± 1.87
Kidney				
SOD, U mg^{-1} protein	479.14 ± 31.66	533.64 ± 59.08	668.64 ± 29.72[a]	658.83 ± 37.95
CAT, μmol min^{-1} mg^{-1} protein	11.63 ± 0.74	11.25 ± 0.76	7.67 ± 0.76[a]	13.10 ± 0.92[aa]
GR, nmol $NADPH_2$ min^{-1} mg^{-1} protein	128.20 ± 6.80	143.61 ± 7.06[a]	178.26 ± 5.69[aa]	155.94 ± 9.26
GPx, nmol GSH min^{-1} mg^{-1} protein	50.65 ± 3.42	59.09 ± 4.22	31.51 ± 3.76[a]	103.26 ± 5.09[aa, ab]

Significant differences between groups are designated as follows:
[a]AES group vs. control group.
[b]AES + Mel group vs. AES group.
[c]AES + Mel group vs. Mel group.

commonly used for diabetes mellitus monitoring. Since many alcohol states are accompanied with liver cirrhosis and glucose intolerance (Isida *et al.*, 2017), the present study was designed to determine whether glycated haemoglobin concentration, as a predictive marker of glycaemia, may by corrected by melatonin administration. Diabetes and ethanol treatment induces oxidative stress in several organs and systems due to alterations in redox status, ROS overproduction and dysfunction of main antioxidant enzymes (Muriach *et al.*, 2006, Sommavilla *et al.*, 2012). Molecular mechanisms of ethanol intoxication and glucose intolerance are interlinked by proinflammatory action (molecular signalling, genes and transcriptional factors) as shown by Barcia *et al.* (2015). In our study, ethanol statistically increased HBA1c levels. Melatonin reduces oxidative stress and HBA1c in diabetes mellitus (Montilla *et al.*, 1998; Sudnikovich *et al.*, 2007). The examined parameters of the present research corroborate this study. Thus, our study proves that similar anti-inflammatory mechanisms exerted by melatonin may play a protective role in acute ethanol intoxication.

The tissue oxidation induced by ethanol is associated with increased production of ROS, wherein the first generated ROS is a superoxide anion (Ponnappa and Rubin, 2000). Due to a high level of oxygen consumption and aerobic metabolism, the kidney and liver are exposed to large amounts of superoxide anion (Somani *et al.*, 1996). Increased production of superoxide anion in these conditions is due to intensification of the conversion of xanthine dehydrogenase to xanthine oxidase (Kato *et al.*, 1990). This is due to an increase in the concentration of NADH produced during the oxidation of both ethanol and its metabolite—acetaldehyde (Yeligar *et al.*, 2012). Production in the liver of acetaldehyde and ROS after ethanol creates a very hostile biochemical environment. Superoxide anion involves SOD to produce the hydrogen peroxide, which in turn can act as an activator of the next enzyme antioxidant defence system—CAT (Correa *et al.*, 2004).

In the present study, we showed that at initial stages of free radical oxidation of lipids concerning dienes conjugation production, and at the last stages of free radical oxidation with MDA production, ethanol increased the intensity of these processes in liver, kidney and muscle. Concomitantly with the increased lipid peroxidation in mice exposed to ethanol, we found the increase in OMP concentration, namely aldehyde and ketonic derivates. Similar dependences were shown by our correlation and regression analysis between OMP concentrations in liver, namely OMP AD and GR activity ($r = 0.87$, $P = 0.027$). Moreover, our data indicate that ethanol modifies the relationship between OMP AD and TBARS ($r = 0.92$, $P = 0.010$) in muscle tissue, and between conjugated dienes and GPx in kidney ($r = 0.91$, $P = 0.013$). Our results are in line with Sönmez *et al.* (2012), who showed that oxidative stress is included in the pathophysiology of renal diseases and caused ethanol treatment and overload ROS production under these conditions. ROS causes glomerular and tubulointerstitial damage by activating and maintaining cytokine release, leukocyte infiltration and fibrosis. Ethanol consumption causes disruption in the structure and function of the kidneys.

The protective effects of melatonin against different diseases include scavenging of ROS in various ischaemia/reperfusion and hypoxia/reoxygenation models (Jou *et al.*, 2007; Petrosillo *et al.*, 2008; Huang *et al.*, 2013; Santofimia-Castaño *et al.*, 2014). High concentrations of melatonin minimize oxidative damage, due to the antioxidant properties of the molecule (Reiter *et al.*, 2003). Scientific evidence suggests that melatonin can inhibit production of NO and formation of peroxynitrite radical, and regulate the activity of nitric oxide synthase. Melatonin also exerts indirect antioxidant actions through promotion of antioxidant enzymatic activity in cells. Some studies have shown that melatonin regulates GSH redox status in brain and liver mitochondria (Reiter *et al.*, 2003). Furthermore, it seems that melatonin may promote *de novo* synthesis of GSH, and preserve the nicotinamide nucleotides NADPH and NADH. The antioxidant properties of melatonin can be explained by two different mechanisms. Melatonin scavenges hydroxyl radicals (Kim *et al.*, 2005), which are considered the most highly reactive ROS and, thus, diminish damage of cell structures, including DNA. Melatonin also reduces oxidative stress by stimulating antioxidant enzymes (Reiter *et al.*, 2003).

The main focus of the antioxidant effect of melatonin is the protection of DNA, proteins and lipids. Firstly, the mechanism of the antioxidant action of melatonin is due to the fact that this indolamine has a pronounced ability to bind free radicals. They are formed by lipid peroxidation hydroxyl radicals and exogenous carcinogens. Secondly, melatonin activates an important intracellular defence factor against free radical damage as an enzyme of glutathione peroxidase. This effectiveness of antioxidant protection is impaired during ethanol intoxication (Sommavilla *et al.*, 2012). We have found significantly increased GPx activity in the liver and muscle during melatonin administration compared to the control group. In the liver and kidney, the protective effects of melatonin were shown in ethanol-intoxicated mice. The ultrastructural and biochemical study by Esrefoglu *et al.* (2012) demonstrated that melatonin prevents ethanol effects in the liver. Antioxidants, such as melatonin and vitamin C, ameliorate ethanol-induced oxidative stress consequences of long-term ethanol administration in rat kidney in rat kidney by scavenging ROS (Sönmez *et al.*, 2012).

In the present study biochemical analyses revealed that ethanol consumption increased MDA levels and SOD and CAT activities significantly in the ethanol consumption groups compared with the control group, while melatonin significantly decreased MDA levels and antioxidant enzyme activities compared with ethanol consumption groups. Thus, our study provides evidence that melatonin might regulate the antioxidant enzyme activity at levels for SOD, CAT and GPx, both under physiological conditions and conditions of elevated oxidative stress caused by ethanol administration.

CONCLUSIONS

Ethanol treatment causes significant changes in WBC count and glycated haemoglobin levels, and significantly destroys antioxidant defence and repair systems in liver, kidney and muscle tissues of mice. Ethanol reduces the antioxidant capacity and leads to exaggerated ROS production and consequent increases in OMPs, diene conjugates and MDA. Melatonin administration facilitates normalization of organism functions by restoring the WBC count, glycated haemoglobin levels and essential limitations of ROS production. Melatonin exerts protective effects on three selected tissues by preventing the intensive lipid peroxidation processes at the initial (diene conjugation production) and terminal stages (MDA concentration). Finally, melatonin significantly reduces the level of aldehyde and ketone protein derivatives.

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CONFLICT OF INTEREST STATEMENT

None.

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