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Melatonin diminishes oxidative stress in plasma, retains erythrocyte resistance and restores white blood cell count after low-dose lipopolysaccharide exposure in mice

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Abstract. The aim of the study was to elucidate the effects of melatonin administration (10 mg/kg, 10 days) in a model of inflammation and oxidative stress induced by low-dose bacterial lipopolysaccharide (LPS, once 150 μg). Assays were carried out in quadruplicate in the control, melatonin (10 mg/kg, 10 days), acute LPS administration (once 150 μg) and LPS plus melatonin groups. Blood morphological examination was performed. Erythrocyte resistance to haemolytic agents, ceruloplasmin, diene conjugates, malondialdehyde, oxidatively modified protein concentrations, total antioxidant capacity and antioxidant enzyme activity in plasma were measured. LPS administration in mice resulted in white blood cell (WBC) depletion, erythrocyte cell membrane impairment and oxidative stress in plasma characterised by lipid and protein oxidative processes, decreased antioxidative defence and augmented ceruloplasmin concentrations. Melatonin treatment provided to LPS-exposed animals restored WBC counts, ameliorated erythrocyte membrane damage and decreased overall oxidative stress in plasma. Melatonin provides multilevel protection in animals exposed to low-dose LPS.

Key words: Melatonin — LPS — Oxidative stress — Lipids oxygenation — Oxidatively modified proteins — Antioxidant enzymes — Ceruloplasmin — Blood — Plasma

Introduction

Systemic inflammation is one way in which an organism can respond to a bacterial infection (Faist et al. 1999). Gram-negative bacteria lipopolysaccharide (LPS) is the most thoroughly studied inducer of such an inflammatory response (Annane et al. 2005; Wang and Quinn 2010). LPS exposure results in the development of various types of endotoxic shock, depending on dose (Cadenas and Cadenas 2002). The complexity of pathological changes caused by LPS is the consequence of two interrelated processes. The first is related to the direct toxic effect of LPS on vital organs and functions, and the second is associated with the development of excessive protective reactions of the organism (Dauphinee and Karsan 2006). The clinical consequences of lipopolysaccharide endotoxin, such as sepsis and septic shock, are among the most frequent causes of patient death (Wichmann et al. 2000).

Recently, risks associated with subclinical endotoxaemia and subsequent low grade inflammation and oxidative stress associated with obesity and diabetes mellitus have been increasingly recognized. Obesity and diabetes mellitus favour endotoxin (especially LPS) translocation across the intestinal

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barrier, leading to its mild increase in concentration in the bloodstream (Cani et al. 2008). In addition, hyperglycaemia, hyperinsulinemia and insulin resistance impair the functionality of polymorphonuclear neutrophils, Kupffer cells, and macrophages, suppressing the bactericidal activity of leukocytes (Yu et al. 2003; Creely et al. 2007). Therefore, diabetic subjects seem to have lower clearance of LPS and consequently, increased LPS concentrations. Finally, diabetic subjects are more susceptible to developing infections (Al-Attas et al. 2009). Serum LPS activity correlates with metabolic syndrome components, such as triglycerides and fasting glucose. Therefore, serum LPS activity combined with common metabolic abnormalities in diabetes mellitus may contribute to the development of macro- and micro-vascular complications (Lassenius et al. 2011).

The spectrum of physiological effects caused by melatonin is quite wide (Melchiorri et al. 1995; Escames et al. 1997; von Gall et al. 2002). The action of this substance is observed at the systemic, tissue, cellular and subcellular levels (Saravanan et al. 2007). This is due to the ability of melatonin to readily dissolve in water and lipids, thus it penetrates through cell membranes and initiates various physiological processes. Melatonin interacts with the membrane receptors MT_1 and MT_2, which are G protein-coupled receptors located in different parts of the central nervous system, the retina of the eye, blood vessels, the gastrointestinal tract, liver, kidneys, skin and immune cells; moreover, melatonin can bind to intracellular proteins such as calmodulin and tubulin (Escames et al. 1997; von Gall et al. 2002). Melatonin reduces both basal and bacterial LPS-induced lipid peroxidation *in vitro*, as was shown by Sewerynek et al. (1995). Pretreatment with melatonin significantly increases antioxidant activities and decreases lipoperoxidation and oxidative protein levels in selected tissues and blood. These results indicate that the significant protective activity of melatonin on oxidative stress and morphological blood parameters induced by LPS might be associated with the antioxidant activity of melatonin.

In our study, we aimed to determine using an *in vivo* experimental mice model, whether single low dose of LPS causes oxidative damage in the blood, and whether melatonin has a protective role on the haematological profile, the resistance of erythrocytes to haemolytic agents and overall oxidative stress in plasma. We hypothesised that melatonin would diminish LPS-induced oxidative injury.

Materials and Methods

Animals and experimental design

The experiments were performed in accordance with the Guidelines of the European Union Council and the current laws in Ukraine, and approved by the Ethical Commission of National State University in Chernihiv (2612/2016). All experiments were performed between 10:00 a.m. and 12:00 p.m. to compensate for the circadian rhythm. Healthy male white Balb/c mice (*Mus musculus*), weighing about 20–30 g and aged about 2–3 months, were used in the experiments. The data were collected from 24 adult animals 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.

Experimental groups

The mice were randomly assigned into four groups: untreated controls ($n = 6$), melatonin-treated ($n = 6$), LPS-treated ($n = 6$) and LPS+melatonin-treated ($n = 6$). Melatonin was given as a daily intraperitoneal injection at a dose of 10 mg/kg 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; Kurhaluk et al. 2017). LPS (*E. coli* serotype 026:B6, Sigma-Aldrich Sp. z.o.o, Poznan, Poland) was injected once intraperitoneally in a 150 μg dose *per* mouse, as described by Yang et al. (2013) and Blanque et al. (1999). Control mice were given 0.9% NaCl intraperitoneally.

Drugs and solutions

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

Sampling

Blood samples (3 ml) were collected from single mouse into tubes containing K_2EDTA. After centrifugation, plasma samples were removed and frozen at –20°C and stored until analysis.

Isolation of erythrocytes

Blood samples were taken from the caudal vein using syringes in less than 1 min and transferred to tubes containing EDTA, then kept on ice until centrifugation at 3,000 × *g* for 10 min. The plasma was removed; the erythrocytes were washed three times with five volumes of saline solution and centrifuged at 3,000 × *g* for 10 min. Haemolysed erythrocytes were used for the determination of osmotic and acid resistance, and plasma was used for the determination 2-thiobarbituric acid reactive substances.

Resistance of erythrocytes to haemolytic reagents

Acid resistance of erythrocytes

The acid resistance of erythrocytes was determined according to the method of Terskov and Gitelson (1957). The method is based on the measurement of erythrocyte dynamic disintegration in 0.1 N HCl used as the haemolytic reagent. Briefly, the duration of HCl action serves as a measure of erythrocyte resistance. Freshly collected blood samples were centrifuged at 3,000 rpm for 10 minutes. The absorbance was read at 540 nm every 30 s after the addition of HCl until the end of haemolysis. Differences in absorbance at the beginning (taken as 100%) and at the end of haemolysis were determined. The acid resistance of erythrocytes as a % of the disintegration of erythrocytes for every period of time was expressed as a curve.

Osmotic resistance of erythrocytes

The Kamyshnikov (2004) method was used to assay the osmotic resistance of erythrocytes. The method is based on the measurement of differences between the osmotic resistance of erythrocytes in a mixture containing different concentrations of sodium chloride and urea. The absorbance of the mixture containing erythrocytes and 0.3 mol/l urea was determined as 100%. The degree of haemolysis in every test tube was measured spectrophotometrically at a wavelength of 540 nm. The haemolysis of erythrocytes (%) in test tubes with different urea concentrations was expressed as a curve.

Haematological profile

K_2EDTA-treated blood was collected and analysed in an automated manner (Abacus Junior Vet, Austria) considering the following parameters: red blood cell count (RBC, 10^6/μl), white blood cell (WBC, 10^3/μl), lymphocytes (10^3/μl), monocytes (10^3/μl), neutrophils (10^3/μl), lymphocytes (%), monocytes (%) and neutrophils (%), 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, %), mean platelet volume (MPV, fl) and platelet distribution width (PDWc, %).

Biochemical assays

Conjugated dienes assay

The level of conjugated dienes was determined according to Kamyshnikov (2004) method. Conjugated dienes groups were determined at absorption peak at the wavelength of 233 nm and expressed in nmol *per* miligram of protein.

Thiobarbituric acid reactive substances (TBARS) assay

TBARS were estimated using the method of Kamyshnikov (2004). TBARS levels were expressed in μmol of malondialdehyde (MDA) *per* mililitre of plasma.

Protein carbonyl derivatives assay

The oxidatively modified protein (OMP) rate was estimated using the reaction detecting carbonyl derivatives of amino acids with 2,4-dinitrophenyl hydrazine (DNFH) as described by Levine et al. (1990) and modified 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 nm and 430 nm and an absorption coefficient 22,000 $M^{-1}{\cdot}cm^{-1}$. Carbonyl groups were determined spectrophotometrically at 370 nm (aldehyde derivatives (AD), OMP_{370}) and 430 nm (ketone derivatives (KD), OMP_{430}), and expressed in nmol *per* millilitre of blood.

Superoxide dismutase activity assay

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity in the blood was determined according to Kostiuk et al. (1990). SOD activity was assessed by its ability to eliminate O_2^- during quercetin auto-oxidation 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* millilitre of blood.

Catalase activity assay

Catalase (CAT, E.C. 1.11.1.6) activity was determined by measuring the decrease in H_2O_2 in the reaction mixture using the method of Koroliuk et al. (1988). One unit of CAT activity is defined as the amount of enzyme required for the decomposition of 1 μmol H_2O_2 *per* minute *per* litre of plasma.

Glutathione reductase activity assay

Glutathione reductase (GR, E.C. 1.6.4.2) activity in the blood was measured according to the method of Glatzle et al. (1974). The enzymatic activity was assayed spectrophotometrically by measuring NADPH consumption. A blank without NADPH was used and the GR activity was expressed as nmol NADPH *per* minute *per* millilitre of blood.

Glutathione peroxidase activity assay

Glutathione peroxidase (GPx, EC 1.11.1.9) activity was determined by the detection of non-enzymatic utilisation of reduced glutathione (GSH) as the reacting substrate at 412 nm after incubation with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) according to the method of Moin (1986). GPx activity is expressed as nmol GSH *per* minute *per* millilitre of blood.

Total antioxidant capacity (TAC) assay

The TAC level in the plasma was estimated spectrophotometrically with Tween 80 oxidation at 532 nm by measuring the TBARS level following the method described by Galaktionova et al. (1998). Tissue inhibits Fe^{2+}/ascorbate-induced oxidation of Tween 80 resulting in a decrease of TBARS level. Absorbance of blank was determined as 100%. The level of TAC in sample (%) was calculated according to the absorbance of blank.

For the quantification of proteins, the Bradford method (1976) was used with bovine serum albumin as the standard. 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 are expressed as mean ± SEM. All variables were tested for a normal distribution using the Kolmogorov-Smirnov and Lilliefors tests ($p > 0.05$) and the homogeneity of variance was checked using Levene's test. The significance of differences in the level of lipid peroxidation, amino acid carbonyl derivatives and antioxidant enzyme activities between the control and experimental groups was examined using one-way analysis of variance (ANOVA) and Bonferonni's post-hoc test. Statistical analysis was carried out on a double ways: the effect of melatonin and LPS was compared with those of the control group, and the combined effect of melatonin and LPS was compared with the data of the melatonin group and the LPS group separately. Differences were considered significant at $p < 0.05$. In addition, the associations between the data from 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

The haematological data are presented in Table 1. Melatonin did not cause statistically significant changes in the blood cell

Table 1. Effects of melatonin, LPS and combined LPS plus melatonin administration on blood morphology in mice

Morphological blood parameters	Group			
	Control	Mel	LPS	LPS+ Mel
RBC (10^6/μl)	7.91 ± 0.29	7.18 ± 0.44	6.40 ± 0.61	7.42 ± 0.18
WBC (10^3/μl)	4.79 ± 0.36	5.93 ± 0.83	3.56 ± 0.85	6.93 ± 0.67^{aa,2}
LYM (10^3/μl)	3.60 ± 0.23	3.94 ± 0.08	2.64 ± 0.58	7.18 ± 0.50^{aa,3}
MON (10^3/μl)	0.18 ± 0.04	0.25 ± 0.52	0.17 ± 0.09	0.20 ± 0.06
NEU (10^3/μl)	1.01 ± 2.26	1.74 ± 0.46	0.75 ± 0.20	1.56 ± 0.18^{aa}
LYM (%)	75.71 ± 2.63	66.38 ± 4.86	72.42 ± 2.24	74.98 ± 2.71
MON (%)	3.70 ± 0.61	4.38 ± 0.52	3.78 ± 1.19	2.68 ± 0.64
NEU (%)	20.57 ± 2.26	29.22 ± 5.27	20.83 ± 2.22	22.32 ± 2.72
Hb (g/dl)	13.54 ± 0.59	12.45 ± 0.55	11.03 ± 1.28	12.67 ± 0.59
HCT (%)	42.70 ± 1.86	39.31 ± 2.46	35.52 ± 3.43	40.55 ± 1.86
MCV (fl)	53.71 ± 0.78	54.83 ± 0.65	55.50 ± 0.67	54.50 ± 0.76
MCH (pg)	17.07 ± 0.20	17.47 ± 0.37	17.03 ± 0.53	17.08 ± 0.23
MCHC (g/dl)	31.74 ± 0.36	31.93 ± 0.68	30.7 ± 0.75	31.28 ± 0.40
RDWc (%)	18.13 ± 0.30	18.0 ± 0.17	18.52 ± 0.19	17.72 ± 0.21^{aa,4}
PLT (10^3/μl)	598.43 ± 39.53	634.33 ± 146.35	536.17 ± 38.32	504.33 ± 61.78
PCT (%)	0.47 ± 0.03	0.49 ± 0.11	0.41 ± 0.03	0.38 ± 0.04
MPV (fl)	7.81 ± 0.05	7.83 ± 0.11	7.57 ± 0.11	7.67 ± 0.17
PDWc (%)	30.91 ± 0.15	30.62 ± 0.20	29.52 ± 0.26^{a,1}	30.50 ± 0.35

Results are expressed as mean ± S.E.M. Differences between experimental groups were analysed by one-way ANOVA and Bonferoni post-hock test; ($n = 6$). Control, control animals; Mel, melatonin administration; LPS, LPS-induced toxicity; LPS+Mel, LPS-induced toxicity and melatonin administration. Significant differences between groups: ^{a} $p < 0.05$ *vs.* Control group, ^{aa} $p < 0.05$ *vs.* LPS group; ^{1} $p = 0.002$, ^{2} $p = 0.022$, ^{3} $p = 0.016$, ^{4} $p = 0.024$.

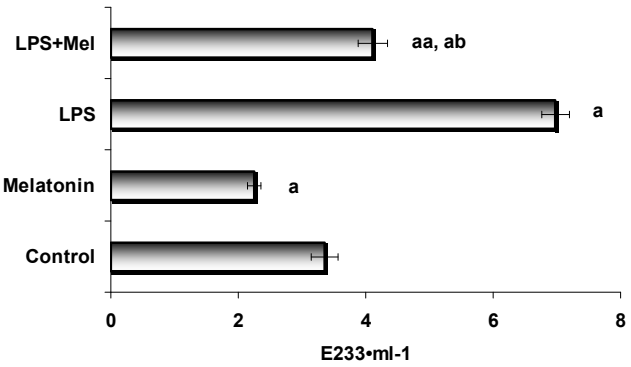

Figure 1. Effect of melatonin (Mel) on conjugated diene contents in plasma during LPS-induced toxicity (E233·ml^{-1}) in mice. Results are expressed as mean ± S.E.M. Differences between experimental groups were analysed by one-way ANOVA and Bonferoni post-hock test; (n = 6). Control, control animals; Mel, melatonin administration; LPS, LPS-induced stress model, LPS+Mel, LPS-induced stress model and melatonin administration. Significant differences between groups: ^{a} $p < 0.05$ *vs.* Control group, ^{aa} $p < 0.05$ *vs.* LPS group, ^{ab} $p < 0.05$ *vs.* Mel group.

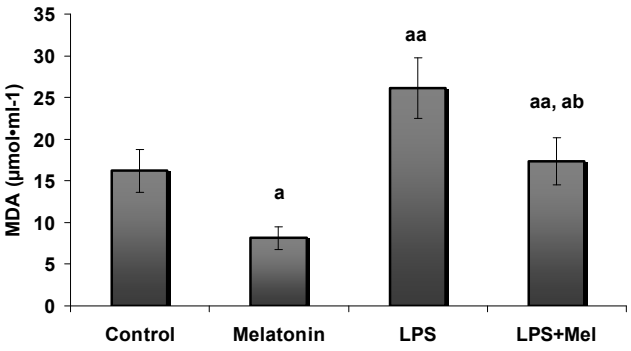

Figure 2. Effect of melatonin on LPS-induced lipid peroxide levels estimated as MDA value (µmol MDA·ml^{-1}) in the plasma of mice. Results are expressed as mean ± S.E.M. Differences between experimental groups were analysed by one-way ANOVA and Bonferoni post-hock test; (n = 6). Significant differences between groups: ^{a} $p < 0.05$ *vs.* Control group, ^{aa} $p < 0.05$ *vs.* LPS group. ^{ab} $p < 0.05$ *vs.* Mel group. For abbreviations see Figure 1.

counts in mice. A single dose of LPS resulted in the depletion of WBC, mostly lymphocytes and neutrophils. Melatonin administration in LPS-exposed mice restored WBC counts compared to the LPS-treated group.

LPS administration was associated with the free radical oxidation of lipids. As this process occurs in several stages, we decided to assess the degree of change at the beginning and at the end of the lipoperoxidation process (LPO). Diene conjugation is considered to be a primary product following the formation of diene conjugates and ketodienes (Fig. 1). Consequently, we estimated the initial substrate accumulation in this stage of free radical oxidation in plasma. After LPS treatment, the concentration of conjugated dienes was significantly higher (F = 16.94, p = 0.000) compared to those observed in control mice. Melatonin statistically decreased the concentration of conjugated dienes in plasma compared to LPS-treated mice. The concentration of MDA (Fig. 2), i.e. the end product of the terminal stages of free radical oxidation of lipids plasma, was significantly higher in plasma in LPS-exposed mice compared to the control group (F = 13.84, p = 0.000). Melatonin treatment in LPS-exposed mice resulted in lower MDA concentrations.

In most cases, LPS toxicity is associated with intensive initial and terminal stages of LPO. Erythrocytes are one of the most sensitive indicators of increased ROS exposure. For this purpose, we carried out the determination of the acid resistance of erythrocytes to haemolytic reagents. The method shows the dynamics of erythrocyte disintegration, which is typically expressed as a curve. The acid resistance of erythrocytes (Fig. 3) was significantly higher after melatonin treatment compared to the response in erythrocytes from control mice. LPS significantly increased the percentage of

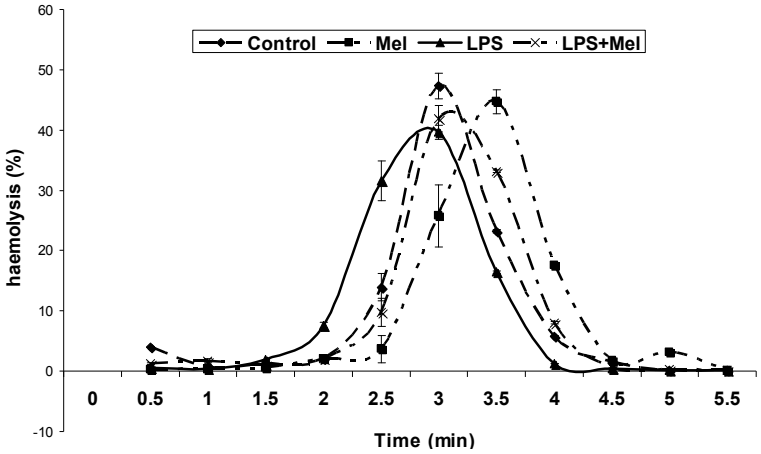

Figure 3. Resistance of erythrocytes to acid (% haemolysed erythrocytes *per* minute) from control, melatonin, LPS and LPS+melatonin-treated mice. Results are expressed as mean ± S.E.M. For abbreviations see Figure 1.

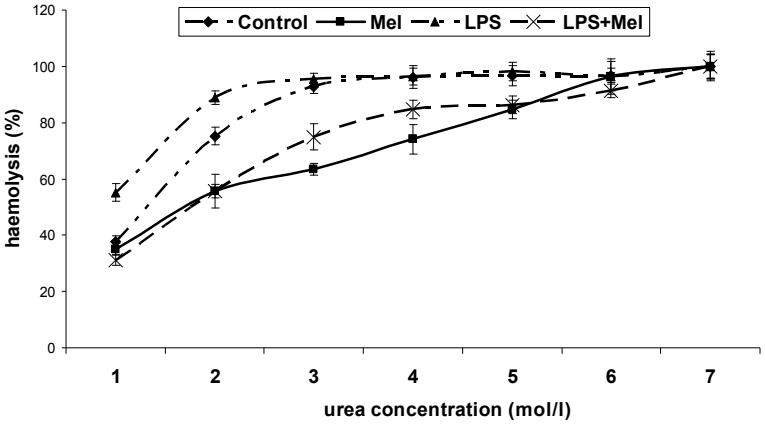

Figure 4. Osmotic resistance of erythrocytes (% of haemolysed erythrocytes in different concentrations of urea) from control, melatonin, LPS and LPS+melatonin-treated mice. Horizontal axis: urea concentration (1: 0.12, 2: 0.135, 3: 0.15, 4: 0.165, 5: 0.18, 6: 0.195, 7: 0.3 mol/l). Results are expressed as mean ± S.E.M. For abbreviations see Figure 1.

haemolysed erythrocytes. Melatonin administration to LPS-treated mice increased the acid resistance of erythrocytes.

The osmotic resistance of erythrocytes was investigated next (Fig. 4). Melatonin moved the curve to the right. On the contrary, the percentage of haemolysed erythrocytes with different concentrations of urea was higher after LPS treatment in mice. Melatonin administration to LPS-exposed mice stabilised erythrocyte membranes.

Oxidative stress products such as free radicals have a high reactivity and damage many cell biostructures, especially proteins. Furthermore, we determined concentration of OMP (Table 2) in plasma ($F = 16.98$, $p = 0.000$ for AD and $F = 12.93$, $p = 0.000$ for KD, respectively), in response to LPS treatment. Melatonin did not affect the OMP concentration. In the LPS group, we observed a 3- to 4-fold elevation in the OMP concentration. Melatonin diminished the effects of LPS. Moreover, melatonin administration did not influence TAC activity. The TAC concentration (Fig. 5) decreased in plasma ($F = 21.05$, $p = 0.000$) after LPS treatment compared to the control group. Melatonin administration to LPS-treated mice augmented the TAC concentration in plasma compared to the LPS group.

The evidence accumulates that pro-inflammatory and oxidative processes are associated with increase in ceruloplasmin concentration in plasma. Ceruloplasmin is a multifunctional protein appearing in acute phase of inflammation. Ceruloplasmin concentration increased in plasma after melatonin treatment (Fig. 6). LPS administration also augmented the ceruloplasmin concentration. Melatonin treatment in LPS-exposed mice lowered the ceruloplasmin concentration in plasma compared to LPS-treated mice.

We observed several interdependences between evoked oxidative stress and antioxidant enzyme activity in response to LPS and melatonin treatment (Table 3). After LPS admin-

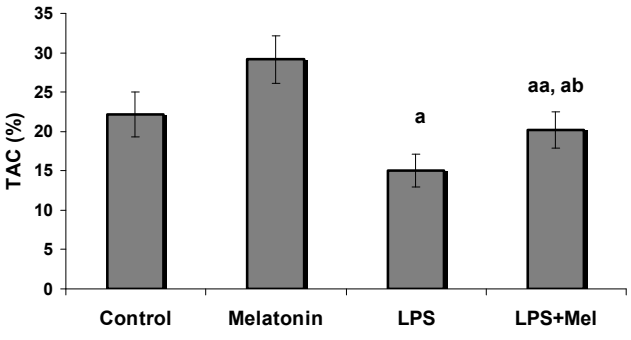

Figure 5. Effect of melatonin (Mel) on the total antioxidant capacity level (%) in plasma during LPS-induced stress in mice. Results are expressed as mean ± S.E.M. Differences between experimental groups were analysed by one-way ANOVA and Bonferoni post-hock test; ($n = 6$). Significant differences between groups: $^{a}p < 0.05$ *vs.* Control group, $^{aa}p < 0.05$ *vs.* LPS group, $^{ab}p < 0.05$ *vs.* Mel group. For abbreviations see Figure 1.

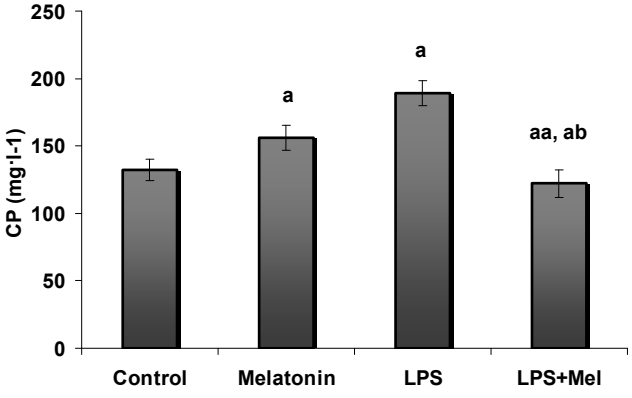

Figure 6. Effect of melatonin on the LPS-induced ceruloplasmin concentration ($mg \cdot l^{-1}$) in mouse plasma. Results are expressed as mean ± S.E.M. Differences between experimental groups were analysed by one-way ANOVA and Bonferoni post-hock test; ($n = 6$). Significant differences between groups: $^{a}p < 0.05$ *vs.* Control group, $^{aa}p < 0.05$ *vs.* LPS group, $^{ab}p < 0.05$ *vs.* Mel group. For abbreviations see Figure 1.

Table 2. Effects of melatonin on protein carbonyl derivatives contents in plasma during LPS-induced stress in mice

Protein carbonyl derivatives	Group			
	Control	Mel	LPS	LPS+ Mel
aldehyde derivative E370·ml	4.22 ± 0.56	3.45 ± 0.23	12.51 ± 1.16 [a]	9.34 ± 1.22 [aa,ab]
ketone derivative E420·ml	3.98 ± 0.22	2.98 ± 0.09	11.25 ± 1.13 [a]	5.16 ± 0.57 [aa,ab]

Results are expressed as mean ± S.E.M. Differences between experimental groups were analysed by one-way ANOVA and Bonferoni post-hock test; (n = 6). Significant differences between groups: [a] $p < 0.05$ *vs.* Control group, [aa] $p < 0.05$ *vs.* LPS group; [ab] $p < 0.05$ *vs.* Mel group. (For abbreviations, see Table 1).

Table 3. Antioxidant enzyme activity in the blood during LPS-induced toxicity and after melatonin (Mel) administration in mice

Parameters	Group			
	Control	Mel	LPS	LPS+ Mel
SOD ($U \cdot ml^{-1}$)	244.56 ± 45.29	342.17 ± 29.37	658.88 ± 58.64[a]	422.71 ± 33.16[aa]
CAT ($\mu mol \cdot min^{-1} \cdot l^{-1}$)	12.22 ± 0.31	9.42 ± 0.39[a]	16.11 ± 1.04[a]	13.21 ± 1.11[aa, ab]
GR (nmol $NADPH_2 \cdot min^{-1} \cdot ml^{-1}$)	88.44 ± 16.22	99.78 ± 17.14	156.55 ± 22.12[a]	195.84 ± 22.16[ab]
GPx (nmol GSH $\cdot min^{-1} \cdot ml^{-1}$)	56.22 ± 6.71	78.64 ± 8.43	118.43 ± 7.11[a]	86.81 ± 7.24[aa]

Results are expressed as mean ± S.E.M. Differences between experimental groups were analysed by one-way ANOVA and Bonferoni post-hock test; (n = 6). Significant differences between groups: [a] $p < 0.05$ *vs.* Control group, [aa] $p < 0.05$ *vs.* LPS group; [ab] $p < 0.05$ *vs.* Mel group. (For abbreviations, see Table 1).

istration, relationships were observed between ceruloplasmin and conjugated dienes (r = 0.93, p = 0.000, y = 159.5400x + 1.8546), ceruloplasmin and OMP AD (r = 0.89, p = 0.001, y = 113.2600x + 1.8114) and TAC and ceruloplasmin (r = 0.90, p = 0.000, y = 5.8840x + 0.1717). Melatonin administration in the LPS-treated group showed an interdependency between TAC and ceruloplasmin (r = 0.87, p = 0.001, y = 4.9035x + 0.1609).

Discussion

In a phase I dose escalation study, melatonin was administered to healthy volunteers at oral doses ranging from 20 to 100 mg per person. Importantly, no adverse reactions other than mild transient drowsiness were reported (Galley et al. 2014). Therefore, the process has already been started to register melatonin as a drug that might be used in various clinical applications. There are several new findings in this study: melatonin 1) diminishes oxidative stress in plasma, 2) retains erythrocytes resistance and 3) restores white blood cell counts after acute low dose LPS exposure in mice.

LPS is the predominant inducer of inflammatory responses of Gram-negative bacteria (Sakaguchi and Furusawa 2006). LPS administration has been extensively accepted as an ideal pharmacological research model characterised by increased release of ROS, neutrophil infiltration and the expression of inflammatory cytokines (Blanque et al. 1999; Su et al. 2014). Low-dose LPS disrupts human hematopoiesis including bone marrow progenitor cell expansion and reduced lymphoid potential (Esplin et al. 2011; Liu et al. 2015). Melatonin has been reported to ameliorate the irradiation-induced decline in peripheral WBC (Koc et al. 2002; Shirazi et al. 2013). The effect was attributed to its ROS scavenging properties (Koc et al. 2002). To the best of our knowledge, we are the first to show that melatonin restores WBC in acute low-dose LPS toxicity. Although the beneficial effects of melatonin have been reported in at least five studies (Sewerynek et al. 1996; Fjærli et al. 1999; Yerer et al. 2004; Alamili et al. 2013; Lansink et al. 2016), the LPS doses administered in these studies were much higher, thus mimicking sepsis state. In our study low-dose LPS was administered to develop low grade inflammation and oxidative stress that are typically associated with obesity and diabetes mellitus.

The MDA concentration (the end product of lipid oxidation), as an oxidative stress marker, was elevated in the plasma of LPS-treated animals compared to the control group. MDA is strongly cytotoxic, resulting in a time-dependent haemolysis (Allegra et al. 2002; Tesoriere et al. 2002), an effect that is inhibited by melatonin (Allegra et al. 2003). An important finding of this study is that melatonin stabilises the erythrocyte membrane and permeability, estimated by erythrocyte haemolysis tests. We have shown that the beneficial effects of melatonin on the oxidative damage induced by low-dose LPS are also present in the initial (diene conjugates) stages of the LPO process. Furthermore, the LPS-induced increase in OMP (AD and KD derivatives) can be modified by melatonin as well.

The supportive effects of melatonin with respect to antioxidant processes in the organism are, to some extent, dependent on the ceruloplasmin concentration. Although ceruloplasmin is synthesised in the liver, 70% of the total quantity that exists in the organism is found in plasma. Therefore, this protein was used to estimate the inflammatory and oxidative status after LPS treatment. Despite what the systematic name of ceruloplasmin might suggest, i.e. Fe(II):oxygen oxidoreductase (ferroxidase I, EC 1.16.3.1), it is a multifunctional protein with broad biological activities (Kamyshnikov 2004).

After LPS administration several interdependencies between ceruloplasmin and conjugated dienes, OMP AD and TAC were observed. After melatonin administration in the LPS-treated group correlation between TAC and ceruloplasmin was demonstrated. Sorenson (1977) formulated the hypothesis that endogenous copper may have protective effect against inflammation. Consequently, an increase in serum concentrations of copper and ceruloplasmin observed in many pathological processes, whether chronic or acute, represents a natural anti-inflammatory response (Sorenson 1977; Goldstein et al. 1982). Consequently, the reduction in ceruloplasmin concentrations seen in response to melatonin may reflect an overall decrease in inflammatory and oxidative status.

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals. The addition of one electron to dioxygen forms the superoxide anion radical ($O_2^{\bullet -}$). Superoxide anion, arising either through metabolic processes or following oxygen "activation" by physical irradiation, is considered the "primary" ROS, and can further interact with other molecules to generate "secondary" ROS, either directly or prevalently through enzyme- or metal-catalysed processes (Valko et al. 2007). Various pathways of ROS formation under different agents have been previously examined. The inductors of inflammatory processes and oxidative stress are toxic hydroxyl radicals. These radicals, released during inflammation, can activate neutrophils and macrophages. These cells release superoxide anion radicals due to the activation of membrane enzyme NADPH-oxidase. The "oxygen burst" caused by the high activity of this enzyme to generate free radicals is the part of the protective mechanism against invading microbial pathogens (Halliwell 2007; Wang and Quinn 2010). Antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), ceruloplasmin (CP), and the reduced and oxidised glutathione ratio) play an important role in protecting the cell from the harmful effects of ROS; in the our study, this effect was strongly modulated by melatonin. Melatonin is known to be a powerful antioxidant (Sönmez et al. 2012). It exhibits direct antioxidant properties, acting as a scavenger of free radicals, indirectly enhances the activity of anti-oxidative enzymes (SOD, CAT and GPx) and can stabilise their concentrations (Watson 2012).

The inflammatory response to LPS was not assessed in this study. However, these measurements have been well-described in animals and humans, and our data are consistent with previous findings (Baker at al. 2014). Therefore, we are confident that our acute LPS dose evoked the typical inflammatory reaction and consequent oxidative stress. Furthermore, we did not measure plasma melatonin concentration in experimental animals.

Conclusion

Low-dose LPS administration in mice results in WBC depletion, erythrocyte cell membrane impairment and oxidative stress in plasma characterised by lipid and protein oxidative processes, decreased antioxidative defence and augmented ceruloplasmin concentrations. Melatonin treatment provided to low-dose LPS-exposed animals restored the WBC count, ameliorated erythrocyte membrane damage and decreased indicators of overall oxidative stress in plasma.

Conflict of interest. None.

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