

Melatonin influences antioxidant homeostasis and basal metabolism in the BALB/c mouse model

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Abstract

OBJECTIVES: Melatonin is a hormone with strong antioxidant activity. It is biosynthesized in the pineal gland and serves in the biological signaling and control of the circadian rhythm. Though there is evidence of beneficial effects of melatonin, the substance was not investigated in greater details associated with specific regulation of oxidative stress in organs and tissues.

DESIGN: The experiment is based on exposure of BALB/c mice to doses from 10 µg to 1mg of melatonin. Mice were euthanized after one and 24 hours, respectively. Biochemical markers in plasma, ferric reducing antioxidant power (FRAP), thiobarbituric acid reactive substances (TBARS) and activity of caspase-3 were examined in selected organs.

RESULTS: We confirmed significant alteration in high-density lipoprotein and total cholesterol and glucose. After one day, levels of glucose and high-density lipoprotein cholesterol were decreased, while total cholesterol increased in a dose dependent manner. FRAP values increased in spleen, liver, kidney, heart, and brain indicating a growing potential of low molecular weight antioxidants 24 hours after exposure. However, TBARS values indicating oxidative stress were elevated in heart, kidney, and liver.

CONCLUSIONS: Despite the antioxidant properties of melatonin, its effect on the organism is more complicated. It influences not only the oxidative homeostasis but also the basal metabolism, as represented by, e.g., cholesterol and glucose. This substance could thus be used for therapeutical purposes such as amelioration of pathologies associated with generation of reactive species or some metabolic dysfunctions.

INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone produced in the pineal gland of vertebrates. The hormone is implicated in the control of sexual activity in animals with seasonal reproduction (Zarazaga *et al.* 2010). In humans, melatonin is involved in sleep and circadian cycle control

(Zhou *et al.* 2012). Owing to control of sleep, it is sometimes administered as a safe drug for treatment of sleep disturbances (Lemoine *et al.* 2012). Three specific receptors of melatonin are known. These are G protein-coupled MT₁, MT₂ and MT₃ membrane receptors. Complex effects of melatonin have not yet been understood. On the other hand, there is evidence of link between MT₁ and

clock gene expression (Imbesi *et al.* 2009). MT₁ receptor is expressed on cerebellar granule cells (Imbesi *et al.*, 2008). Besides the circadian rhythm control, melatonin acts as an antagonist of nitric oxide production in both isolated cells and viable organisms (Silva *et al.* 2007). MT₂ receptor is involved in the nitric oxide regulation (Tunstall *et al.* 2011). Interaction with the retinoic acid related orphan receptor $\alpha 1$ is another biological effect of melatonin (Hill *et al.* 2009). Due to its chemical structure, melatonin is a potent antioxidant relieving from oxidative burden by the so called suicidal (irreversible) scavenging of reactive oxygen species (Maharaj *et al.* 2007). Products of melatonin oxidation, 6-hydroxymelatonin and N-acetyl-N-formyl-5-methoxykynurenamine, act as antioxidants as well. Owing to the regulatory and antioxidant potency of melatonin, it is considered as a promising compound for treatment of neurodegenerative disorders associated with oxidative stress (Pohanka 2011). The beneficial effect of melatonin was confirmed by many investigators engaged in the treatment of, e.g., cancer (Orendas *et al.* 2012) or obesity (Nduhirabandi *et al.* 2012).

Implication of melatonin in the regulation of antioxidants levels remains unclear as some controversial findings have been made. Besides the above reported antioxidant action of melatonin, pro-oxidant activities of melatonin were also recognized in proteins of human erythrocytes (Dikmernoglu *et al.* 2008). In order to reveal the link between melatonin and oxidative stress, we aimed our experiment to investigate selected markers of oxidative stress and antioxidants in organs of laboratory mice exposed to melatonin. We hypothesized that evidence of the melatonin action can help to evaluate the potency of melatonin for pharmacological purposes. Besides the oxidative stress, biochemical markers were evaluated for a better understanding of the melatonin toxicity.

MATERIAL AND METHODS

Experimental animals

Female BALB/c mice weighing 20 ± 2 g and two months old (BioTest, Konarovice, Czech Republic) were used. The BALB/c mice were chosen as a suitable model due to two facts. Firstly, the BALB/c mice are well for research of oxidative stress (Nagato *et al.* 2012). Secondly, we can compare results with findings in previous experiment (Pohanka *et al.* 2011b). The animals were kept in an air conditioned room with temperature 22 ± 2 °C, humidity $50 \pm 10\%$, and 12-hour light period. Food and water were supplied without any restriction. The whole experiment was conducted in the vivarium of the Faculty of Military Health Sciences (Hradec Kralove, Czech Republic) and was permitted and supervised by the Ministry of Defense ethical commission (Czech Republic).

In total, seven groups of five animals were established. The animals received melatonin (Sigma-Aldrich, St. Louis, MO, USA) as a suspension in saline solution

administered subcutaneously. Controls received physiological saline only. The suspension was applied in the total volume of 1 μ l per 1 g of body weight into musculature of the rear limb. Animals were sacrificed in carbon dioxide narcosis by cutting the carotid artery. The dose of melatonin and time of euthanization is clearly shown in the following scheme:

- Controls – physiological saline only
- Melatonin 10 μ g/kg, euthanasia after 1 hour
- Melatonin 10 μ g/kg, euthanasia after 24 hours
- Melatonin 100 μ g/kg, euthanasia after 1 hour
- Melatonin 100 μ g/kg, euthanasia after 24 hours
- Melatonin 1 mg/kg, euthanasia after 1 hour
- Melatonin 1 mg/kg, euthanasia after 24 hours

Tissue and plasma samples

Samples of the liver, kidney, spleen, heart, telencephalon part of the brain and blood were collected. Blood was sampled into heparinized tubes and centrifuged for 10 minutes at $2,000 \times g$. After that, plasma was separated and used for the assay of biochemical markers. A cut from the organs was taken and then thrown into phosphate buffered saline (PBS) (1 ml of PBS was given per 100 mg of the organ cut and homogenized using a stainless steel mixer (Ultra-Turrax, Ika, Werke, Staufen, Germany). The mixer was adjusted up to 8,000 rotations per minute and it was immersed into the PBS with the organ cut. It was intensively mixed per one minute. The crude homogenate was used as a sample for the next assay. After the mixing, the samples were stored at -70 °C or used immediately. The homogenization procedure was used in compliance with the before introduced protocol (Pohanka *et al.* 2011 a, b).

Ex vivo assays

Total protein, superoxide dismutase and caspase-3 activity were measured using TP0100, SOD and CASP3C kits from Sigma-Aldrich. Thiobarbituric acid reactive substances (TBARS) and ferric reducing antioxidant power (FRAP) were assayed in compliance with previously optimized protocols (Pohanka *et al.* 2011 a, b). Molar values of TBARS and FRAP or the activity of caspase-3 were related to total protein. The biochemical analysis of plasma markers was performed using an automated analyzer (Spotchem™ EZ SP-4430, Arkray, Japan). Glucose, blood urea nitrogen, total bilirubin, alanine aminotransferase, alkaline phosphatase, creatinine, total protein, albumin, calcium, inorganic phosphate, magnesium, lactate dehydrogenase, gamma glutamyl-transferase, creatinine phosphokinase, total and high-density lipoprotein (HDL-C) cholesterol and amylase were also measured using the automated analyzer.

Data processing and statistics

All markers were calculated per gram of protein. Origin 8 SR2 (OriginLab Corporation, Northampton, MA, USA) was used for Bonferroni significance testing. Both $p=0.05$ and 0.01 probability levels were calculated

considering the group size of 5 specimens. Mean \pm standard error of mean was calculated for each marker and experimental group.

RESULTS AND DISCUSSION

Biochemical markers

The animals were healthy till the end of the experiment without clinical signs of poisoning. Considering the biochemical plasma markers, alanine aminotransferase, alkaline phosphatase, creatinine, total protein, albumin, calcium, inorganic phosphate, magnesium, lactate dehydrogenase, gamma glutamyltransferase, creatinine phosphokinase and amylase were not significantly altered (data not shown). Owing to the aforementioned markers, we can neglect pertinent hepatotoxic effect of melatonin. The finding is in a good compliance with literature search (Kurus *et al.* 2009; Pohanka 2011). On the other hand, blood urea nitrogen, glucose, HDL-C and total cholesterol were altered when compared to the control. Blood urea nitrogen is shown in Figure 1. The marker indicates function of the renal system. Once kidney function becomes deteriorated, the marker is elevated (Murray *et al.* 2011). We found no effect of melatonin on urea level in the group of animals euthanized one hour after their exposure. The opposite was true one day after exposure. No alteration in blood urea nitrogen was observed in groups dosed 10 and 100 $\mu\text{g}/\text{kg}$. However, the upper dose, 1 mg/kg, caused a significant ($P = 0.01$) increase of the marker up to the double value of the control. Owing to the marker importance, we can infer adverse effects of melatonin on kidneys when given in the dose of 1 mg/kg. The effect arisen after melatonin elimination as the half time of melatonin is quite short. For example, the half time is 1.8 – 2.1 hours in humans (Gooneratne *et al.* 2011). It is probable that melatonin in the high dose can initiate some adverse effects lasting after its full elimination. This finding is surprising as search of literature provided only data about protective effects of melatonin on kidneys. In an example, there is evidence about melatonin protection of kidneys during ischemic injury (Patschan *et al.* 2012), nephropathy induced by mechlorethamine (Kunak *et al.* 2011), and autoimmune idiopathic membranous nephropathy (Wu *et al.* 2011). We can conclude this part of experiment by a statement that overdosing by melatonin can be risky for kidney homeostasis. On the other hand, doses taken for sleeping regulation are deeply under the mentioned risky dose.

Levels of glucose (Figure 2), HDL-C (Figure 3) and total cholesterol (Figure 4) were significantly altered following melatonin administration. Glucose decreased in a dose dependent manner after both one and 24 hours. The mechanism action of melatonin on glycaemia is not clear. It is probably linked with melatonin receptors as some types of melatonin receptor isotypes are associated with an increased risk of diabetes mellitus in humans (Vlassi *et al.* 2012). We can appoint at

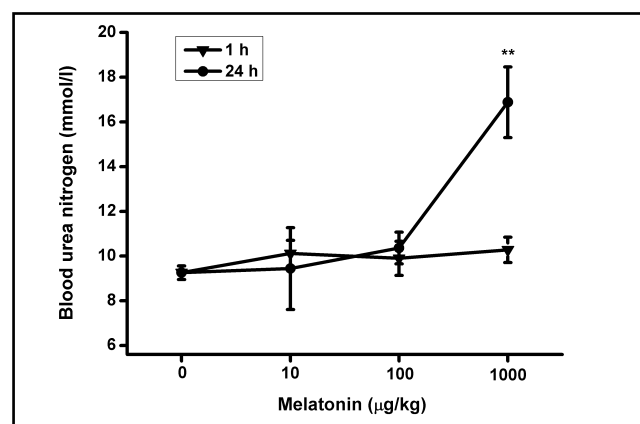


Fig. 1. Blood urea nitrogen in plasma sample \pm S.E.M.

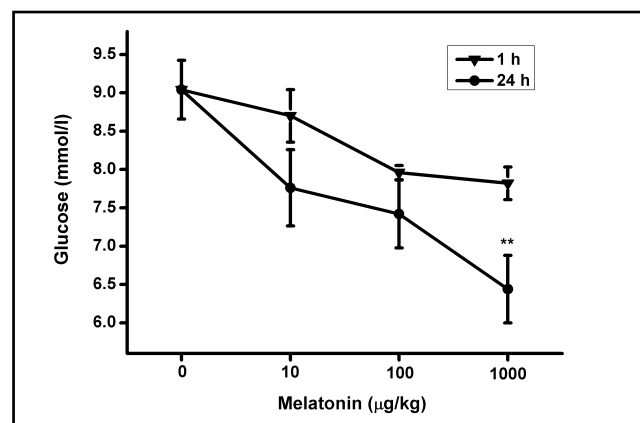


Fig. 2. Glucose in plasma sample \pm S.E.M.

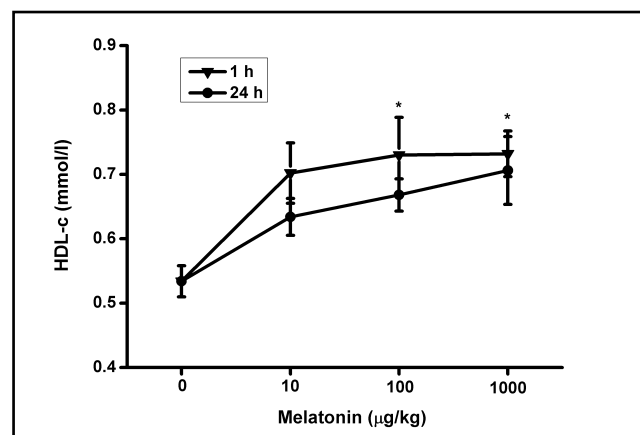


Fig. 3. High density lipoprotein cholesterol (HDL-c) in plasma sample \pm S.E.M.

the fact that the effect of melatonin on the glucose level can be considered beneficial as diabetes is a current worldwide epidemic and melatonin would be suitable for amelioration of diabetic rise in the glucose level. This conclusion is in compliance with other reports (Korkmaz *et al.* 2012; Oliveira *et al.* 2012; Cuesta *et al.* 2012). Total cholesterol decreased in a dose-response manner after melatonin as well as the above-mentioned

glucose. Contrary to the glucose, a greater decrease of total cholesterol occurred one hour after administration of melatonin and the decrease was lower one day after exposure. The improvement in the cholesterol level after melatonin was in compliance with the effect of melatonin on the metabolic syndrome revealed by Kitagawa *et al.* (2011). HDL-C increased in a dose-dependent manner in both intervals. However, the alteration was higher one hour after exposure. Considering the importance of lipoproteins in the body, HDL-C is beneficial and its increased level is commonly considered as a factor associated with lower incidence of vascular diseases including coronary heart disease (Siri-Tarino 2011). For this reason, melatonin is potent enough to improve the HDL-C levels. This finding was

also reported by, e.g., Tunes *et al.* (2002). Similarly to blood urea nitrogen, the discussed markers glucose, HDL-C and total cholesterol were elevated after the period of elimination of melatonin from the body. For this reason, the regulatory effect of melatonin is supposed rather than some direct action of the compound.

Caspase

Caspases are a group of cysteine proteases playing an important role in apoptosis. We chose to assay caspase-3 in tissues as it executes apoptosis initiated by both mitochondrial way via caspase-9 and extra-cellular via the Fas receptor and caspase-8 (Martin 2001). Our experiment provided evidence of ambiguous effects on apoptotic processes. From the examined organs, the kidneys (Figure 5A), liver (Figure 5B) and brain (Figure 5C) had elevated caspase-3 levels with more pronounced effects after one hour and lower activities after one day. Contrary to the above-mentioned organs, the heart (Figure 5D) and spleen (Figure 5E) had decreased levels of caspase-3 in a dose-response manner. Surprisingly, the caspase-3 activity decreased following melatonin administration in the heart and spleen and the lowering was more extensive after one day. It seems that caspase-3 is regulated in a different way in the spleen and heart than in the kidneys, liver, and brain.

The pro-apoptotic effect of melatonin was surprising as melatonin was reported as an inhibitor of Bax and an anti-inflammatory agent (Wang *et al.* 2012; Liu *et al.* 2012). On the other hand, Wang *et al.* (2012) proved melatonin-mediated induction of Apaf-1 expression and

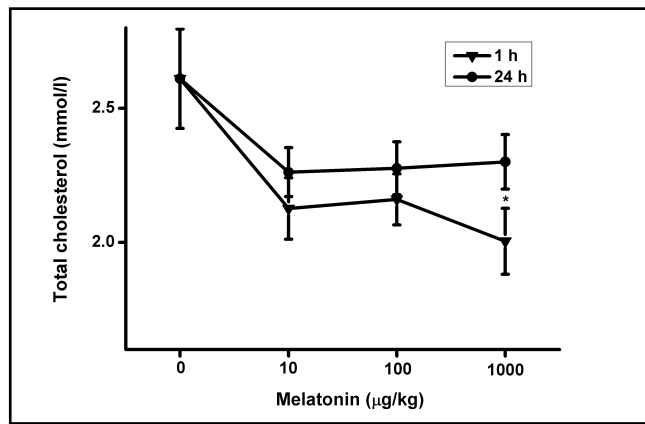


Fig. 4. Total cholesterol in plasma sample ± standard error of mean.

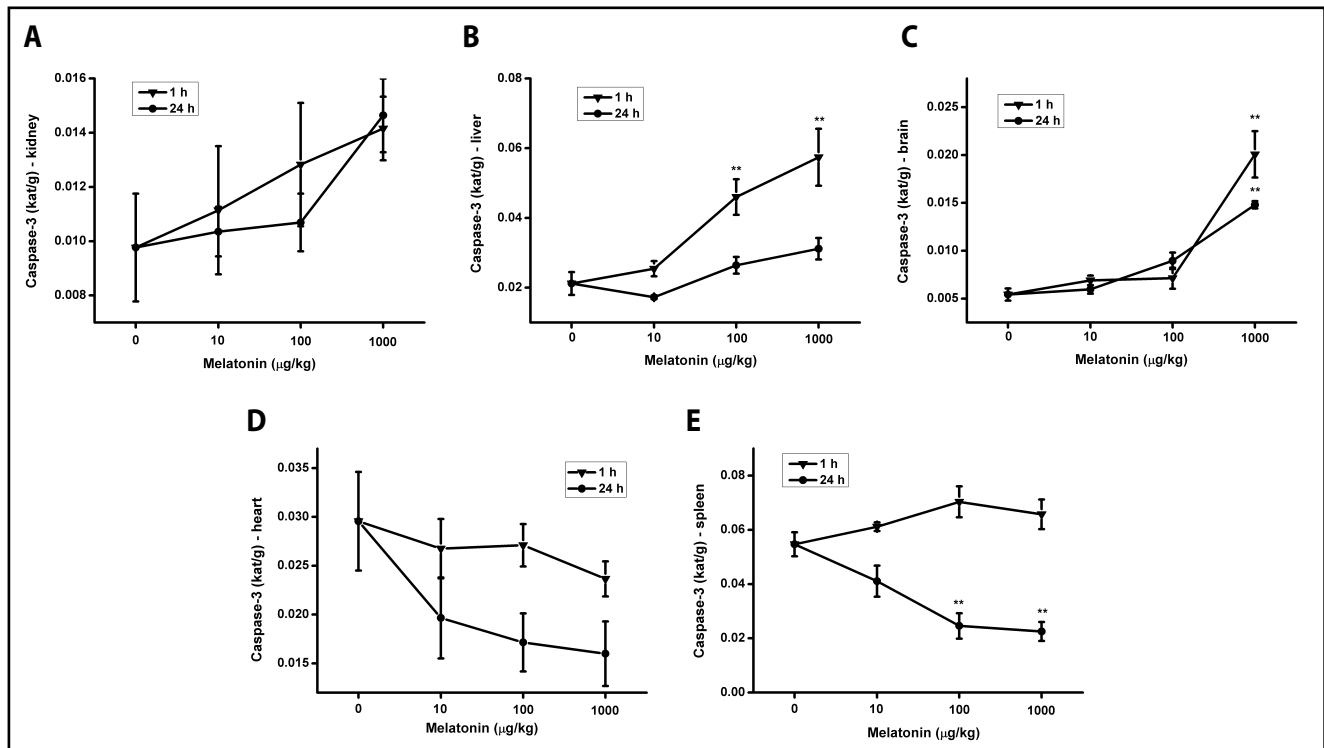


Fig. 5. Caspase-3 activity per gram of protein ± S.E.M. in kidney, liver, brain, heart and spleen.

then induction of apoptosis via cytochrome c release in breast cancer cells. We have no evidence about regulation of apoptosis controlled with melatonin. However, tissue specific action of melatonin observed here may be caused by a dominant presence of either Apaf-1 or Bax and Fas receptor.

Oxidative balance

Oxidative balance was investigated using FRAP and TBARS levels in the examined tissues (Pohanka *et al.* 2009). The FRAP level indicates ability of the organism to maintain the level of low molecular weight antioxidants. It should be emphasized that the administered level of melatonin is quite small in comparison with level of endogenous antioxidants. Though FRAP value can be directly influenced by the administered melatonin, percentage change in total antioxidant capacity is irrelevant due to high antioxidant potency of tissues (Pohanka *et al.* 2009). Alteration in the found FRAP levels should be inferred as alteration in keeping of antioxidant homeostasis.

Contrary to the FRAP, the TBARS method is based on measuring the malondialdehyde level. The malondialdehyde is a terminal product of lipid peroxidation produced in low amount under physiological conditions. Its production is accelerated by presence of reactive species (Lykkesfeldt 2007).

The FRAP value was not altered in any of the examined organs in animals euthanized one hour after melatonin application. However, all tested organs had increased FRAP values in a dose-response manner one

day after exposure (Figure 6 A–E). Melatonin is one of the most potent antioxidants as it undergoes irreversible oxidation into 6-hydroxymelatonin and N-acetyl-N-formyl-5-methoxykynurenamine, i.e. antioxidants as well (Tan *et al.* 2007, Pohanka 2011). Application of melatonin presented here, however, did not cause a significant elevation of the FRAP value shortly after injection. This fact indicates that melatonin administered in the chosen doses had no relevant effect on the antioxidant potential in the body. So the recognized effects should be attributed to melatonin hormonal and regulative properties rather than its antioxidant properties. It should be emphasized that the chosen doses applied to the animals cover the doses expected for humans as melatonin is commercially available as pills containing from 0.3 to 20 mg of melatonin with the most frequently used dose of 2.5–7.5 mg (McArthur and Budden 1998). Production of endogenous melatonin is quite low with changes during the day. It is expected that healthy persons produce approximately 30 µg of melatonin daily (Fellenberg *et al.* 1980). Owing to the above-discussed half time of melatonin (Gooneratne *et al.* 2011), it is expected that the mice examined in the present experiment were free of exogenous melatonin after one day. Surprisingly, the effect of melatonin on the examined organs was more extensive after one day than after one hour. We can infer that the effect of melatonin on antioxidants in the body is more extensive than can be expected from the dose and its antioxidant properties. The effect is probably linked with a cascade of melatonin-associated hormonal actions.

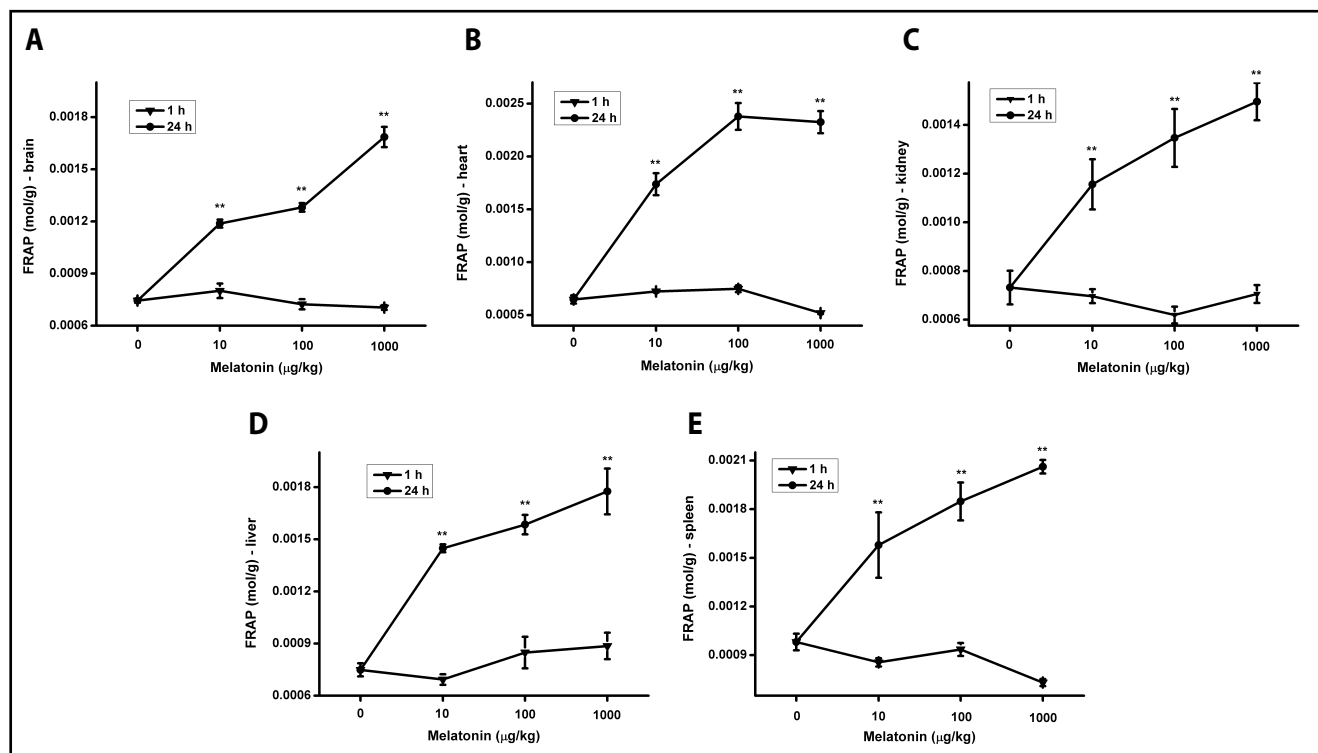


Fig. 6. Ferric reducing antioxidant power (FRAP) per gram of protein \pm standard error of mean in kidney, liver, brain, heart and spleen.

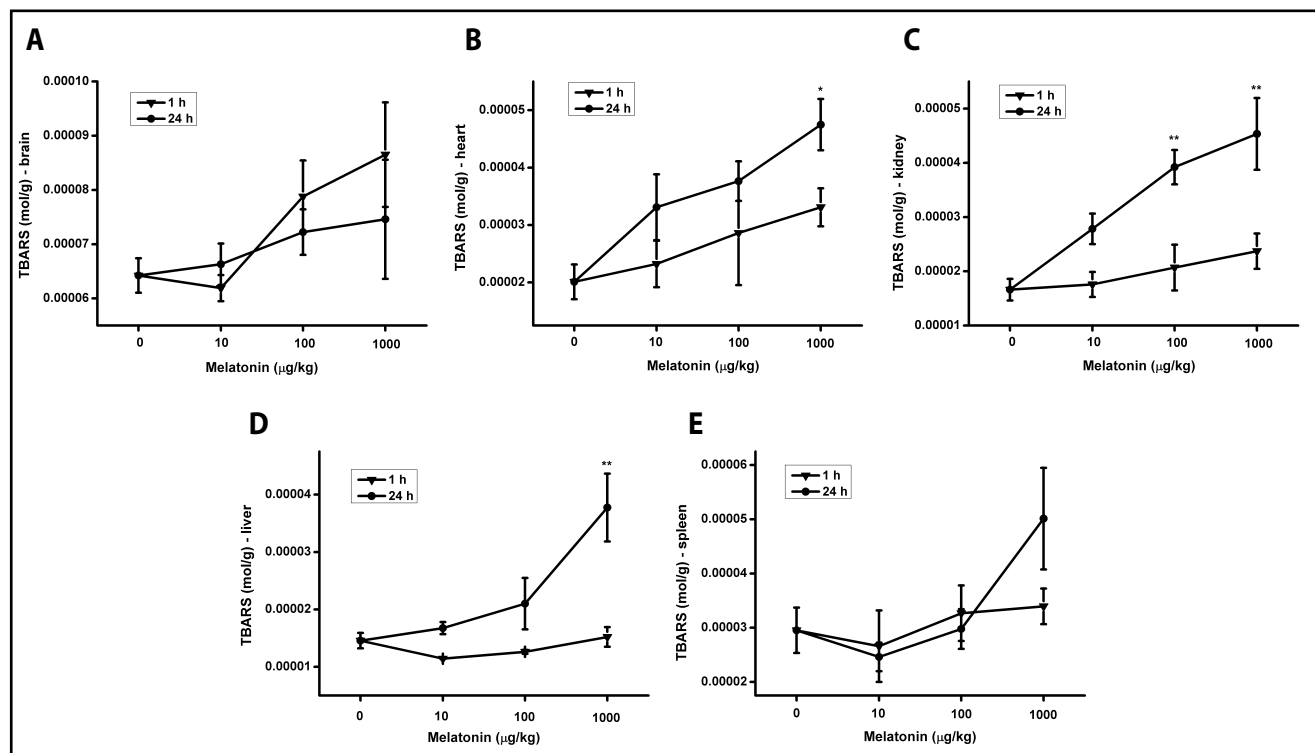


Fig. 7. Thiobarbituric acid reactive substances (TBARS) per gram of protein \pm standard error of mean in kidney, liver, brain, heart and spleen.

The TBARS values are shown in Figure 7 A–E. Despite expectation from the above discussed FRAP values, the TBARS levels were also elevated one day after melatonin application. The levels of TBARS were significantly increased in a dose-dependent manner in the heart, kidney and liver tissues one day after application. The increase in the brain and spleen was insignificant at both intervals. The results point at the fact that melatonin can exert action as a pro-oxidative agent. Evidence of such a finding is not surprising as it was described in some papers. For example, Dikmenoglu *et al.* (2008) described link between the release of malondialdehyde and application of melatonin in human erythrocytes. On the other hand, the effect reported by Dikmenoglu *et al.* was not in a dose-response manner. In other experiments, laboratory data provided evidence of melatonin protection against malondialdehyde. Melatonin ameliorated ischemic detrimental oxidative consequences in the rat model via reduction of TBARS (Watanabe *et al.* 2011). Elsewhere, melatonin decreased the plasmatic value of TBARS within physiological values in Wistar rats (Pohanka *et al.* 2011c). The level of plasmatic TBARS can be, however, altered due to the bilirubin level without any appearance of oxidative stress (Pyles *et al.* 1993). For this reason, the TBARS is more reliable for assay of malondialdehyde in tissues than in plasma. Findings reported here support the necessity to be cautious when scoring melatonin effects and using this substance as an antioxidant. As shown, both antioxidant and pro-oxidant effects can occur. The enhancement in the level of antioxidants as indicated by the

FRAP value was demonstrated here presented, however, in lower melatonin doses than the pro-oxidant effect.

CONCLUSIONS

Though melatonin is known as a potent antioxidant, its contribution to the body homeostasis is wider. It is able to alter the basal metabolism, to act as a pro-oxidant and enhance the antioxidant level in organs. As the greatest alteration and effects were found later at the time when melatonin was already eliminated from the body it can be inferred that the effect of melatonin is rather regulative and linked to its hormonal action.

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Potential Conflicts of Interest: None disclosed.

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