Constant darkness conditions modulate the effects of melatonin and luzindole on the antioxidant enzyme activities and levels of retinol and α-tocopherol in rats

Svetlana Kalinina, Viktor Ilyukha, Evgeniy Khizhkin, Irina Baishnikova, Ekaterina Antonova, and Artem Morozov

Institute of Biology of the Karelian Research Centre of the Russian Academy of Sciences (IB KarRC RAS), Pushkinskaya Str., 11, Petrozavodsk, Karelia, 185610, Russian Federation

Address correspondence and requests for materials to Svetlana Kalinina, cvetnick@yandex.ru

Abstract

This study was conducted to evaluate the effects of both exogenous melatonin and melatonin receptor antagonist luzindole on the activities of antioxidant enzymes (AOE) (superoxide dismutase, SOD; catalase, CAT) and the level of low-molecular antioxidant vitamins (retinol, α-tocopherol) in male Wistar rats kept in normal light conditions (LD 12:12) or constant darkness (DD). In LD, while melatonin had no influence on the studied antioxidants, luzindole caused an increase in retinol and a decrease in α-tocopherol contents in the liver compared to the control. In DD, with no influence on AOE activities, both drugs exerted similar effects on the liver retinol and kidney α-tocopherol contents, increasing them in comparison with control. Exposing the animals to DD induced an increase in kidney SOD activity and in liver retinol content. Moreover, DD-mel rats had higher SOD activity in the liver and kidney and a higher retinol level in the liver compared to LD-mel ones; DD-luz rats had a higher liver retinol content compared to LD-luz ones. Liver retinol level seems to be the most sensitive to influence of DD, melatonin and luzindole; the data are probably connected with the involvement of vitamin A in the regulation of circadian rhythms.

Keywords: constant darkness, melatonin, luzindole, antioxidant enzymes, retinol, α-tocopherol.

List of abbreviations

AOE	— antioxidant enzymes;
ATRA	— all-trans-retinoic acid;
CAT	— catalase;
DD	— constant darkness, 24 hours dark;
GPx	— glutathione peroxidase;
HPLC	— high performance liquid chromatography;
LD	— normal light conditions, 12 hours light : 12 hours dark;
LD (DD)-control	— group of rats received placebo;
LD (DD)-mel	— group of rats received melatonin;
LD (DD)-luz	— group of rats received luzindole;
MT1 and MT2	— melatonin receptors;
ROS	— reactive oxygen species;
RZR/RORα and NR1F2 (RZR/RORβ)	— nuclear orphan receptors;
SCN	— suprachiasmatic nucleus;
SOD	— superoxide dismutase;
U	— enzyme activity unit.

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Author's information: Svetlana Kalinina, PhD, Head of Laboratory, orcid.org/0000-0003-1906-092X; Viktor Ilyukha, Dr. of Sci. in Biology, Director of Institute, orcid.org/0000-0002-7085-4154; Evgeniy Khizhkin, PhD, Senior Researcher, orcid.org/0000-0001-7831-8025; Irina Baishnikova, PhD, Senior Researcher, orcid.org/0000-0001-5064-3731; Ekaterina Antonova, PhD, Researcher, orcid.org/0000-0002-4740-2141; Artem Morozov, Assistant, orcid.org/0000-0001-7840-939X

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Introduction

Cellular antioxidant responses are crucial for both redox signaling and redox damage. Antioxidant molecules are able to react with free radicals and oxidant species which may behave as deleterious and toxic products, involved in the dysfunction of cells and tissues (Espinoza-Diez et al., 2015). The part of the cellular defense against oxidative stress is such antioxidant enzymes (AOE) as superoxide dismutases (SOD, EC 1.15.1.1), which catalyze the dismutation of the extremely toxic superoxide radical into potentially less toxic hydrogen peroxide; catalase (CAT, EC 1.11.1.6), which stops the formation of the hydroxyl radical by converting hydrogen peroxide to oxygen and water; and glutathione peroxidase (GPx), which metabolizes hydrogen peroxide and lipid hydroperoxides, etc. Among low molecular-weight antioxidants, the lipid-soluble substances such as retinol (one of the major forms of vitamin A) and α-tocopherol (the main and most active component of vitamin E) also act as direct scavengers of reactive oxygen species (ROS). Besides the lipoperoxyl radical-scavenging activity of retinol, it is an essential micronutrient for several biological processes, including vision, embryonic development, cell differentiation, growth and development, and regulation of the immune system (Owusu and Ross, 2016). α-Tocopherol localizes mainly at cell membranes and suppresses the formation of lipid hydroperoxides.

These enzymatic and nonenzymatic antioxidants are necessary for sustaining life by maintaining a delicate intracellular redox balance and minimizing undesirable cellular damage caused by ROS (Espinoza-Diez et al., 2015). Besides the regulation of antioxidants by the redox status of the cell, evidence for their hormonal modulation also exists, and the study of such regulation becomes very important for physiological and therapeutic approaches (Feingold, Longhurst and Colby, 1993; Bolzan, Brown, Goya and Bianchi, 1995; Mayo et al., 2002; Fernández et al., 2005).

It is well known that AOE activities exhibit an endogenous rhythm with a nighttime rise under normal light/dark (LD) conditions that is considered to be correlated with physiological melatonin levels (Benot et al., 1998; Albarrán et al., 2001; Mayo et al., 2002). It remains unclear whether circadian rhythms of retinol and α-tocopherol exist in tissues. In human blood plasma the daily changes of retinol and α-tocopherol are negligible (Nierenberg and Stukel, 1987). However, the synthesis and signaling of all-*trans*-retinoic acid (ATRA), an active metabolite of retinol, exhibit diurnal changes in the pineal gland (Ashton, Stoney, Ransom and McCaffery, 2018).

Melatonin (N-acetyl-5-methoxytryptamine) is the pineal hormone, which is produced and secreted with a circadian rhythm (low level during the day, or in light, and peak values at night, or in darkness) (Tapia-Osorio, Salgado-Delgado, Angeles-Castellanos and Escobar, 2013). Chronobiotic effects of melatonin, modulation of sleep processes and influences on bone growth and osteoporosis are mediated through the interaction of melatonin with at least two G-protein coupled membrane-bound melatonin receptors MT1 and MT2 or indirectly with nuclear orphan receptors (namely, RZR/RORα and NR1F2 (RZR/RORβ)) (Dubocovich et al., 1998; Dubocovich, Rivera-Bermudez, Gerdin and Masana, 2003).

Besides such well-known functions of melatonin as its involvement in circadian rhythm regulation and in the modulation of a variety of neural and endocrine functions, it also acts as the most powerful antioxidant. Due to the fact that melatonin can readily pass through cell membranes and has access to all sub-cellular organelles, this indoleamine and its metabolic derivates are able to function as free radical scavengers (at high concentrations) by receptor-independent pathway (Tan et al., 2015). Also, melatonin can induce the gene expression and the activities of AOE (at physiological concentrations and under non-oxidative conditions), and this ability is receptor-mediated (Antolín et al., 1996; Kotler et al., 1998; Liu and Ng, 2000; Mayo et al., 2002) through RORα (Fang et al., 2019) and MT1 (Barberino et al., 2017). However, it is not fully elucidated whether melatonin might affect not only the enzymatic antioxidants but also the level of low molecular-weight ones such as retinol and α-tocopherol. Previously (Sergina et al., 2013) we have noted the increase in the level of both these substances in the kidney and heart of raccoon dogs (*Nyctereutes procyonoides*, Carnivora, Mammalia) treated with a subcutaneous continuous-release melatonin implant.

To study the receptor-mediated actions of melatonin, the melatonin receptor antagonist luzindole (2-benzyl-N-acetyltryptamine) is widely used — it is considered a non-selective ligand, although it has a 15- to 25-fold higher affinity for the MT2 melatonin receptor as compared to the MT1 receptor (Dubocovich et al., 1998). It was shown that luzindole blocks melatonin-mediated phase advances of circadian rhythms (Dubocovich et al., 1998), exerts antidepressant-like effects in the C3H/HeN mouse (Dubocovich, Mogilnicka and Areso, 1990), exhibits antioxidant actions in vitro (Mathes, Wolf and Rensing, 2008) and reduces the level of lipid peroxidation in vitro (Requintina and Oxenkrug, 2007). Moreover, luzindole does not block the antioxidant effects of melatonin (Behan, McDonald, Darlington and Stone, 1999). It remains unclear whether luzindole acts as an antioxidant in vivo and whether it influences the level of antioxidants.

Although it is generally known that constant conditions (light or darkness) strongly influence circadian rhythmicity and melatonin secretion (Tapia-Osorio, Salgado-Delgado, Angeles-Castellanos and Escobar,

2013), little information is available concerning changes in antioxidant level of mammals following darkness exposure (Delibas, Tuzmen, Yonden and Altuntas, 2002; Pang et al., 2008). Moreover, it is not known whether the altered lighting conditions — for example, constant darkness (DD) — can affect the potent antioxidant effects of melatonin and luzindole in vivo. Exposure of mammals to continuous light (LL) or DD chronically attenuates internal 24-hr rhythms, leading to detrimental effects on multiple biological processes. In DD the circadian rhythm of melatonin persists, but it is determined to be free-running as a result of a steady expansion of its secretion durations, while LL rats show an arryhtmic temporal pattern (Tapia-Osorio, Salgado-Delgado, Angeles-Castellanos and Escobar, 2013). In LD 12:12 conditions the melatonin receptor density in the suprachiasmatic nucleus (SCN) was shown to be specifically reduced during the night, while in DD it did not show any variation throughout the 24 h subjective day and night (Gauer, Masson-Pevet, Stehle and Pevet, 1994).

The aim of this study was to evaluate the effects of both exogenous melatonin and melatonin receptor antagonist luzindole on the activities of AOE (SOD, CAT) as well as retinol and α-tocopherol levels in rats exposed to LD 12:12 or DD conditions.

Materials and methods

ETHICAL PROCEDURES

The research was carried out using the equipment of the Core Facility of the Karelian Research Centre of the Russian Academy of Sciences and according to EU Directive 2010/63/EU for animal experiments with the special permission of the Local Ethics Committee of the Institute of Biology. All efforts were made to minimize the number of animals and their suffering.

SUBJECTS AND EXPERIMENTAL DESIGN

Seven-month-old male Wistar rats weighing 450–500 g were housed at constant temperature (23 ± 1 °C) with laboratory food and water *ad libitum*. Animals were synchronized for 14 days to a 12:12 light/dark cycle (LD, lights on at 07:00, light intensity of 200 lx). After that, a portion of the rats was kept under LD conditions, while another portion was exposed to constant darkness (DD) beginning at the time of lights off for 14 days. Then, rats kept under either LD or DD conditions were randomly divided into three groups (n=3–6 in each group): LD (DD)-control (received placebo); LD (DD)-mel (received melatonin), and LD (DD)-luz (received luzindole). After 14 days the rats were terminated by decapitation at 10.00 after anesthesia with ether. All treatments of the DD rats were performed under a dim red light (640–700 nm, <2 lx) within 3 min.

DRUGS AND DOSES

The drugs used were melatonin (N-acetyl-5-methoxytryptamine) (Sigma, USA) and melatonin receptor antagonist luzindole (2-benzyl-N-acetyltryptamine) (Bachem AG, Switzerland). For each drug, 10 mg were dissolved in a minimal amount of 95 % ethanol and further diluted to 100 ml distilled water to yield stock solution, which was then diluted in 1 liter tap water (final ethanol concentration< 0.1 %). The dose of both melatonin and luzindole was 0.22 mg/kg BW of rat. Control rats received tap water containing 0.0001 % ethanol. All drugs were given to the rats with drinking water at night for 14 days. The last administration of drugs was given the day before death.

TISSUE COLLECTION

After decapitation, the liver and kidney samples were immediately placed on ice for transportation to the laboratory, where samples were stored at –80 °C awaiting analyses. All samples were analyzed in triplicate.

DETERMINATION OF ANTIOXIDANT ENZYMES ACTIVITIES AND CONTENTS OF RETINOL AND Α-TOCOPHEROL

Antioxidant enzyme activities

For determination of AOE activities, tissue samples were homogenized in 0.05 M phosphate buffer, pH 7.0, and centrifuged at 6000 × g for 15 min.

Superoxide dismutase (EC 1.15.1.1)

The total SOD activity was measured by the adrenochrome method based on the spontaneous autoxidation of epinephrine with the formation of product with an absorbance peak at 480 nm (Misra and Fridovich, 1972). This reaction depends on the presence of superoxide anions and is specifically inhibited by SOD. The amount of enzyme that caused 50 % inhibition of epinephrine autoxidation is defined as 1 unit (U). SOD activity was expressed as U per mg protein after normalization with estimated total protein in milligrams in the respective tissues.

Catalase (EC 1.11.1.6)

CAT activity was evaluated by measuring the decrease in H_2O_2 concentration at 240 nm (Bears and Sizes, 1952). One enzyme unit (U) is defined as the amount of CAT capable of transforming 1.0 μmol of H_2O_2 for a minute. CAT activity was expressed as U per mg protein after normalization with estimated total protein in milligrams in the respective tissues.

Total protein assay

Results for AOE activities were standardized to total soluble protein content in tissue homogenates. Total tissue

PHYSIOLOGY

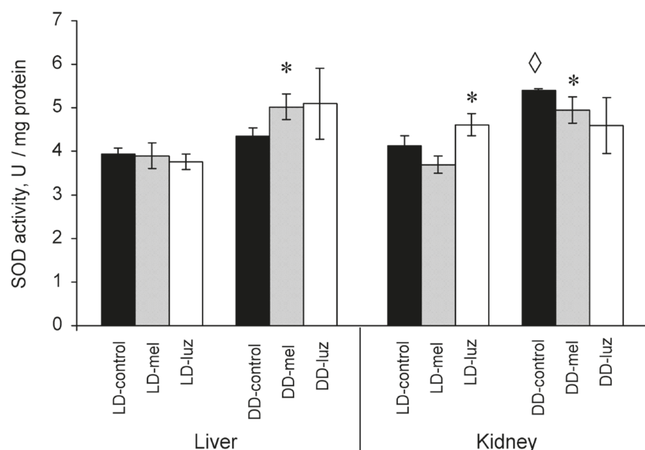

Fig. 1. The activity of SOD in both melatonin- and luzindole-treated rats, exposed to either normal light conditions 12:12 (LD) or constant darkness (DD). Results (in U/ mg protein) are expressed as mean ± SEM. Significant difference with: ◊ LD-control, * LD-mel rats (p<0.05, Kruskall-Wallis test).

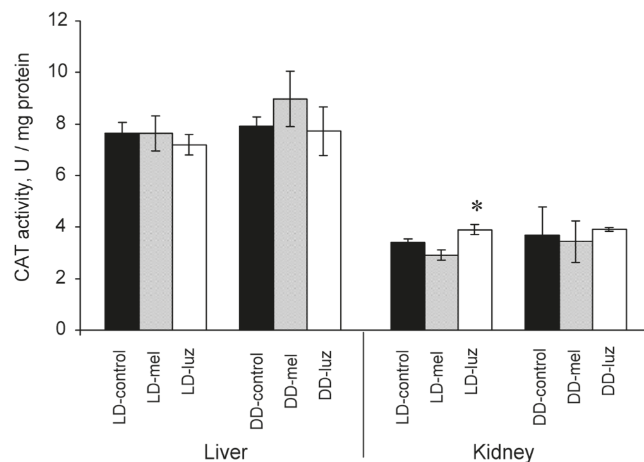

Fig. 2. The activity of CAT in both melatonin- and luzindole-treated rats, exposed to either normal light conditions 12:12 (LD) or constant darkness (DD). Results (in U/ mg protein) are expressed as mean ± SEM. * Significant difference with LD-mel rats (p<0.05, Kruskall-Wallis test).

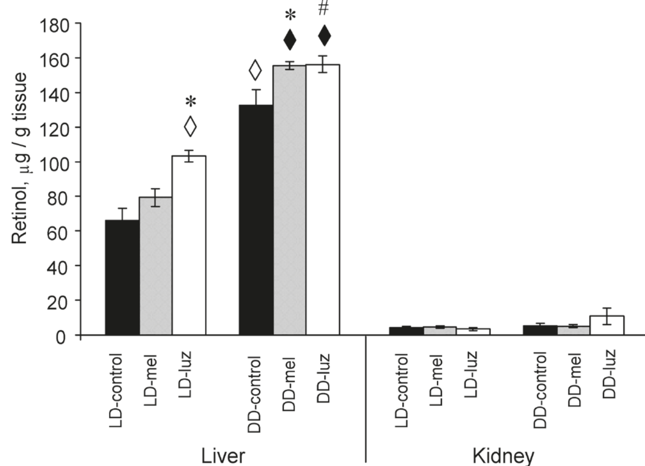

Fig. 3. The retinol content in both melatonin- and luzindole-treated rats, exposed to either normal light conditions 12:12 (LD) or constant darkness (DD). Results (in μg/g wet tissue) are expressed as mean ± SEM. Significant difference with: ◊ LD-control, ♦ DD-control, * LD-mel, ♯ LD-luz rats (p<0.05, Kruskall-Wallis test).

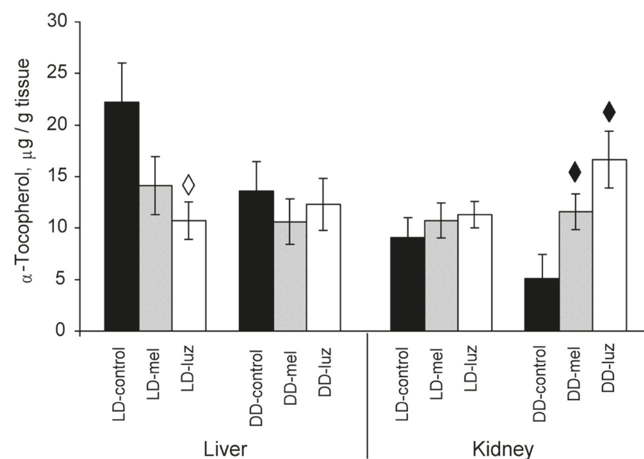

Fig. 4. The α-tocopherol content in both melatonin- and luzindole-treated rats, exposed to either normal light conditions 12:12 (LD) or constant darkness (DD). Results (in μg/g wet tissue) are expressed as mean ± SEM. Significant difference with: ◊ LD-control, ♦ DD-control rats (p<0.05, Kruskall-Wallis test).

protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Retinol and α-tocopherol levels

The contents of retinol and α-tocopherol in the tissues were determined by HPLC. Proteins in the samples were precipitated by ethanol. Retinol and α-tocopherol were extracted by n-hexane. Chromatographic separation was carried out by microcolumn chromatography with a UV detector with n-hexane and isopropanol as an eluent (98.5:1.5). The eluate was monitored at 292 nm for α-tocopherol and 324 nm for retinol, and the substances were identified by retention time compared with pure standards (MP Biomedicals, USA).

STATISTICAL ANALYSIS

All the collected and calculated numerical data were transformed into SI units and processed statistically as mean ± standard error of the mean. Statistical analysis was done using the Kruskal–Wallis analysis of variance with the Mann–Whitney *U post hoc* testing as appropriate. A *p*-value of <0.05 was taken as significant. Relationships between light conditions, drugs and studied values were analyzed by means of the Pearson's and Spearman's correlation coefficients. The statistical analyses were performed using Sigma-Stat 2.03 (SPSS Science Software Ltd., USA), while figures were prepared using Grapher 7.0 (Golden Software Inc., USA).

Results

INFLUENCE OF DD CONDITIONS ON STUDIED PARAMETERS

Results of the study are presented in Figures 1–4. DD-control rats had higher kidney SOD activity (Fig. 1) and higher liver retinol content (Fig. 3) than LD-control ones. No significant differences in liver SOD activity, in both liver and kidney CAT activities, in kidney retinol level and in both liver and kidney α-tocopherol contents were found between LD- and DD-control groups (Figs. 1–4).

INFLUENCE OF BOTH MELATONIN AND LUZINDOLE ON STUDIED PARAMETERS IN RATS EXPOSED TO LD CONDITIONS

No influence of melatonin on studied antioxidants in LD-rats was observed in our study (Figs. 1–4). Treatment of the LD-rats with luzindole had no effect on AOE activities but caused an increase in retinol and a decrease in α-tocopherol contents in the liver in comparison with the control (Figs. 3, 4). Also LD-luz rats had higher kidney AOE activities and higher liver retinol content than LD-mel ones (Figs. 1–3).

INFLUENCE OF BOTH MELATONIN AND LUZINDOLE ON STUDIED PARAMETERS IN RATS EXPOSED TO DD CONDITIONS

In DD-rats, along with having no effect on AOE activities, both melatonin and luzindole induced an increase in the liver retinol content and in the kidney α-tocopherol level compared with DD-control (Figs. 1–4).

INFLUENCE OF DD CONDITIONS ON STUDIED PARAMETERS IN RATS TREATED WITH MELATONIN AND LUZINDOLE

DD-mel rats had higher SOD activities in the liver and kidney and a higher retinol level in the liver compared to LD-mel ones (Figs. 1, 3). DD-luz rats had a higher liver retinol content compared to LD-luz ones (Fig. 3).

CORRELATIONS

Table 1 shows correlation coefficients between light conditions, drugs, and studied parameters. Only significant correlations are presented. The results are mostly similar for the Pearson and Spearman coefficients. Most coefficients of correlation were positive. The highest correlation (positive) occurred between liver retinol content and light conditions (Pearson: r=0.88; Spearman: r=0.84; p=0.000). No correlations were observed between the drugs and AOE activity, nor between either light conditions or drug and CAT activity.

Table 1. Correlations and significance levels (p) in rats between light conditions, drugs, and studied values

Parameter correlation	Pearson		Spearman	
	r	p	r	p
Liver retinol level — light conditions	0.88	0.000	0.84	0.000
Liver retinol level — liver SOD activity	0.62	0.001	0.56	0.007
Liver SOD activity — liver CAT activity	0.53	0.008	0.46	0.027
Liver SOD activity — light conditions	0.62	0.001	0.62	0.003
Kidney SOD activity — light conditions	0.56	0.005	0.52	0.013
Kidney α-tocopherol level — drug	0.60	0.002	0.61	0.003
Liver α-tocopherol level — drug	−0.43	0.036	−0.44	0.034
Kidney retinol level — kidney α-tocopherol level	0.49	0.016		
Liver retinol level — liver α-tocopherol level	−0.43	0.034		
Kidney retinol level — light conditions	0.42	0.042		
Liver retinol level — kidney α-tocopherol level			0.42	0.047

Discussion

The significance of melatonin and melatonin receptor antagonist luzindole in the regulation of AOE activities and retinol and α-tocopherol levels in Wistar rats was evaluated through studies on the effects of exogenously administered drugs during rat exposure to normal light conditions (LD 12:12) or constant darkness (DD).

In DD conditions the clock gene expression in the SCN does not show a 24-h rhythmicity because the clock is not entrained to the environmental lighting cycle (Okamura et al., 1999). Although a circadian rhythm of melatonin with higher values during the subjective night and lower values during the subjective day persists in DD, a smoothing of the melatonin secretion rhythm per day is observed—the day portion slightly increases and the night portion slightly decreases in comparison with the LD regime, but the total concentration of the hormone per day in DD does not exceed that in LD (Tapia-Osorio, Salgado-Delgado, Angeles-Castellanos and Escobar, 2013).

Little information is available concerning the levels of tissue antioxidants following darkness exposure

(Delibas, Tuzmen, Yonden and Altuntas, 2002; Pang et al., 2008). It is well known that under normal LD conditions the AOE activities exhibit endogenous rhythms with a nighttime rise that is considered to be correlated with physiological melatonin levels (Benot et al., 1998; Albarrán et al., 2001; Mayo et al., 2002). In DD the circadian rhythm of melatonin is determined to be free-running, therefore in DD conditions, circadian rhythms of AOE activities could differ from those in a normal LD cycle. We have noted the higher kidney SOD activity in DD-control rats compared to LD-control ones. This is in accordance with earlier observations by Delibas N. et al. (2002), the increase in SOD and GPx activities in the hippocampus of rats exposed to DD in comparison with control ones.

Although the SOD activity was shown to be higher in the kidneys of DD-control rats in comparison with LD-control ones, the activity of another studied enzyme — CAT — did not change. The balance between the activities of SOD and CAT is necessary to provide adequate antioxidant protection. The increase in SOD activity, which dismutates the superoxide anion, causes an increase in the hydrogen peroxide concentration, which if not sufficiently removed by CAT or GPx (not determined in our study) will produce hydroxyl radicals that are highly toxic for the cell (Antolín et al., 1996). However, although the imbalance between the antioxidant enzymes may cause cell injury (Toborek, Kopieczna-Grzebieniak, Drózdz and Wieczorek, 1995), the protective role of SOD is widely accepted even when other AOEs do not change (Warner et al., 1993). Moreover, SOD seems to be more sensitive to hormonal changes (including changes in melatonin level) than CAT or GPx (Kotler et al., 1998; Barp et al., 2002).

Previous studies have suggested that melatonin could induce the gene expression and the activity of AOEs such as SOD, CAT and GPx, and this effect is considered to be receptor-mediated through MT1 (Barberino et al., 2017) and RORα (Fang et al., 2019). However, in our study we did not find a statistically significant effect of exogenous melatonin on AOE activities in LD-rats, but DD-mel rats exhibited higher SOD activities in the liver and kidney compared to LD-mel ones. This illustrates that changes in physiological levels of melatonin are adequate to alter this component of the tissue antioxidative defense. Probably it could be due to alteration in circadian rhythms of melatonin and therefore of SOD activity in DD conditions.

We also detected a higher liver retinol content in DD-control rats compared to LD-control ones. The liver is known to play an important role in vitamin A homeostasis, because in this organ retinol can follow several metabolic pathways, including oxidation to ATRA (Wolf, 2001). When the plasma concentration of ATRA is increased, there is an accompanying reduction of circulating levels of retinol, suggesting that ATRA may have a regulatory effect on retinol metabolism in vivo (Wang, Krinsky and Russell, 1993). The DD conditions may influence the homeostasis of vitamin A by decreasing circulatory levels of ATRA, as was shown in mice (Pang et al., 2008) and increasing liver retinol content, as was observed in rats in our study.

Although data on the melatonin influence on the levels of retinol and α-tocopherol in tissues are lacking in the literature, the protective effect of melatonin against oxidative stress related to restoration of retinol availability was shown in rats treated with melatonin after heroin administration (Cemek et al., 2011). Besides that, melatonin at various concentrations combined with α-tocopherol decreases lipid peroxidation in human placental mitochondria with the effect being additive (Milczarek et al., 2010). In our study, in contrast to LD conditions, where there was no effect of melatonin on the retinol and α-tocopherol contents, in DD melatonin induced an increase in liver retinol content and in the kidney α-tocopherol level compared with the DD-control. Also, we have noted a higher liver retinol content in DD-mel rats compared to LD-mel animals. The increase in kidney vitamin E content after melatonin administration observed in our study may be a result of α-tocopherol mobilization from adipose and other tissues as a consequence of antioxidant metabolic adaptation of the organism. Moreover, melatonin may recycle several oxidized antioxidants including vitamin C, α-tocopherol, glutathione, and NADH, and these antioxidants reportedly also recycle the melatonin neutral radical (Tan et al., 2015).

Most studies of luzindole action in the organism revolve around it being a melatonin receptor antagonist, but others have indicated that luzindole has a function of its own — for example, protection of rat retinal photoreceptors from light damage (Sugawara et al., 1998). Moreover, luzindole's antioxidant effect is considered to exceed that of melatonin (Oxenkrug and Requintina, 2005). Unfortunately, data on the antioxidant actions of luzindole are scarce in the literature and have been revealed only in vitro (Requintina and Oxenkrug, 2007; Mathes, Wolf and Rensing, 2008), therefore it remains unclear whether this substance acts as an antioxidant in vivo. It was shown that luzindole does not suppress the antioxidant effects of melatonin (Behan, McDonald, Darlington and Stone, 1999) and in a model of melatonin plus luzindole co-administration, radical scavenging may be far more intense than with melatonin alone, but in the experiments where luzindole is usually given without melatonin, results may be influenced by its antioxidant properties (Mathes, Wolf and Rensing, 2008).

As in the case of melatonin, luzindole administration to LD- and DD-rats had no effect on AOE activities in comparison with corresponding controls. How-

ever, in LD conditions this drug caused an increase in retinol and a decrease in α-tocopherol contents in the liver compared to the control group. According to the antagonism between retinol and α-tocopherol reported previously in the literature (Olivares, Rey, Daza and Lopez-Bote, 2009), this result would be expected. In DD conditions luzindole induced an increase in liver retinol content and in the kidney α-tocopherol level compared to the control. Although no significant differences in studied values were found between melatonin- and luzindole-treated rats in DD, in LD conditions the luzindole-treated rats had higher kidney AOE activities and a higher liver retinol level than the melatonin-treated ones. According to recently published data (Barberino et al., 2017; Fang et al., 2019), it could be hypothesized that MT2 blocking by luzindole can increase the affinity of melatonin to MT1 and potentiate its antioxidant actions.

In conclusion, our results indicate that DD exerts influence only on the kidney SOD activity and the liver retinol level and in several cases significantly modulates the effects of exogenous melatonin and melatonin receptor antagonist luzindole on the studied antioxidants mainly by increasing their activity or content. In the case of melatonin, these parameters are the liver and kidney SOD activities and liver retinol, and in the case of luzindole it is liver retinol content. Probably due to the important role of ATRA in regulation of circadian rhythms (Pang et al., 2008; Sherman et al., 2012), the liver retinol level seems to be the most sensitive to the influence of DD, melatonin and luzindole. The mechanisms of effects of these factors on studied parameters need to be further investigated.

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