MELATONIN REGULATES OXIDATIVE STRESS INITIATED BY FREUND'S COMPLETE ADJUVANT

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Summary: Melatonin is a hormone with strong antioxidant properties. In this experiment, Freund's complete adjuvant was used as a stressogenic substance given to laboratory outbred mice, whereas melatonin was investigated as a protectant against the stressogenic effect. Levels of low molecular weight antioxidants, thiobarbituric acid reactive substances, and tumor necrosis factor α and activity of glutathione reductase were determined in blood from the animals. Surprisingly, melatonin was not involved in direct regulation of antioxidants, thiobarbituric acid reactive substances and tumor necrosis factor α. On the other hand, melatonin regulated glutathione reductase activity. We can conclude on regulation of metabolism caused by melatonin in the model. The effect was more important than the expected regulation of immunity and basal oxidative homeostasis.

Keywords: *Antioxidant; Oxidative stress; Melatonin; Reactive oxygen species; Epiphysis; Hormone*

Introduction

Melatonin is a hormone from pineal gland. Under physiological conditions, melatonin regulates circadian biological rhythms in vertebrates (1, 2) including mammals (3, 4). Currently, melatonin is not pharmacologically unknown compound as it is distributed like a drug or food supplement regulating sleep. Clinical trials on melatonin applications are running as well (5, 6).

Beside hormonal effect, melatonin is known for its ability to quench reactive oxygen and nitrogen species in course of its antioxidant effect (7). Melatonin is called terminal or suicidal antioxidant that indicates irreversible process of the molecule oxidation. Not only melatonin but also products of melatonin's oxidation, 6-hydroxymelatonin, 3-hydroxymelatonin and N-acetyl-N-formyl-5-methoxykynurenamine, are also antioxidants (8, 9). Because of the antioxidant properties, melatonin can be considered as a compound suitable for suppression of oxidative stress and generation of reactive oxygen and nitrogen species.

In the present manuscript, melatonin was chosen as a low molecular weight antioxidant able to reduce initiated oxidative stress caused by Freund's complete adjuvant. The adjuvant is typically used in immunization experiments because it is able to pronounce immunity response. However, the adjuvant is also effective enough to initiate oxidative stress in animal models (10–13). Because melatonin is a strong antioxidant, we assume that it will be potent enough to resolve oxidative stress initiated by Freund's complete adjuvant. Efficacy of melatonin to suppress oxidative stress is hypothesized and researched in this paper.

Material and Methods

Experiment on laboratory animals

In a total, 600 female mice (standard white outbred, ICR, strain) were purchased from Velaz (Unetice, Czech Republic) and they used in the experiment. The eight weeks old mice weighted 18 ± 2 g. Manipulation with the animals as well as the whole experiment were permitted and supervised by the ethical committee at Faculty of Military Health Sciences (Hradec Kralove, Czech Republic). The mice were accommodated in an animal house with temperature 22 ± 2 °C, humidity 50 ± 10% and light/dark period each 12 hours provided during the whole experiment.

Prior to the experiment beginning, melatonin (grade for analytical purposes) and Freund's complete adjuvant were received from Sigma-Aldrich (Saint Louis, Missouri, USA). Freund's complete adjuvant had standard composition 1 mg of *Mycobacterium tuberculosis* (H 37RA; American type of culture collection 25177) per 0.85 ml of paraffin oil and 0.15 ml mannide monooleate.

The animals were divided into 15 groups each 40 animals. First group served as a control and the animals

received 100 µl saline only. The second and third groups received Freund's complete adjuvant in an amount 20 µl respective 50 µl per an animal. Fourth to seventh groups received melatonin (in a dose 1 – 10 – 100 – 1000 µg/kg of body weight). Melatonin was applied as a solution in saline in total volume 100 µl. Animals in the eight to eleventh groups received the same dose of melatonin like above; however, the animals received 20 µl of Freund's complete adjuvant as well. The last four groups (12–15) had also dose of melatonin 1 – 10 – 100 – 1000 µg/kg but they were exposed to 50 µl of Freund's complete adjuvant. The both melatonin and the adjuvant were given intramuscularly into rear limb. 10 animals from each group was sacrificed one day after experiment beginning. Another 10 animals per a day were sacrificed in second, third and fourth day. The sacrificing was done under CO_2 anesthesia by cutting of jugular vein and blood was collected into tubes with lithium heparin (Dialab, Prague, Czech Republic). Blood was centrifuged at 1,000×g for 5 minutes and plasma was separated from mass of erythrocytes.

Ex vivo assays

Low molecular weight antioxidants were assayed using Ferric Reducing Antioxidant Power (FRAP) method, Free Radicals (FR) method, chromatography of reduced (GSH) and oxidized (GSSG) glutathione, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The assays were done in compliance with the referred papers (14, 15). Level of malondialdehyde was measured by Thiobarbituric Acid Reactive Substances (TBARS) method in compliance with the referred protocols (14, 15). Glutathione reductase (GR) assays was performed earlier and the quoted protocols were used here (16, 17). Level of tumor necrosis factor alpha (TNF-α) in plasma was assayed using indirect Enzyme Linked Immunosorbent Assay (ELISA) kit by Sigma-Aldrich.

Statistics

The achieved experimental data were processed by Origin 8 Pro (OriginLab Corporation, Northampton, MA, USA) software. One-way ANOVA with Bonferroni test were applied and significance of differences between experimental groups (n = 10) was tested on probability levels P 0.05 and 0.01.

Results and Discussion

During the experiment, no decease occurred prior to euthanasia. The animals had normal behavior and no visual difference between the tested groups was found. Examination of plasmatic TNF-α did not show the significant effect of melatonin either alone or its effect to modulation of inflammation initiated by Freund's complete adjuvant. This is a surprising finding because an anti-inflammatory effect of melatonin was expected regarding to cited literature (9, 18, 19). Freund's complete adjuvant causes inflammatory reaction as obvious from Figure 1. The effect was increasing during the time of experiment and level of TNF α was doubled in the end of experiment when compared to the controls. The increase of TNF α was in dose response manner as well which emphasized plausibility of the finding. On the other hand, the increase was not extensive enough to be comparable with inflammation during serious initialization of immune system during infections or sepsis (20). It can be reason why effect melatonin on TNF α was not revealed.

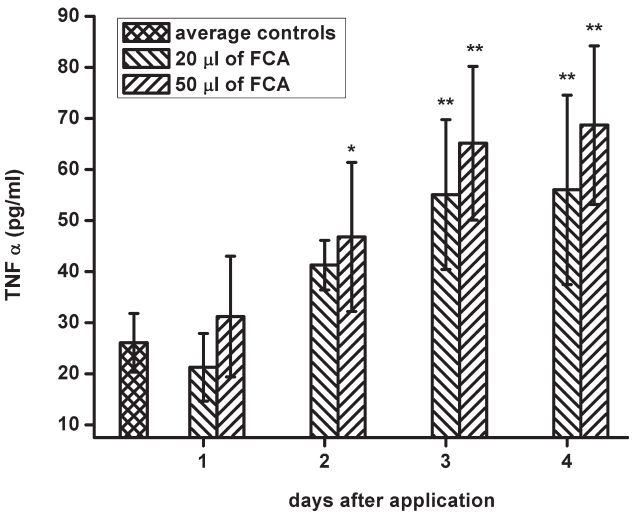

Fig. 1: Level of tumor necrosis factor α (TNF α) in plasma of mice treated with Freund's complete adjuvant (FCA). Error bars indicate standard deviation for n = 10. Significance against controls is marked by * respective ** for significance level 0.05 respective 0.01.

In the tested blood cells, significant effect of melatonin on glutathione reductase activity was proved (Table 1). No significant effect of the both Freund's complete adjuvant and melatonin on glutathione reductase activity was proved in animals sacrificed one respective two days after experiment beginning. Freund's complete adjuvant caused significant increase of glutathione reductase level in blood cells three and four days after experiment beginning. Melatonin significantly reduced the increase of glutathione reductase level caused by Freund's complete adjuvant. Except of the lowest dose 1 mg/kg, melatonin returned glutathione reductase activity to level insignificant to the controls; however, some minor and insignificant increase was observed even for the highest doses of melatonin. Glutathione reductase is an enzyme involved in protection against oxidative stress and its activity is typically increased during stress conditions (21–23). The data can be interpreted as a proved stressogenic effect of Freund's complete adjuvant and amelioration of the effect by melatonin.

Tab. 1: Glutathione reductase activity values (kat/g) in blood cells.

Group	Day 1	Day 2	Day 3	Day 4
controls	3.76 ± 0.31	3.62 ± 0.38	3.78 ± 0.22	3.82 ± 0.25
FCA – 20 µl	3.79 ± 0.33	3.81 ± 0.41	4.30 ± 0.34 (**)	4.36 ± 0.30 (*)
FCA – 50 µl	3.69 ± 0.29	3.83 ± 0.36	4.34 ± 0.43 (**)	4.52 ± 0.55 (**)
M 1 µg/kg	3.81 ± 0.31	3.81 ± 0.21	3.81 ± 0.24	3.71 ± 0.32
M 10 µg/kg	3.77 ± 0.24	3.83 ± 0.36	3.79 ± 0.26	4.00 ± 0.39
M 100 µg/kg	3.82 ± 0.25	3.87 ± 0.29	3.85 ± 0.30	3.75 ± 0.32
M 1000 µg/kg	3.79 ± 0.35	3.92 ± 0.15	3.92 ± 0.20	3.94 ± 0.25
M 1 µg/kg + FCA – 20 µl	3.86 ± 0.31	3.80 ± 0.26	4.26 ± 0.37 (*)	4.41 ± 0.34 (*)
M 10 µg/kg + FCA – 20 µl	3.87 ± 0.44	3.77 ± 0.22	3.95 ± 0.22	4.24 ± 0.43
M 100 µg/kg + FCA – 20 µl	3.81 ± 0.24	3.74 ± 0.18	3.87 ± 0.21	4.12 ± 0.36
M 1000 µg/kg + FCA – 20 µl	3.78 ± 0.22	3.84 ± 0.23	3.98 ± 0.27	3.82 ± 0.24
M 1 µg/kg + FCA – 50 µl	3.81 ± 0.24	3.85 ± 0.27	4.25 ± 0.31 (*)	4.50 ± 0.54 (**)
M 10 µg/kg + FCA – 50 µl	3.75 ± 0.29	3.76 ± 0.19	3.98 ± 0.27	4.40 ± 0.31
M 100 µg/kg + FCA – 50 µl	3.85 ± 0.24	3.88 ± 0.23	3.83 ± 0.24	4.23 ± 0.40
M 1000 µg/kg + FCA – 50 µl	3.94 ± 0.25	3.92 ± 0.31	3.89 ± 0.32	3.96 ± 0.41

M – melatonin; FCA – Freund's complete adjuvant; significance against controls: * (P = 0.05) and ** (P = 0.01)

Tests for low molecular weight antioxidants including GSH/GSSG levels and test for TBARS indicating peroxidation of lipids did not bring a significant change (data not shown). Low molecular weight antioxidants are necessary for covering of oxidative stress as they can directly react with reactive oxygen species (24, 25). GSH/GSSG levels and their ration have an important value as well (26). TBARS level serves as a direct proof of lipid peroxidation resulting in creation of malondialdehyde (27, 28). The TBARS value becomes typically increased once an oxidative insult is uncovered by antioxidants (29, 30). Owing to the experimental data, we can infer that Freund's complete adjuvant caused stressogenic effect; however, the effect was not extensive enough to causes uncovered oxidative insult (regarding to TBARS value) or depletion of antioxidants. Melatonin did not worsen oxidative balance despite it has significant role in decrease of glutathione reductase activity. It seems that melatonin suppressed the stressogenic effect of Freund's complete adjuvant by another way than regulation of immunity or direct effect on low molecular weight antioxidants. Considering examination of TBARS and low molecular weight antioxidants in the blood, melatonin acts as an anti-stressogenic rather than stressogenic compound. The effect is, however, hardly traceable.

It is not clear how melatonin influences level of glutathione reductase. It is probably not based on its antioxidant properties because it has no effect on low molecular weight antioxidant homeostasis. Regulation of metabolism is probably the more plausible hypothesis. Impact of melatonin on enzymes including antioxidant enzymes was reported in some works (31). It is also potent to enhance adipose tissue (32) and even basic metabolism via glucose (33). Metabolizing of Freund's complete adjuvant can be considered as a melatonin effect proved here. The antioxidant effect of melatonin revealed in this experiment is actually based on detoxification rather than common involvement in antioxidant barriers.

Conclusion

Melatonin is the both a low molecular weight antioxidant and a hormone. In the present experiment, we proved regulatory effect of melatonin during mild stressogenic conditions. It appears that melatonin was able to modulate stressogenic reaction by metabolism rather than direct involvement in regulation of immunity or antioxidant barriers.

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