Physiol. Res. 50: 595-602, 2001

Does Exogenous Melatonin Influence the Free Radicals Metabolism and Pain Sensation in Rat?

I. PEKÁRKOVÁ[1], S. PARARA[1], V. HOLEČEK[3], P. STOPKA[2], L. TREFIL[3], J. RACEK[3] , R. ROKYTA[1]

[1]Department of Normal, Pathological and Clinical Physiology, Third Faculty of Medicine, Charles University, Prague, [2]Institute of Inorganic Chemistry, Czech Academy of Sciences, Řež near Prague, [3]Department of Clinical Biochemistry and Laboratory Diagnostics, Faculty of Medicine, Charles University, Pilsen, Czech Republic

Received October 12, 2000
Accepted February 28, 2001

Summary

Melatonin has been shown to play a role in antioxidative defence. We therefore studied its effect on oxidative damage to the rat cerebral cortex evoked by painful stimulation and immobilization-induced stress. Moreover, the effect of melatonin on chronic pain perception was examined. Rats were injected with either a high dose of melatonin (100 mg/kg i.p.) or a vehicle for five days and were subjected to painful stimulation or immobilization stress 30 min after the treatment. To determine the degree of oxidative stress, the levels of free radicals, thiobarbituric acid reactive substances (TBARS) as indicators of lipid peroxidation and glutathione peroxidase (GSHPx) were estimated in somatosensory cortex. Pain perception was measured by the tail-flick and plantar test. Melatonin reduced the level of TBARS previously increased by painful stimulation. Melatonin also exhibited a slight analgesic effect in those animals exposed to painful stimulation but its role in free radical scavenging did not contribute to this effect.

Key words

Melatonin • Oxidative stress • Pain • Stress • Free radicals

Introduction

Repeated painful stimulation by the constriction of the hind limbs (repeated microtrauma) results in a pain syndrome similar to the causalgia syndrome. It is one of the most stressful types of pain, typically accompanied by unbearable burning hyperalgesia, allodynia, paresthesia, dysesthesia, often associated with reflex sympathetic dystrophy (autonomic dysfunction manifested by vasomotor, sudosecretory and trophic disorders often accompanied by edema). It does not respond to the usual analgesic treatment. Therefore, intensive efforts of scientists is focused on the determination of principles and effective treatment of this kind of pain.

It was found in our previous research in the field of pain that after repeated painful stimulation there is an apparent increase in oxidative stress (OS) in the rat cerebral cortex, which persists for more than two weeks (Rokyta *et al.* 1995, 1996). Long-lasting pain was also shown to increase the generation of superoxide or nitric oxide and peroxynitrite due to activation of AMPA and NMDA receptors (Reiter *et al.* 1997, Gilad *et al.* 1997).

Fax +420 2 24920590
E-mail physres@biomed.cas.cz http://www. biomed.cas.cz/physiolres

Therefore, our approach was to decrease the pain-induced oxidative stress with an antioxidant and to follow the impact of such treatment on pain perception.

Pain is accompanied by negative emotions and stress perception and thus one of our experiments was performed to determine how deeply is stress involved in the pain-induced OS phenomenon. To ascertain, if the simple repeated stressful stimulation is also able to elicit OS in the somatosensory cortex and thus to contribute to the increase in OS which had been detected after repeated painful stimulation, we decided to employ the model of immobilization stress.

It has been repeatedly reported that pineal hormone melatonin (N-acetyl-5-methoxytryptamine) may function as an antioxidant (Pieri *et al*. 1994, Reiter *et al*. 1997). Melatonin is produced at night and besides its role in biological rhythm coordination it has a multi-regulatory role in endocrine, immune and nervous systems (Solár 1999). As an antioxidant, melatonin has been reported to function as a scavenger of hydroxyl, peroxyl and superoxide radicals (after melatonin's metabolization to indolyl radical) (Tan *et al.* 1993, Pieri *et al.* 1994, Marshall *et al.* 1996, Abuja *et al.* 1997). It has amphophilic character (soluble both in lipids and water), which means that it can easily cross the blood-brain-barrier and enter any cell without the need of melatonin receptors, although they are present in many cells. In the brain tissue, melatonin effectively protects against lipid peroxidation (Carneiro *et al.* 1998). Except of its direct, receptor-independent scavenging effects, it may also act indirectly by stimulating enzymes involved in the antioxidative defense cascade, e.g. glutathione-peroxidase (Barlow-Walden *et al.* 1995).

A possible analgesic effect of melatonin has been suggested in patients suffering less from pain during the night when melatonin levels are high, than during the day. Prolonged latencies of pain threshold have been measured in healthy subjects during the dark phase of the photoperiod, while pinealectomy abolishes this dark-phase analgesia (Lakin *et al.* 1981) and application of melatonin is able to restore it (John *et al.* 1994). The exact mechanism of the melatonin analgesic effect is not yet fully explained, but there is the assumption that it may interfere with μ-opioid and GABA receptors, because antagonists of these receptors block the analgesic effect of melatonin (Kavaliers 1989, Golombek *et al.* 1991). Melatonin is also able to influence pain perception threshold *via* inhibition of prostaglandin E_2 synthesis (Cuzzocrea *et al.* 1999).

The question remains whether the scavenging properties of melatonin may contribute to the changes in pain perception. It might be concerned especially in the cases of pain connected with widespread tissue damage, inflammation and hyperalgesia. Lasting pain stimulates AMPA and NMDA receptors, which leads to superoxide or nitric oxide and peroxynitrite generation (Reiter *et al.* 1997, Gilad *et al.* 1997)

Material and Methods

Animals

All animal procedures were in strict accordance with the Helsinki Declaration and guidelines of the Ethics Committee of the International Association for The Study of Pain (Zimmermann 1983). They were approved by the Animal Care Committee of the Third Faculty of Medicine, Charles University, Prague. The number and suffering of the animals were reduced to a minimum.

Fourty-eight male Wistar rats (235±15 g body weight; Velaz, Prague, Czech Republic) were used. The animals were housed in plexiglass cages with 4 animals per cage, they received standard laboratory food and water *ad libitum*. The animal room was windowless with automatic temperature (22±2 °C), constant humidity and controlled lighting conditions with 12 h of light and 12 h of darkness per day (light turned on at 06:00 h and off at 18:00 h). All experiments were preceded by one week of acclimatization.

Experimental paradigm

Animals were divided into 6 groups with 8 animals per group. In all animals, the pain threshold was measured on the first day (before any treatment) and on the last day (after all treatments had been applied) of the experiment. In between, every day during 5 days of the experiment, animals were injected with melatonin (100 mg/kg, i.p. in 2 ml volume) or vehicle (2 ml of physiological saline i.p.) at 07:30 h. The animals were either left to rest for 30 min after the injection or were subjected to painful manipulation or immobilization. The various groups of animals were assigned according to the treatment as follows:

Group 1. Controls: no stimulation + vehicle injection
Group 2. Melatonin: no stimulation + melatonin injection
Group 3. Painful stimulation + vehicle injection
Group 4. Painful stimulation + melatonin injection
Group 5. Immobilization stress + vehicle injection (vehicle injection was followed by immobilization stress)

Group 6. Immobilization stress + melatonin injection (melatonin injection was followed by immobilization stress)

Drug treatments

Pilot studies with lower doses of melatonin (1, 5, 10, 30, 60 and 100 mg/kg) revealed that only the highest dose of melatonin was effective to induce statistically significant OS reduction (Konečná *et al.* 2000). Melatonin (Sigma-Aldrich, Prague, Czech Republic) was dissolved in ethanol and the solution was diluted with 0.9 % saline to make a 2 % ethanol in saline solution. The vehicle-treated animals received 2 % ethanol in saline solution only.

Painful stimulation

The model of repeated acute peripheral painful stimulation was used (Rokyta *et al.* 1987). The animal was immobilized in a plexiglass box and both its hind limbs were clamped with metal clamps for a 5 min interval. This procedure was performed at the same day time period (8:00 h) on 5 consecutive days. By this method the terminal branches of peroneal, sural and tibial nerves and also the autonomous nervous system of the hind limbs were affected.

Non-painful stimulation – immobilization stress

Animals were placed into tight plexiglass cages with apertures for breathing, limbs and tail. They were left there for 5 min without any possibility of movement. This was repeated at the same period of the day (8:00 h) for 5 consecutive days.

Pain threshold measurements

Two methods were used for measuring changes in pain perception.

The tail-flick test (TF) (Tail Flick Analgesia Meter Model 33, IITC Inc., Life Science Instruments, USA) measures the latency period from the application of a thermal stimulus (light ray) onto the distal part of the tail, until the withdrawal reaction, i.e. the jerk of its tail when the stimulation reaches the pain threshold.

Plantar test (PT) (Ugo Basile Plantar Test 7370, UGO BASILE, Italy) is another method of measuring the response to thermal stimulation. It consists of a movable light generator, which the operator manipulates under a glass pane. The rats are placed on this pane in a 3-compartment plexiglass enclosure. The withdrawal latency of the animal's paws is automatically measured.

For higher objectivity and better comparability, the percentage difference of withdrawal latencies between the first and the fifth day of the experiment was assessed. The absolute values of withdrawal latencies vary from 3.5 to 6.5 seconds in the TF test and from 6 to 10 seconds in PT.

Sample preparation

On the last day of the experiment (the 5th day), after all the treatments and procedures had been applied, the animals were anesthetized with 5 % ketamine in a dose of 90 mg/kg (Narkamon inj., Léčiva, Czech Republic) and 2 % xylazine in a dose of 15 mg/kg (Rometar inj., Léčiva, Czech Republic). The skull was then opened and two large (50-60 mg) and two small (10-20 mg) samples of somatosensory brain cortex were removed, one from each hemisphere. The animals were sacrificed immediately afterwards. The large brain cortex samples were homogenized in 2 % butylated hydroxytoluene (BHT)/physiological solution buffer (to stop further oxidation processes) diluted 20 times and frozen in liquid nitrogen. To the small samples, 5,5-dimethyl-1-pyrroline N-oxide (DMPO) spin trapping substance was added in the dose of 7 µl/10 mg of tissue and they were also frozen in liquid nitrogen. The samples were stored in the refrigerator under –80 °C temperature.

Chemical reagents

Melatonin 97 %, ethanol 96 %, butylated hydroxytoluene (BHT), 5,5 dimethyl-1-pyrroline-N-oxide (DMPO), were obtained from Sigma-Aldrich, Prague, Czech Republic, whereas BIOXYTECH® LPO-586 TH Assay (colorimetric assay for lipid peroxidation markers) was from OXIS International, Portland, Oregon, USA, EURO-IR, Český Těšín, Czech Republic, RAD-SEL, Randox, EURO-IR, Břeclav, Czech Republic.

TBARS assay

TBARS (thiobarbituric acid reactive substances – mostly malonyl dialdehyde (98 %) and 4-hydroxyalkenals) produced during acid hydrolysis of lipoperoxide in investigated samples react with thiobarbituric acid forming a colored substance which is extracted into N-butanol and detected by spectrophotometry (535 nm).

GSHPx assay

GSHPx (glutathione-peroxidase) acts as catalyst of glutathione oxidation with kumen hydroperoxide. In the presence of glutathione-reductase and NADPH,

oxidized glutathione is reduced immediately, with the parallel oxidation of NADPH to NADP. The missing absorption of NADPH at 340 nm is detected.

Spectrophotometric automatic analyzer HITACHI 717 was used to evaluate the samples in both assays.

Free radical determination

The method of electron paramagnetic resonance was used. The principle of this method is based on detection of high-frequency radiation (9-10 GHz) absorption of any paramagnetic substance (in our case the molecule with an unpaired electron) placed into a strong magnetic field.

Free radicals in the sample are stabilized by the addition of spin trapping substance 5,5-dimethyl-1-pyrroline N-oxide (DMPO). They were measured with a spectrophotometer working in the X-range with magnetic modulation at 100 kHz, using a digital record. The records were evaluated and the concentration of detected radicals was counted using a special graphic program "Genplot" on personal computer.

The qualitatively and quantitatively detected radicals were nitroxyl radical ($NO^{\bullet}$) and hydroxyl radical ($HO^{\bullet}$).

Statistical analysis

All data were analyzed by one-way ANOVA, if the F values were significant, the Student Newmal-Keuls test was used for comparing the groups. The level of significance was accepted at $P<0.05$. Data are presented as mean values ± S.E.M. The significant difference between the means ($P<0.05$) is labeled with an asterisk.

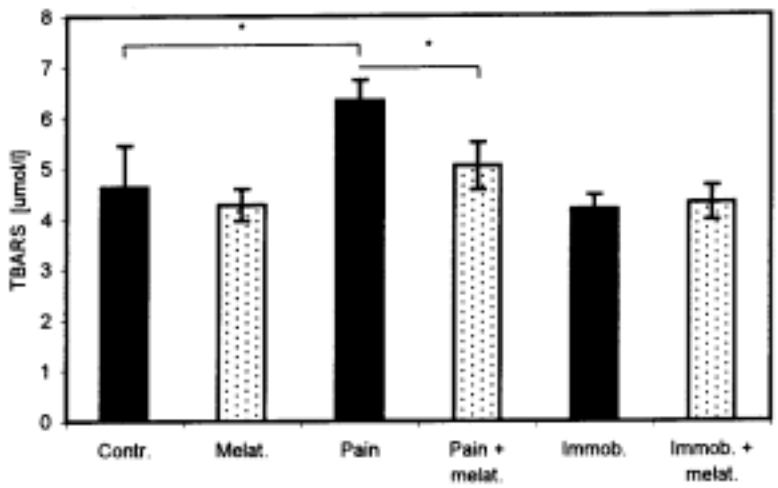
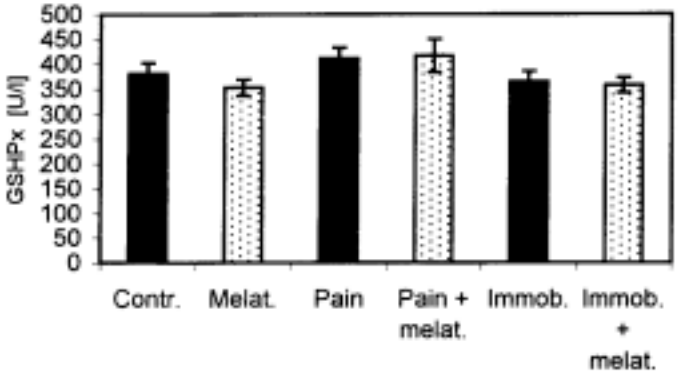


Fig. 1. *TBARS in somatosensory cortex after 5 days of stimulation. Repeated painful stimulation (5 days) increased the degree of lipo-peroxidation (TBARS concentration) in somatosensory cerebral cortex, melatonin in the dose 5x100 mg/kg i.p. (injected 30 minutes before the stimulation) was able to predict the pain-induced lipoperoxidation significantly. Immobilisation stress had no influence of the level of lipoperoxidation in this experiment. Neither in controls nor in immobilised groups melatonin decreased the end-lipoperoxidation products level.*

Fig. 2. *The activity of glutathione peroxidase in the somatosensory cortex after 5 days of painful stimulation. There was a tendency to increase in the activity of GSHPx after 5 days of repeated painful stimulation, but this difference was not significant. Melatonin, injected according the experimental scheme, did not influence the activity of GSHPx in any case. The GSHPx activity after 5 days of the immobilisation stress did not change.*



Results

Effect of painful stimulation or immobilization on oxidative stress indicators

Significant increase of TBARS was found only after repeated painful stimulation (Fig. 1). GSHPx also increased significantly only after the painful stimulation (Fig. 2). The concentration of $NO^{\bullet}$ was decreased after painful stimulation (Fig. 3). Hydroxyl radical levels did not vary significantly, but there was a decreasing trend after the painful stimulation (Fig. 4). The levels of TBARS and GSHPx did not change significantly after immobilization as compared to the controls (Figs 1 and 2).

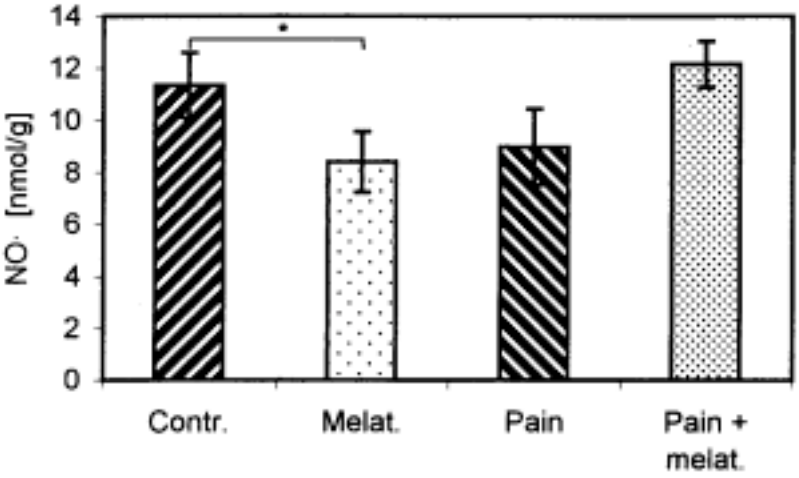


Fig. 3. *Concentration of nitroxyl radicals after 5 days of painful stimulation. In controls, there was detected rather high concentration of NO· in the cortex samples even without any intervention. After repeated painful stimulation there was found significant decrease of NO· in tested samples. Melatonin injected to the control animals is probably able to act as NO· scavenger, being injected before painful stimulation, it does not change the NO· existing level significantly (comparing to controls).*

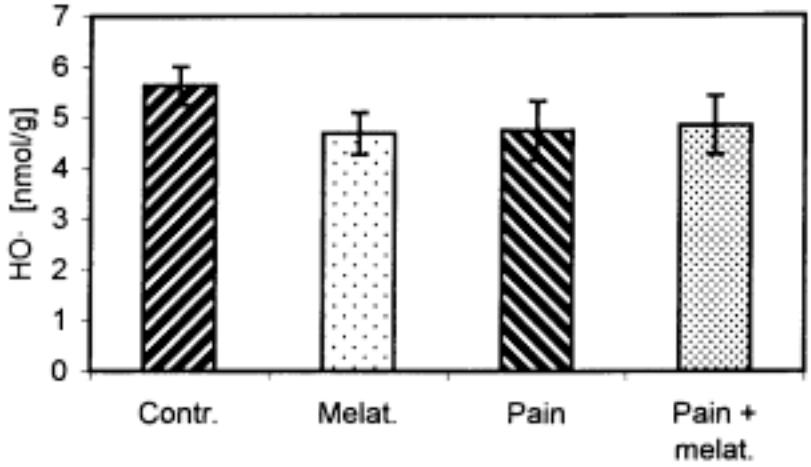


Fig. 4. *Concentration of the hydroxyl radicals after 5 days of painful stimulation. The HO· concentration is decreased after 5 days of painful stimulation. HO· scavenging effect of melatonin was statistically significant only in control animals.*

Effect of melatonin on OS indicators

Melatonin, applied according the above mentioned scheme, did not significantly reduce the level of TBARS or GSHPx in the controls (Figs 1 and 2), but it significantly decreased $NO^{\bullet}$ (Fig.3)and $OH^{\bullet}$ levels (Fig. 4).

Effect of melatonin on OS indicators affected by pain or immobilization

Melatonin significantly decreased TBARS concentration in painfully stimulated animals (Fig. 1). No significant change was found in GSHPx activity after melatonin treatment (Fig. 2). $NO^{\bullet}$ and $HO^{\bullet}$ concentrations after painful stimulation were not affected by melatonin (Figs 3 and 4). Melatonin did not significantly influence any OS indicators in immobilization stress group (Figs 1 and 2)

Effect of melatonin on pain perception

Repeated 5-min painful stimulation applied for 5 consecutive days decreased both TF and PT latencies by about 12 %. This indicates that the pain threshold was decreased and the pain perception was more intensive. Melatonin seemed to exert certain analgesic effect (a

decrease of TF and PT, after 5 days of melatonin injection just before each painful stimulation was smaller by about 6%, the pain threshold tended to be prolonged again). If the animals had only undergone the 5-min immobilization stress for 5 days, their TF and PT latencies increased by about 20 % (TF) and 13 % (PT). The repeated injection of melatonin did not significantly affect the latencies in this situation (Figs 5 and 6).

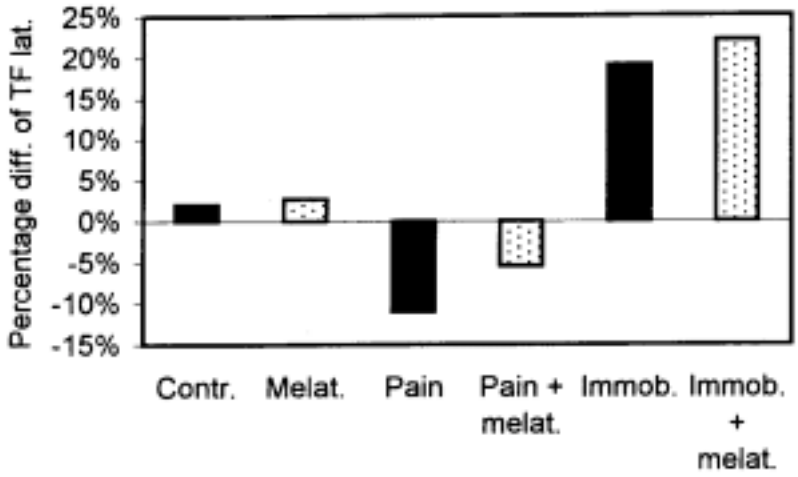


Fig. 5. *Changes of tail-flick latency between the first and the last day of the experiment. For better graphical comparability, there is shown the percentage difference of TF latencies between the first and the last day of the experiment on the y axis.The absolute value in the rat varies from 3,5 to 6,5 seconds. Repeated painful stimulation (5 days) decreased TF latency.Melatonin had a tendency to attenuate it - that means increase the pain threshold and act analgetically. Immobilisation caused stress analgesia, melatonin had no significant effect in this case.*

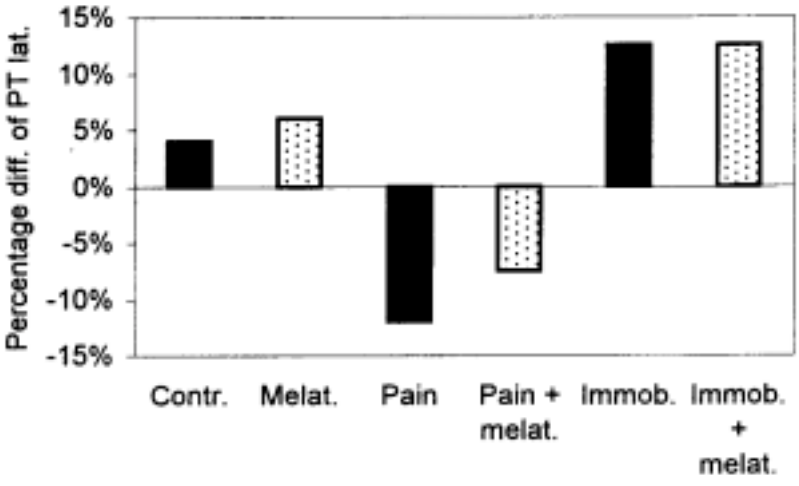


Fig. 6. *Changes of plantar test latency between the first and the last day of the experiment. For better graphical comparability, there is shown the percentage difference of PT latencies between the first and the last day of the experiment on the y axis. The absolute value in the rat varies from 8 to 10 seconds. Repeated painful stimulation (5 days) decreased PT latency. Melatonin had a tendency to reinstate it towards the resting level, that means increase the pain threshold and act analgetically. Immobilisation caused stress analgesia, melatonin had no significant effect in this case.*

Discussion

The present results have shown that repeated peripheral painful stimulation is able to act as a promoter of OS mechanisms causing damage to biomolecules (represented by lipoperoxidation in our experiments) (Rokyta *et al.* 1996). If this stimulatory process is long enough, the results of oxidative damage even in the cortical pain perception areas can be detected. On the contrary, repeated painless stress induced no significant change of TBARS levels in our experiments. This shows that stress is probably not involved in OS promotion in somatosensory cerebral cortex and suggests that another mechanism might responsible. The reason for persisting increase in TBARS concentration in the studied CNS areas still remains unclear. One possible explanation might be the increasing neuronal metabolism during pain perception, which could intensify the oxidative processes. Another hypothesis may concern the production of kinins during painful stimulation. Among other functions, kinins can augment pain signaling *via* their own receptors, which are present not only in the periphery but also in

central nervous system neurons. Kinins also stimulate the neural and neuroglial tissues, thus inducing the synthesis and release of other proinflammatory mediators such as prostanoids, cytokines and cytotoxine-like reactive oxygen species and nitric oxide (Walker *et al.* 1995). Another hypothesis speculates that the activation of AMPA and NMDA receptors during persisting pain could result in nitric oxide and peroxynitryl anion generation (NMDA receptor stimulation) or superoxide radical generation (AMPA receptor stimulation) (Gilad *et al.* 1997). Exogenous melatonin participated in antioxidative stress defense, but only in extremely high doses. The effective dose (100 mg/kg i.p.) was 20-100 fold higher than the dose used for treating circadian rhythm disorders in humans (1 or 5 mg/kg *per os*). The complex role of melatonin in the prevention of oxidative stress damage in the cerebral cortex *in vivo* seems to be more based upon blocking of lipoperoxidation than upon GSHPx stimulation. It is possible that the time period of melatonin administration (5 days) was not long enough to stimulate GSHPx production. Another interesting fact resulting from our experiments was that melatonin seemed to scavenge free radicals more effectively in the controls than in animals subjected to painful stimulation.

As far as our results of direct measurement of free radicals are concerned, we can speculate that the decrease in their concentration after 5 days of painful stimulation is caused by the reaction of superoxide, generated during painful stimulation, with the other radicals, which seem to be present in rather high concentrations in the cerebral cortex even before the stimulation (as it can be deduced from the electron paramagnetic resonance results of the control group). Their reaction with superoxide could result in the reduction of free radicals which had been detected. The production of other cytotoxines, e.g. peroxynitric acid, could be a phenomenon accompanying the reduction of this radical. In the case that the control animals are injected with melatonin, it could immediately act as a scavenger of the already existing free radicals and lower their concentration. On the other hand, when more radicals are produced because of the painful stimulation, melatonin could be consumed for their neutralization and the level of radicals in the sample does not appear to decline dramatically.

The analgesic effect of melatonin was clear in both types of tests employed. In TF test, melatonin decreased the hyperalgesia after repeated painful stimulation by about 8.5 %. The mechanism of melatonin analgesic action is not clear yet. There exists a theory that it can interfere with opiates on μ-opioid receptors, potentiate the inhibitory effects of GABA (Kavaliers 1989, Golombek *et al.* 1991) or inhibit prostaglandin E_2 synthesis (Cuzzocrea *et al.* 1999). The hypothesis of its analgesic effect *via* free radicals scavenging seems to be less probable. The phenomenon of prolonged withdrawal latency after repeated stressful stimulation (demonstrated in our experiments) was earlier described as the stress-induced analgesia (Bodnar *et al.* 1980, Mahajan *et al.* 1997).

Our findings suggest that exogenous melatonin is able to influence ROS metabolism in the rat cerebral cortex and to attenuate the oxidative stress damage, but its effectiveness *in vivo* is not potent enough to be considered as a perspective drug in the field of antioxidative CNS defense.

Acknowledgements

Supported by IGA Ministry of Health of the Czech Republic (grant 4665-3/1998).

References

ABUJA PM, LIEBMANN P, HAYN M, SCHAUENSTEIN K, ESTERBAUER H.: Antioxidant role of melatonin in lipid peroxidation of human LDL. *FEBS Lett* **413**: 289-293, 1997.

BARLOW-WALDEN LR, REITER RJ, ABE M, PABLOS MI, MENDELEZ-PELAEZ A, CHEN LD, POEGGELER B: Melatonin stimulates brain glutathione peroxidase activity. *Neurochem Int* **26**: 497-502, 1995.

BODNAR RJ, KELLY DD, BRUTUS M, GLUSMAN M: Stress-induced analgesia: neural and hormonal determinants. *Neurosci Biobehav Rev* **4:** 87-100, 1980.

CARNEIRO RCG, REITER RJ: Melatonin protects against lipid peroxidation induced by δ-aminolevulinic acid in rat cerebellum, cortex and hippocampus. *Neuroscience* **82**: 293-299, 1998.

CUZZOCREA S, CONSTANTINO G, MAZZON E, CAPUTI AP: Regulation of prostaglandin production in carrageenan-induced pleurisy by melatonin. *J Pineal Res* **21**: 9-14, 1999.

GILAD E., CUZZOCREA S, ZINGARELLI B, SALZMAN A.L, SZABÓ C: Melatonin is a scavenger of peroxynitrite. *Life Sci* **60:** PL169-PL174, 1997.

GOLOMBEK DA, ESCOLAR E, BURIN LJ, DE BRITO-SANCHÉZ MG, CARDINALI DP: Time dependent melatonin analgesia in mice: Inhibition by opiate or benzodiazepine antagonism. *Eur J Pharmacol* **194**: 25-30, 1991.

JOHN TM, BROWN MC, WIDEMAN L, BROWN GM: Melatonin replacement nullifies the effect of light-induced functional pinealectomy on nociceptive rhythm in the rat. *Physiol Behav* **55**: 735-739, 1994.

KAVALIERS M: Funaltrexamine disrupts the day-night rhythm of nociception in mice. *Brain Res Bull* **22**: 783-785, 1989.

KONEČNÁ I, PARARA S, STOPKA P., HOLEČEK V, TREFIL L, RACEK J, ROKYTA R: An attempt to influence reactive oxygen species in rat brain cortex after painful stimulation with exogenous melatonin. *Physiol Res* **49**: P24, 2000.

LAKIN CH, MILLER CH, STOTT ML, WINTERS WD: Involvement of the pineal gland and melatonin in murine analgesia. *Life Sci* **29**: 2543-2551, 1981.

MAHAJAN AS, MISHRA TK, CHAKRABARTY K, CHAKRABARTY AS: Immobilisation stress induced analgesia in diabetic rats. *Indian J Physiol Pharmacol* **41**: 275-279, 1997.

MARSHALL KA, REITER RJ, POEGGELER B, ARUOMA OI, HALIWELL B: Evaluation of the antioxidant activity of melatonin in vitro. *Free Radic Biol Med* **21**:307-315, 1996.

PIERI C, MARRA M., MORONI F, DECCHIONI R, MARCHESELLI F: Melatonin: a peroxyl radical scavenger more effective than vitamin E. *Life Sci* **55:** 271-276, 1994.

REITER RJ, TANG L, GARCIA JJ, MUÑOS-HOYOS A: Pharmacological actions of melatonin in oxygen radical pathophysiology. *Life Sci* **60**: 2255-2271, 1997.

ROKYTA R, KUBÍK V, KORPANCOVÁ J: Development of different parts of thalamus. Electrophysiological and biochemical study. In: *Ontogenesis of the Brain, vol 4*. TROJAN S, ŠŤASTNÝ F (eds), Universitas Carolina Pragensis, Prague 1987, pp 199-202.

ROKYTA R, HOLEČEK V, RACEK J: Les changements des radicaux libres dans l'écorce cérébrale aprés la stimulation douloureuse chez le rat. *Arch Physiol Biochem* **103**: 26, 1995.

ROKYTA R, HOLEČEK V, KREJČOVÁ J, PULS M, RACEK J, ŠŤASTNÁ H, YAMAMOTOVÁ A.: The changes of free radicals after nociceptive stimulation. *Physiol Res* **45**: P29, 1996.

SOLÁR P: Wide-spectrum effects of melatonin: its application in therapy of tumors (in Slovak). *Čs Fyziol* **48:** 27-40, 1999.

TAN DX, CHEN LD, POEGGELER B, MANCHESTER LC, REITER RJ: Melatonin: a potent, endogenous hydroxyl radical scavenger. *Endocrine J* **1:** 57-60, 1993.

WALKER K, PERKINS M, DRAY A: Kinins and kinin receptors in the nervous system. *Neurochem Int* **26**: 1-16, 1995.

ZIMMERMANN M: Ethical guidlines for investigation of experimental pain in conscious animals. *Pain* **16**: 109-110, 1983.

Reprint requests

I. Pekárková, M.D., Department of Neurology, Rudolf and Stefanie Hospital in Benešov, Máchova 400, 257 01 Benešov, Czech Republic. Fax +420 2 24923827. e-mail: ivanapekarkova@seznam.cz