

Melatonin Reduces Oxidative Stress Induced by Chronic Exposure of Microwave Radiation from Mobile Phones in Rat Brain

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Purpose: The aim of the study was to evaluate the intensity of oxidative stress in the brain of animals chronically exposed to mobile phones and potential protective effects of melatonin in reducing oxidative stress and brain injury. **Materials and methods:** Experiments were performed on Wistar rats exposed to microwave radiation during 20, 40 and 60 days. Four groups were formed: I group (control)- animals treated by saline, intraperitoneally (i.p.) applied daily during follow up, II group (Mel)- rats treated daily with melatonin (2 mg kg⁻¹ body weight i.p.), III group (MWs)- microwave exposed rats, IV group (MWs + Mel)- MWs exposed rats treated with melatonin (2 mg kg⁻¹ body weight i.p.). The microwave radiation was produced by a mobile test phone (SAR = 0.043–0.135 W/kg). **Results:** A significant increase in the brain tissue malondialdehyde (MDA) and carbonyl group concentration was registered during exposure. Decreased activity of catalase (CAT) and increased activity of xanthine oxidase (XO) remained after 40 and 60 days of exposure to mobile phones. Melatonin treatment significantly prevented the increase in the MDA content and XO activity in the brain tissue after 40 days of exposure while it was unable to prevent the decrease of CAT activity and increase of carbonyl group contents. **Conclusion:** We demonstrated two important findings; that mobile phones caused oxidative damage biochemically by increasing the levels of MDA, carbonyl groups, XO activity and decreasing CAT activity; and that treatment with the melatonin significantly prevented oxidative damage in the brain.

INTRODUCTION

Our knowledge about mobile telephones and their effects on human health as well as on natural environment is still inefficient because this modern technology is in constant development and spread. The fast-growing, second generation mobile phone system, global system for mobile communications (GSM), has become the world's largest mobile telecommunications system, with its one billion subscribers in over 200 countries.¹⁾ The dangers from electromagnetic field (EMF) exposure by using cellular phones are huge because their effects on human health and environment are not visible in short-time period. The grow-

ing concern about possible effects on the brain after microwave (MW) exposure from mobile phones, especially in children, have been discussed in many countries because of increasing usage of wireless communication systems.²⁾

Microwaves, electromagnetic waves ranging in frequency from 300 MHz to 300 GHz, are increasingly present in our daily environment, for example, in microwave ovens, satellite, and radio/TV transmission, cellular-phone transmitters/receivers, video display terminals, occupational use, and medical exposure through diathermy. Exposure to high density microwaves can cause detrimental effects on the central nervous system, testis, cardiovascular system, hematopoietic system, uteroplacental function.³⁾ Various nonthermal effects of MWs from mobile phones on the central nervous system, including permeability of the blood-brain barrier (BBB), neuronal electrical activity and increase in calcium ion efflux, neurotransmitter balance, cognitive function, and sleep have recently been reviewed.^{4,5)} It has been reported that increased incidence of brain tumors and acoustic neurinoma is correlated with exposure to mobile phone MWs depending on duration of mobile phone use.⁶⁾ There is growing evidence that suggests possible

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relationship of MW exposure immune response in human lymphocytes.⁷⁾ Numbered health effects of MWs exposure are related to thermal effect.⁸⁾ Formation of reactive oxygen species (ROS) and increased oxidative stress may be involved in the action of microwave radiation on the biological system.⁹⁾

Oxidative stress has been implicated in many brain disorders.¹⁰⁾ Antioxidant enzymes, superoxide dismutase (SOD) convert $O_2^{\bullet-}$ into a reactive oxygen intermediate H_2O_2 , catalase (CAT) detoxifies H_2O_2 and glutathione peroxidase (GPx) catalyses the breakdown of H_2O_2 and lipid hydroperoxides to non-toxic products. ROS also cause injury by reacting with biomolecules, such as lipids, proteins and nucleic acids, as well as by depleting enzymatic and/or nonenzymatic anti-oxidants in the brain.¹¹⁾ Anti-oxidant treatments in animals and humans could be beneficial in preventing or reducing some complications of microwave radiation.¹²⁾ One of the possible effects is probably increasing of xanthine oxidase (XO) activity, which produces uric acid and ROS during the catabolism of purines.¹²⁾

Melatonin (N-acetyl-5-methoxy-tryptamine) is a neurohormone synthesized in and released from the pineal gland during the dark period. Its effects are mediated through the activation of two melatonin G-protein-coupled receptors, MT_1 and MT_2 .¹³⁾ In addition to its neuroendocrine functions, melatonin seems to exert psychotropic effects in rodents, such as sedative, analgesic, anticonvulsant, hypnotic, and anxiolytic effects.¹⁴⁾ Melatonin (a) protects nuclear and mitochondrial DNA, membrane lipids, and cytosolic proteins from oxidative damage; (b) it blocks oxidative mediators that initiate the neuroinflammatory response after traumatic brain injury, e.g. by reducing NF- κ B activation; (c) interact with central γ -aminobutyric acid (GABA) neurotransmission; (d) it detoxifies ROS and nitrogen species (RNS); (e) it stimulates antioxidative enzymes; (f) it improves oxidative phosphorylation and stabilizes neuronal membranes.^{15,16)} Melatonin protective actions against these adverse changes are believed to stem from its direct free radical scavenging and indirect antioxidant activities, possibly from its ability to limit free radical generation at the mitochondrial level and because of yet-undefined functions.

The purpose of the present study is to evaluate the intensity of oxidative stress in the brain of animals chronically exposed to mobile phones and potential protective effects of melatonin in reducing oxidative stress brain injury.

MATERIALS AND METHODS

Chemicals

The reagents were purchased from Sigma (St. Louis, MO, USA) and were of the highest commercial grade available. All used chemicals were of analytical grade. All drug solutions were prepared on the day of experiment. Animals used for procedure were treated in strict accordance with the NIH

Guide for Care and Use of Laboratory Animals (1985).

Animal model

Experiments were performed on 84 adult male Wistar Albino rats (6–8 weeks old, 150 g), bred at the Vivarium of the Institute of Biomedical Research, Medical faculty, Nis, under conventional laboratory conditions. All animals were suited in the same room without near sources of EMF, from the cages. All animals were housed collectively (7 animals in each cages $30 \times 40 \times 40$ cm – W $\times$ L $\times$ H). The rats were kept in a pure (i.e. lacking any metallic fittings) polycarbonate cage and given ad libitum access to standard laboratory food and water. The housing room was maintained at 24°C with $42 \pm 5\%$ relative humidity and had a 12–12-h light–dark cycle (light on 06:00–18:00 h).

All animal experiments had been approved by the Animal Ethics Board of the Medical Faculty in Nis and were performed according to these guidelines.

Experimental design

The animals were allocated into four experimental groups. Each group consisted of 21 animals, situated in 3 cages by 7 animals in each). *I group (control)*—animals treated by saline, intraperitoneally (i.p.) applicated everyday during follow up, *II group (Mel)*—everyday treated rats with melatonin (2 mg kg^{-1} body weight i.p.), *III group (MWs)*—MWs exposed rats, *IV group (MWs + Mel)*—MWs exposed rats treated with melatonin (2 mg kg^{-1} body weight i.p.)

Microwave exposure

The animals were exposed by microwave radiation for 20, 40 and 60 days (4 h/day during light period). The microwave radiation was produced by a mobile test phone (model NOKIA 3110; Nokia Mobile Phones Ltd.) connected to a Communication Test Set PCDK with PC and appropriate software module. MW exposure was performed in the same room where all animals were housed. The two mobile test phones and PC module were situated at the wooden desk with rubber surface. The desk was placed at distant part of the room, 5 meters from housed rats. Every four hours two cages from MWs and MWs + Mel group were transferred to the desk. Mobile test phone, as a source of MW radiation, was put in each cage. Mobile phone was put in small perforated polycarbonated cell in the center of the cage in order to prevent damage that could be caused by rats. Two false mobile phones were put in the control and Mel group cages in the same time. After four hours of MWs exposure, the same procedure was applied to the next two cages of investigated groups. In the present study, an electromagnetic near-field signal for GSM (Global System for Mobile communication) at 900 MHz with continuous wave, mobile phone system was used. Electromagnetic fields parameters in cage were measured several times during experimental exposure. Electrical field was estimated by EM field meter

AARONIA SPECTRAN HF6080 with $E = 9.88 \text{ V/m}$ to 18.356 V/m and magnetical field $B = 4.68 \mu\text{T}$ to $8.69 \mu\text{T}$. The whole-body specific energy absorption (SAR) rate was estimated as $0.043\text{--}0.135 \text{ W/kg}$ using data for a rotating ellipsoidal model of a rat.

Melatonin is applicated intraperitoneally every morning at 08.00 AM. According to the research of Drago *et al.*¹⁷⁾ Melatonin is intraperitoneally applied daily in the dose of 2 mg/kg thus avoiding its effects as neurotransmitter and/or neuromodulator.

Seven animals from each group were sacrificed after 20, 40 and 60 days. Rats were anesthetized with ketamine HCl (50 mg/kg), administered intraperitoneally (i.p.), before sacrificing each rat and removing the brain. For estimation of oxidative stress the brain tissue was cut in small pieces and homogenized in ice-cold water, by using a homogenizer (IKA® Works de Brasil Ltda Taquara, RJ 22713-00). The homogenates ($10\% \text{ w/v}$) were centrifuged at $1500 \times g$ for 10 min. at 4°C .

Biochemical analysis

Determination of MDA. The intensity of lipid peroxidation in the brain tissue was spectrophotometrically measured, based on the thiobarbituric (TBA) response products from Ohkawa *et al.*¹⁸⁾ Homogenate absorption was measured at 532 nm . Malondialdehyde (MDA)–lipid peroxidation end product, concentration was expressed per mg/protein , using the molecular extinction coefficient of MDA ($1.56 \times 10^{-5} \text{ mol cm}^{-1}$).

Determination of protein oxidation. Carbonyl group concentration, as the level of oxidative modified proteins, was determined spectrophotometrically¹⁹⁾ using 2,4 dinitrophenylhydrazine (DPNH), a traditional carbonyl reagent. Reactive carbonyl derivatives were calculated using the DPNH molar extinction coefficient at 370 nm ($22 \times 10^3 \text{ L/mol/cm}$) and expressed in $\mu\text{mol/g}$ of protein.

Determination of CAT activity. The activity of catalase was measured spectrophotometrically at 405 nm by a method described by Goth²⁰⁾ in which serum or homogenate was incubated in H_2O_2 substrate and the enzymatic reaction was stopped by ammonium molybdate. Activity was expressed in micromoles per mg ($\mu\text{M mg}^{-1}$) protein.

Determination of XO activity. The activity of xanthine oxidase enzyme in brain homogenate was estimated by the amount of uric acid produced for fixed time interval. In brief, reaction mixture containing 0.1 ml brain homogenate and 0.1 M Tris/HCl buffer, $\text{pH } 7.4$, in a final volume of 2.5 ml was pre-incubated for 15 min at 37°C . The reaction was started by addition of 0.5 ml of 0.6 mM xanthine. The oxygen-dependent xanthine oxidase activity was estimated by the increase of the uric acid content as a result of incubation of this reaction mixture for 30 min at 37°C . The uric acid content was calculated by the increase in the absorbency at 293 nm against blank, which was run parallel with the

reaction mixture without xanthine. Molar extinction coefficient of $7.6 \times 10^{-3} \text{ M cm}^{-1}$ was used for this purpose.²¹⁾ XO activity was also expressed as $\text{U/mg tissue protein}$ in brain homogenate.

Determination of proteins. Brain proteins were determined according to Lowry's method,²²⁾ using bovine serum albumin as standard.

Statistical analysis

Data were analysed using a commercially available statistics software package (SPSS® for Windows, v. 9.0, Chicago, USA). Results were presented as means $\pm$ SD. Statistical significance was determined at level of $p < 0.05$ using the Student's t-test.

RESULTS

In the MWs group, 20 days of exposure to mobile phone produced a significant increase in the brain tissue MDA content ($p < 0.001$), an index for lipid peroxidation, when compared with control and Mel group. The significant increase in the brain tissue MDA content remained after 40 and 60 days of exposure to mobile phones when compared with control, Mel and MWs + Mel group ($p < 0.001$) (Fig. 1). Melatonin treatment (MWs + Mel group) significantly prevented the increase in the MDA content of the brain tissue after 40 days ($p < 0.01$) and 60 days ($p < 0.001$) of exposure to mobile phones when compared with MWs groups (Fig. 1).

Microwave radiation produced a significant increase of carbonyl groups content, an index for oxydative modification of proteins, in brain after 20 and 40 days ($p < 0.01$), as well as after 60 days ($p < 0.001$) of exposure to mobile phone, when compared with control and Mel group (Fig. 2). Melatonin treatment (MWs + Mel group) did not prevent the

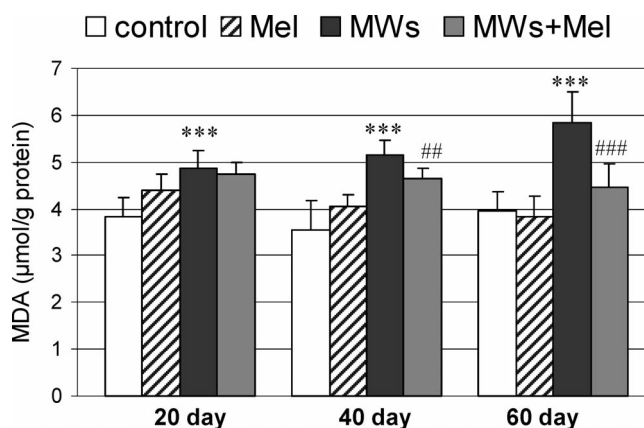


Fig. 1. The effects of melatonin (Mel) on lipid peroxidation (MDA level- $\mu\text{mol/g}$ protein) in the rat brain exposed to microwave radiation. *** $p < 0.001$ (vs. control); ## $p < 0.01$ (vs. MWs); ### $p < 0.001$ (vs. MWs).

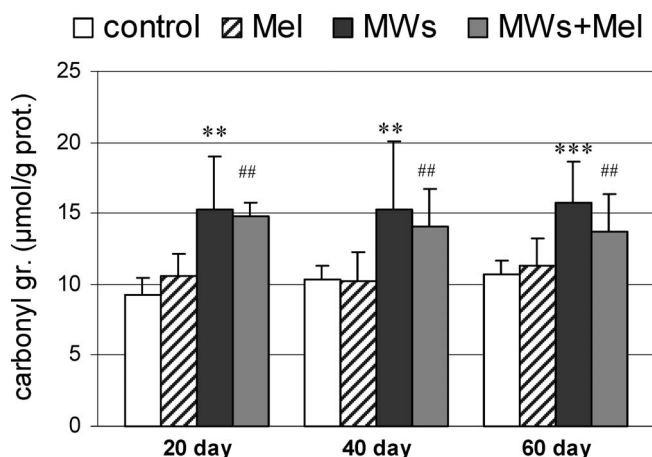


Fig. 2. Concentration of carbonyl groups (μmol/g protein) in the rat brain exposed to microwave radiation (MW). ** $p < 0.01$; *** $p < 0.001$ (vs. control and Mel); ## $p < 0.01$ (vs control and Mel).

increase in the carbonyl groups content of the brain tissue during exposure to mobile phones when compared to MWs group during observed period of 60 days (Fig. 2). The carbonyl group content remained significantly higher in MWs + Mel group compared to control and Mel groups during whole period of exposure to mobile phones ($p < 0.01$).

Catalase activity was significantly decreased in brain of MWs group after 20, 40 and 60 days ($p < 0.001$) of exposure to mobile phone, when compared with control and Mel group (Fig. 3). Melatonin treatment (MWs + Mel group) did not prevent the depletion of CAT activity produced by microwave radiation after 20, 40 and 60 days of mobile phones exposure when compared to MWs group (Fig. 3).

As shown in Fig. 4, the activity of XO is significantly reduced by melatonin administration (Mel group) after 40 and 60 days when compared to control ($p < 0.01$). Exposure to mobile phone induced a significant increase in brain XO

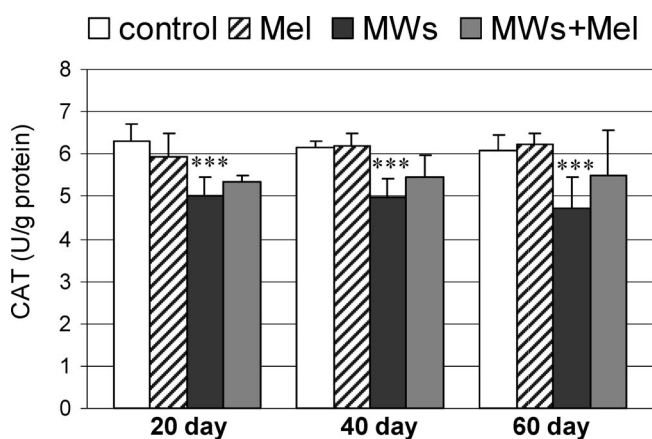


Fig. 3. The activity of enzyme catalase (CAT-U/g protein) in control, Mel, MWs and MWs + Mel groups. *** $p < 0.001$ (vs. control and Mel).

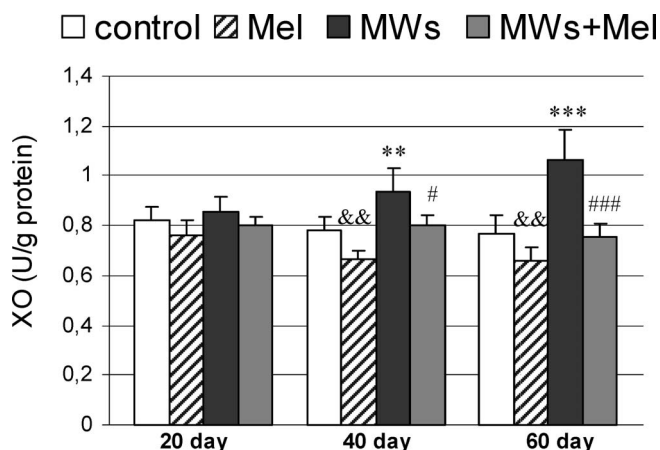


Fig. 4. Xanthine oxidase (XO) activities (U/g protein) in brain tissues of rats. ** $p < 0.01$; *** $p < 0.001$ (vs. control and Mel); # $p < 0.05$; ### $p < 0.001$ (vs. MWs); && $p < 0.01$ (vs. control).

activity after 40 days ($p < 0.01$) and 60 days ($p < 0.001$) when compared to control and Mel group. The increase in brain tissue XO activity produced by mobile phone was significantly prevented by melatonin application after 40 ($p < 0.05$) and 60 days ($p < 0.001$) when compared to MWs group (Fig. 4).

DISCUSSION

Oxidative insults resulting from either an excessive generation of ROS or the deterioration of antioxidant defense capacity has been closely linked to the pathogenesis of neuronal dysfunction or death.²³⁾ The nervous system is particularly vulnerable to ROS due to its high metabolic rate, its deficient oxidant defense mechanisms and its diminished cellular turn over. Salford *et al.*²⁴⁾ first reported the evidence for neuronal damage caused by nonthermal microwave exposure. Mobile phones with emission of 900 MHz MWs could be sources of neuronal damage. The brain may absorb emitted MWs radiation more than other internal organs because mobile phones are generally used near the brain. In particular, a variety of neurological effects have been postulated to MWs, including headaches attributable to use of mobile phones and changes in sleep patterns.²⁵⁾

Lipid peroxidation and exposure to mobile phones

In this study, increased levels of lipid peroxidation were found in the brain of rats exposed to the 900 MHz microwave radiation (MW) during observed periods of 60 days (Fig. 1). This result suggests that the microwave can induce brain damage of exposed rats by increasing oxidative stress and lipid peroxidation. The similar findings were presented by Ilhan *et al.*¹²⁾ They found that malonaldehyde (MDA) levels in rat brain tissues was increased significantly after exposure to mobile phone (SAR = 0.25 W/kg, for one hour/

day for seven days).

There are both direct and indirect effects of lipid peroxidation on neuronal damage. The direct effects include the loss of fluidity, a decrease in electrical resistance, a depression in protein mobility in the membrane and increased phospholipid exchange between the bilayers of the membrane.²⁶⁾ The breakdown of cell membranes in the worst case causes inactivation of membrane-bound enzymes and loss of decompartmentalization, events that are catastrophic to the normal function of cells. Indirect effects of lipid peroxidation are possibly less apparent but likely no less detrimental. The aldehydes that are produced as a consequence of lipid peroxidation are biologically active. One of these aldehydes - MDA has been shown to cross-link and aggregate membrane proteins. It also leads to oxidation of polyunsaturated fatty acids and thus serves as a reliable marker of oxidative stress mediated lipid peroxidation in brain rats.⁹⁾ In general, the complex reactions of free radicals, aldehydes, and other products of lipid peroxidation lead to the destruction of major membrane proteins.

The present study was also designed to explore the protective effects of a well-known free radical scavenger melatonin, on MW-induced brain oxidative injury. Treatment with melatonin prevented the high MDA level in the brain after the MW-induced oxidative injury. Melatonin treatment (MWs + Mel group) significantly prevented the increase in the MDA content of the brain tissue, after 40 and 60 days, but not after 20 days of exposure to mobile phones when compared with MWs groups (Fig. 1).

The peroxidation of membrane lipid also seems to be in the realm of melatonin's antioxidative actions and melatonin is to be a very potent protector against lipid peroxidation in vivo. Melatonin, a lipophilic compound²⁷⁾ is potent cellular antioxidant. It can both directly scavenge free radicals²⁸⁾ and indirectly induce antioxidant enzymes via a specific melatonin receptor.²⁹⁾ For example, melatonin directly scavenges singlet oxygen, hydrogen peroxide and the devastatingly toxic hydroxyl radical.³⁰⁾

Melatonin molecule is capable to catch $\cdot\text{OH}$ and form cyclic 3-hydroxymelatonin (cyclic 3-OHM) excreted in the urine of rodents and man.³¹⁾ In this sense, melatonin can be categorized as the first line of defensive molecule. Melatonin interacts with peroxynitrite (ONOO^-) with the resultant formation of 6-hydroxymelatonin. Indeed, 6-hydroxymelatonin was found to reduce lipid peroxidation in the liver, muscle and brain of rats induced by oxidative stress.³²⁾ The product that results from the melatonin and H_2O_2 interaction is N^1 -acetyl- N^2 -formyl-5-methoxykynuramine (AFMK).³³⁾ In specific experiments, AFMK reduced DNA damage and also limited the destruction of lipids from their exposure to H_2O_2 .^{33,34)} Indirect evidence suggested that melatonin neutralized singlet oxygen ($^1\text{O}_2$) in the brain.³⁵⁾

Thereby, these multiple actions of melatonin protect cells from ROS-mediated lipid peroxidation, protein destruction

and nuclear DNA damage, under conditions of MWs radiation. This is in line with observation that reduction of melatonin contents in the brain is consequence of chronic exposure to EMF.³⁶⁾

Oxidative modification of proteins and MWs

Oxidative modification of proteins, assessed based on carbonyl group content, can be an early marker of oxidative brain damage induced by microwave radiation. In the brain of experimental rats exposed to MWs, significant increase of carbonyl group content was registered compared to control and Mel group, during whole observed periods (Fig. 2).

Whereas all amino acid residues in proteins are capable of being damaged by free radicals, it generally seems that the process of protein aggregation may relate to the ability of the $\cdot\text{OH}$ to form cross linkages.³⁷⁾ As a consequence of the conformational changes in proteins resulting from their interactions with free radicals, they become increasingly vulnerable to proteolytic degradation. Thus, protein denaturation increases the likelihood that a molecule will be enzymatically degraded. As a result, when oxygen radical production is elevated it is presumed that the oxidation of proteins is proportionally increased. Protein degradation compromises the metabolic efficiency of cells. With aging of cells, their enzymes, including proteases and antioxidant proteins, and smaller antioxidant molecules become progressively inactivated due to the failure of the antioxidant systems to overcome the constant influx of ROS. Consequently, the accumulation of free radical-induced carbonylated proteins accelerates, indicating the age or state when cells in the individual become increasingly more susceptible to ROS-mediated damage.³⁸⁾

The present study was not able to show the protective effects of a melatonin in reduction of raised carbonyl contents, in MW-induced brain oxidative injury (Fig. 2). This indicates that although melatonin is a potent protector against protein oxidation either by the directly scavenging radicals or indirectly by some other antioxidative means,⁹⁾ oxidative modification of proteins also occurs by reactions with aldehydes produced during lipid peroxidation. Thereafter, end products of lipid peroxidation such as MDA and 4-hydroxy-2-nonenal (HNE) as well as products from polyunsaturated fatty acids cause protein damage.³⁹⁾ It seems that MWs induce pathophysiological conditions that we can see in aging process.

Activity of antioxidant enzymes in brain of MW exposed rats

Decreasing of catalase (CAT) activity in brain tissue in MW exposed rats was registered during whole observed period (Fig. 3). The decreased CAT activity in brain indicates a high degree of oxidative stress resulting in the increased endogenous H_2O_2 after MW exposure. It suggests an over consumption of enzyme related to increased produ-

ction of H_2O_2 in vivo.

Administration of melatonin did not prevent the depletion of CAT activity in brain tissue exposed to MWs, but showed the tendency to raise CAT activity (Fig. 3). Melatonin stimulates several important antioxidative enzymes (CAT, SOD and GSH-Px).²⁸⁾ The activity of antioxidative enzyme-CAT, which plays a major role in maintaining low steady-state concentrations of H_2O_2 , thereby reducing highly toxic OH generation, is normally stimulated by melatonin. These indirect antioxidative actions of melatonin might certainly magnify its protective actions in the brain tissue under conditions of high oxidative stress, but effects on decreased activity of CAT in brain did not count as protective effect in state of mobile phone's MWs exposure.

Activity of prooxidant enzymes in brain of MW exposed rats

The present study was also designed to estimate the effects of MWs on xanthine oxidase (XO) activity in rats' brain tissue. The main finding was that exposure to mobile phone did not immediately induce a significant change in brain XO activity. The increase in brain XO activity was visible after prolonged period of exposure such as 40 and 60 days (Fig. 4).

As XO can reduce molecular oxygen to superoxide and hydrogen peroxide, XO is thought to be one of the key enzymes producing reactive oxygen species. Xanthine dehydrogenase (XDH) can be converted reversibly to xanthine oxidase (XO) by oxidation of cysteine residues or irreversibly by limited proteolysis. XO has high reactivity toward O_2 but negligible reactivity toward NAD^+ . Increased activity of xanthine oxidase to dehydrogenase form increases production of free radicals and lipid peroxidation can lead to gout and mutagenesis. Superoxide anion, hydrogen peroxide and hydroxyl radical are formed as by-products in reaction. Inhibitors of xanthine oxidase reaction have therapeutic application in treating of gout and hepatic injury.⁴⁰⁾

In this animal model application of melatonin induced significant reduction of XO activity both in control group and MWs group. Interestingly, this effect is like MDA contents reduction also visible only after prolonged period of exposure such as 40 and 60 days (Fig. 4).

There are a few data in literature about ability of melatonin to decrease XO activity in brain. These results demonstrate that the antioxidant action of melatonin is principally related to decreasing of pro-oxidant enzymes activity such as XO.⁴¹⁾

Observation that melatonin application exerts protective effects only after prolonged period of 40 days showed existence of some other adding effects of melatonin action. It indicates that effects of melatonin are closely related to melatonin's protection against lipid peroxidation and DNA damage in nucleus of neuronal cells, maybe even for some transcriptional effects. This is in line with recent data that

melatonin protects against mitochondrial DNA mutation and deletion caused by oxidative damage and apoptosis, via its ability to strongly inhibit cytochrome C release from the mitochondria and to suppress the activity of caspase 3 as well as to stabilize the mitochondrial membrane potential thereby protecting against neuronal death.⁴²⁾

This study provides two important findings relating to oxidative stress in the brain of animals exposed to mobile phones. Firstly, we demonstrated that mobile phones caused oxidative damage biochemically by increasing the levels of MDA, carbonyl groups and XO activity and decreased CAT activity. Secondly, intraperitoneal treatment with the melatonin as an antioxidant and a potent free radical scavenger agent, significantly prevented oxidative damage in the brain tissue.

Although present scientific information does not indicate the need for any special precautions for use of mobile phones, if individuals are concerned, they might choose to limit their own EMF exposure by limiting the length of calls, or using "hands-free" devices to keep mobile phones away from the head and body.

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