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The Effects of Melatonin on Oxidative Stress Parameters and DNA Fragmentation in Testicular Tissue of Rats Exposed to Microwave Radiation*

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Abstract

Background. Microwaves from mobile phones are one of the environmental toxicants that are capable of compromising male fertility by inducing oxidative stress and apoptosis in the testes. Melatonin is a lipophilic tryptophan indole amine and a potent antioxidant.

Objectives. The aim of the study was to evaluate the effect of melatonin treatment on oxidative stress parameters and DNA fragmentation in the testicular tissue of rats exposed to microwave radiation (4 h/day).

Material and Methods. Adult Wistar rats were divided in 4 groups: I – treated with saline; II – treated with melatonin; III – exposed to microwaves; IV – exposed to microwaves and treated with melatonin. The melatonin (2 mg/kg ip) was administered daily. The animals were sacrificed after 20, 40 and 60 days.

Results. Melatonin treatment prevented previously registered increases in malondialdehyde after only 20 days. Furthermore, it reversed the effects of microwave exposure on xanthine oxidase (after 40 days) and acid-DNase activity (after 20 days). However, neither protein carbonyl content nor catalase and alkaline DNase activity were changed due to melatonin treatment.

Conclusions. Melatonin exerts potent antioxidant effects in the testes of rats exposed to microwaves by decreasing the intensity of oxidative stress; it also reduces DNA fragmentation (*Adv Clin Exp Med* 2015, 24, 3, 429–436).

Key words: melatonin, microwave radiation, testicular tissue, oxidative stress, DNase.

The use of electrical devices that produce microwave radiation (TV sets, mobile phones, PC monitors, etc.) has for several decades been an integral part of everyday life in developed societies. However, in the past few years there have been growing public concerns about the effects of

chronic exposure to microwave radiation (MWs), especially when it comes to its potentially harmful effect on reproductive health [1].

The testes are exposed to high power density mobile phone radiation, due to the fact that mobile phones are often carried in trouser pockets.

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It is well known that in addition to damage caused by thermal effects, MW exposure can induce severe changes at the cellular and molecular levels, such as single and double strand DNA breaks, protein conformation alterations, increases in oxidative stress [2] and apoptosis [3].

Oxidative stress is thought to be one of the key mechanisms of cell injury caused by MW exposure. Lipid peroxidation and oxidative modification of protein molecules are the most important mechanisms of oxidative damage in tissues. Malondialdehyde (MDA) increases and changes in catalase activity in testes, kidneys and skin have previously been reported after exposure to MWs [4–6]. Xanthine oxidase (XO), an enzyme able to produce reactive oxygen species (ROS), is reported to be elevated in the brain tissue after exposure to MWs [5, 7]. Secondary products of lipid peroxidation, particularly MDA, have large-scale cytotoxic effects. Chemical reactions between biomolecules such as proteins, DNA, phospholipids and MDA cause covalent modification of those biomolecules and lead to consequent cell membrane injury and intracellular macromolecule alterations [8].

Increased production of free radicals during exposure to microwave radiation is usually associated with DNA damage in tissues [9, 10]. In order to limit the effects of oxidative stress, cells can respond by committing suicide, and in this case the ROS produced by mitochondria can act as a trigger for apoptotic cell death through the activation of caspases [11]. One of the main biochemical events during apoptosis is degradation of DNA, quantified through enzyme activity (alkaline and acid DNase); their activity is considered to actually reflect post-apoptotic DNA destruction in tissue.

Melatonin is a lipophilic tryptophan, indoleamine and neurohormone. In humans, melatonin is primarily synthesized and released from the pineal gland during the night, because its synthesis and secretion are inhibited by electromagnetic radiation in the visible light range of frequencies. Melatonin acts as antioxidant [12] and immunostimulant, and regulates mRNA levels for some specific proteins. It plays a role in the prevention of cancer, neurodegenerative diseases and diabetes complications [13].

The aim of the study was to evaluate the effect of melatonin on oxidative stress parameters (such as MDA, protein carbonyl formation, catalase and xanthine oxidase activity) and DNA fragmentation (alkaline and acid DNase activity) in the testicular tissue of rats exposed to microwave radiation.

Material and Methods

Chemicals

The reagents used were of the highest commercial grade available. The chemicals used for this experiment were of analytical grade. Drug solutions were prepared on the day of the experiment.

Animals

The animals used for the procedure were treated in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals (1985).

The experiments were performed on 84 adult male Wistar albino rats (6–8 weeks old, 150 g), bred *in vivarium* of the Biomedical Research Institute at the Faculty of Medicine of the University of Nis, Serbia, under conventional laboratory conditions. The control animals and study group animals were collectively housed in polycarbonate cages 30 × 40 × 40 cm (W×L×H) and had *ad libitum* access to standard laboratory food and tap water. The room in which they were housed was maintained at 24°C with 42 ± 5% relative humidity and was on a 12:12-h light/dark cycle (exposure to light from 06:00 AM to 06:00 PM).

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

MW Exposure

The animals were exposed to MWs for 20, 40 and 60 days (4 h per day during the light period). The MWs were produced by a Nokia 3110 mobile test phone (Nokia Mobile Phones Ltd.) The test phone was connected to a computer with an appropriate software module by a communication test set PC development kit. During MW exposure, 8 rats were able to move freely in a 100% plastic cage without any metallic fittings. A continuous-wave near-field electromagnetic signal (900 MHz) for the Global System for Mobile Communication (GSM) was used for the purposes of the experiment. Electromagnetic field parameters within the cage were measured several times during the experimental exposure by means of an electromagnetic field meter (Spectran HF6080, Aaronia AG, Germany). Electric field intensity (E) ranged from 9.88 V/m to 18.356 V/m, and magnetic field intensity (B) ranged from 4.68 µT to 8.69 µT. The whole-body specific energy absorption rate (SAR) was estimated at 0.043–0.135 W/kg using data for a rotating ellipsoidal rat model.

Experimental Design

The animals were divided in 4 experimental groups. Each group consisted of 21 animals housed in 3 cages (7 animals in each). Group I (the control group) consisted of rats treated with 0.9 % saline; Group II (Mel) consisted of rats treated with melatonin (2 mg per kg⁻¹ body weight by IP injection); Group III (MWs) comprised rats exposed to MWs; and Group IV (MWs + Mel) was made up of rats exposed to MWs and treated with melatonin (2 mg per kg⁻¹ body weight by IP injection). The melatonin was administered every morning at 08.00 AM, as proposed by Drago et al. [14]. The melatonin was administered intraperitoneally in a single dose of 2 mg/kg⁻¹ in order to avoid the neuromodulatory effect of melatonin. In the control rats, isotonic saline solution (equal to the volume of melatonin) was given intraperitoneally every day during the follow-up.

Seven animals from each group were sacrificed after 20, 40 and 60 days from the beginning of the experiment. The rats were anesthetized by ketamine HCl (50 mg/kg), which was administered intraperitoneally. Before sacrificing each rat, the testes were surgically removed from the scrotum.

Tissue Sampling

To assess oxidative stress and DNA fragmentation parameters, the testicular tissue was first cut into small pieces and then homogenized in ice-cold water, by means of a homogenizer (IKA® Works de Brasil Ltda Taquara, RJ, Brazil). Homogenates (10% w/v) were centrifuged at 1500 × g for 10 min at 4°C.

Biochemical Analysis

Determination of MDA

Malondialdehyde in the testicular tissue was determined spectrophotometrically, based on the chemical reaction between thiobarbituric acid (TBA) and MDA, as described by Ohkawa et al. [15]. Homogenate absorption was read at 532 nm. The MDA concentration was expressed as nanomoles per mg of protein, using the MDA molecular absorbance coefficient (1.56 × 10⁻⁵ mol cm⁻¹).

Determination of Protein Oxidation

Protein carbonyl content, used as a quantification of oxidative modified proteins, was determined spectrophotometrically [16] using 2,4-dinitrophenylhydrazine. Reactive carbonyl derivatives were assessed by using the DPNH molar extinction coefficient at 370 nm (22 × 10³ L/mol/cm) and expressed as μmol/g of protein.

Determination of CAT Activity

Catalase activity was measured spectrophotometrically at 405 nm as described by Góth [17]. In this method, the homogenates were incubated in an H₂O₂ substrate and the enzymatic reaction was stopped by adding ammonium molybdate. Activity was expressed as micromoles per mg (μM mg⁻¹) of protein.

Determination of XO Activity

Xanthine oxidase activity in the homogenates was estimated by the amount of uric acid produced for a fixed time interval. In brief, a reaction mixture containing 0.1 mL of testis homogenate and 0.1 M Tris/HCl buffer, 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 adding 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 incubating this reaction mixture for 30 min at 37°C. The uric acid content was calculated by the increase in absorbency at 293 nm in comparison with a homogenate/buffer mixture that was run parallel with the reaction mixture but without xanthine. A molar extinction coefficient of 7.6 × 10⁻³ M cm⁻¹ was used for this test [18]. XO activity was expressed as U/mg tissue protein in testis homogenate.

Determination of Alkaline DNase (DNase I) and Acid DNase (DNase II) Activity

Alkaline and acid DNase activity were determined as described by Bartholeyns et al. [19]. In this method, DNA was used as a substrate. Alkaline DNase activity was determined at optimum pH = 7.4 using Tris-HCl buffer, with the addition of Mg²⁺ ions as the activator; and acid DNase activity was determined using acetate buffer at optimum pH = 5.0.

Determination of Proteins

Testicular proteins were determined according to Lowry's method [20], using bovine serum albumin as the standard.

Statistical Analysis

The results were presented as means ± SD. The data were analyzed using a one-way ANOVA carried out using SPSS® Statistics software for Windows (v. 20.0.0, IBM, NY, USA). *Post-hoc* Tukey's test was used. Statistical significance was set at p < 0.05.

Results

MDA levels in the testicular tissue of rats exposed to MWs were significantly higher in comparison to the control group and the Mel group ($p < 0.001$, Fig. 1). Reduced MDA levels were found in the testicular tissue of animals that were both irradiated and treated with melatonin in comparison to animals that were irradiated but did not receive melatonin treatment, after 20 days ($p < 0.05$), 40 days ($p < 0.001$) and 60 days ($p < 0.01$) (Fig. 1).

Protein carbonyl content in the testicular tissue of rats exposed to MWs was significantly

higher as compared to the control group and the Mel group after 40 days ($p < 0.05$) and 60 days ($p < 0.001$) (Fig. 2). Melatonin administration to rats exposed to MWs (the MWs + Mel group) did not prevent the increase of protein carbonyls content in the testicular tissue (Fig. 2).

Catalase activity was found to be decreased in the testicular tissue of rats exposed to MWs in comparison to the control group and the Mel group ($p < 0.05$) (Fig. 3). Melatonin administration to animals exposed to MWs (the MWs + Mel group) caused a slight, but not statistically significant, increase in catalase activity (Fig. 3).

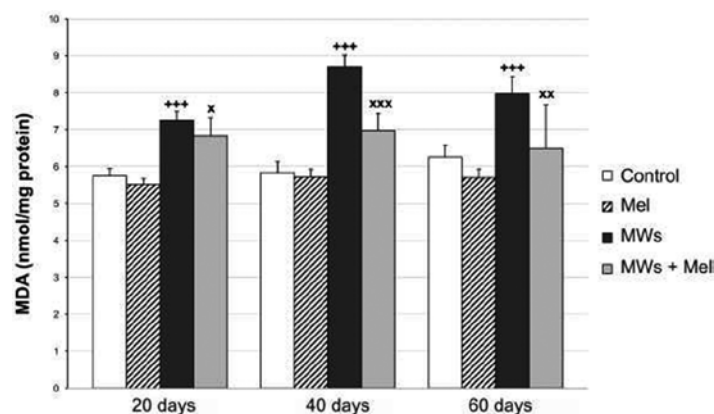


Fig. 1. The effect of melatonin (Mel) on lipid peroxidation (MDA level – nmol/mg of protein) in the testicular tissue of rats exposed to MWs.

*** $p < 0.001$ (vs. control and Mel);

^x $p < 0.05$ (vs. MWs);

^{xx} $p < 0.01$ (vs. MWs);

^{xxx} $p < 0.001$ (vs. MWs)

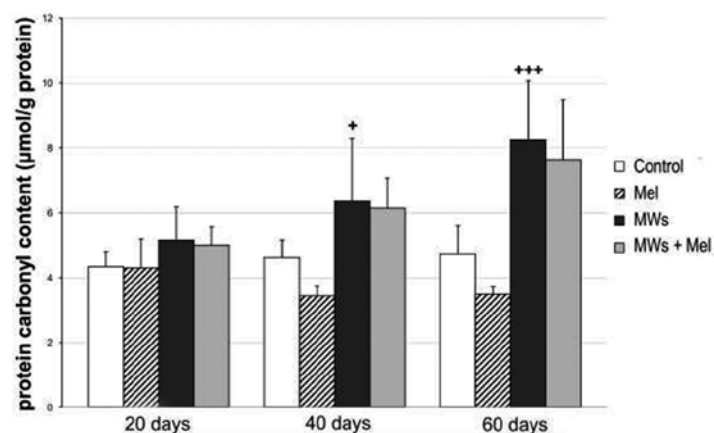


Fig. 2. The concentration of protein carbonyl groups (µmol/g protein) in the testicular tissue of rats exposed to MWs.

⁺ $p < 0.05$ (vs. control and Mel);

^{xxx} $p < 0.001$ (vs. control and Mel)

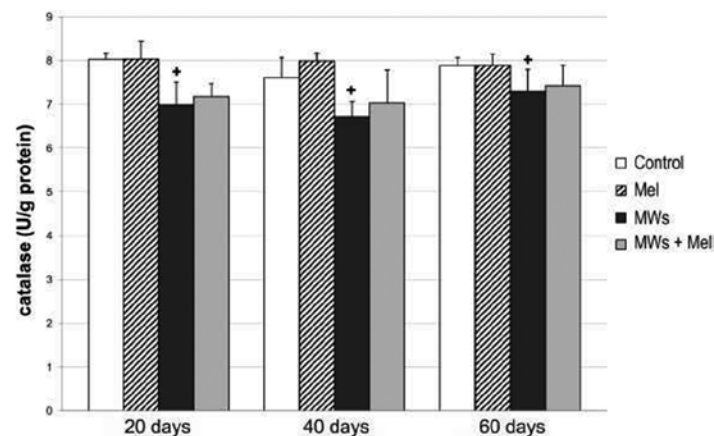


Fig. 3. Catalase activity (CAT–U/g protein) in control, Mel, MWs and MWs + Mel groups.

⁺ $p < 0.05$ (vs. control and Mel)

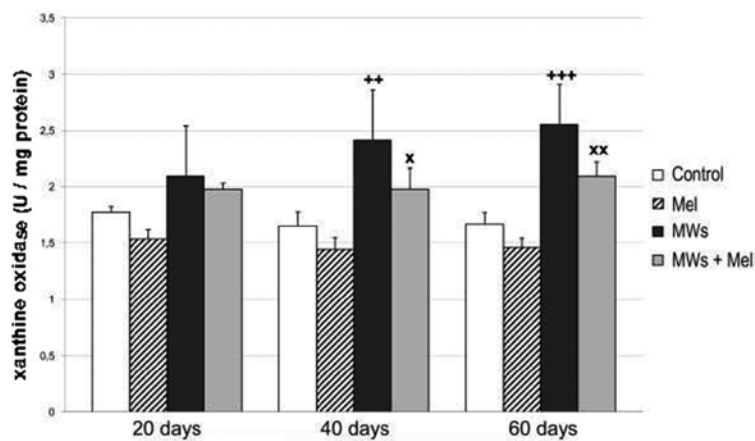


Fig. 4. Xanthine oxidase (XO) activity (U/g protein) in the testicular tissue of rats exposed to MWs.

⁺⁺p < 0.01 (vs. control and Mel);

⁺⁺⁺p < 0.001 (vs. control and Mel);

^xp < 0.05 (vs. control and Mel);

^{xx}p < 0.01 (vs. MWs)

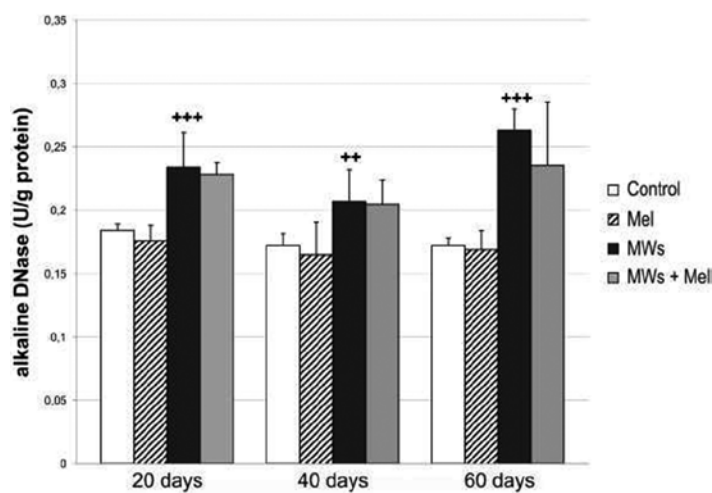


Fig. 5. Alkaline DNase (DNase I) in the testicular tissue of rats exposed to MWs.

⁺⁺p < 0.01 (vs. control and Mel);

⁺⁺⁺p < 0.001 (vs. control and Mel)

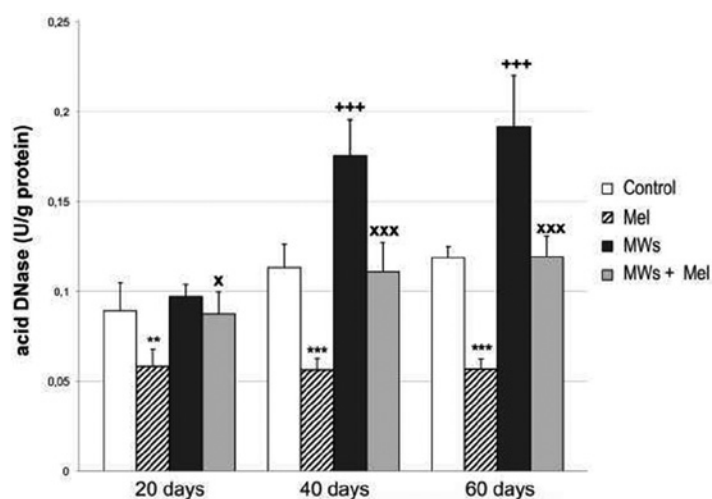


Fig. 6. Acid DNase (DNase II) activity in the testicular tissue of rats exposed to MWs.

⁺⁺⁺p < 0.001 (vs. control and Mel);

^xp < 0.05; ^{xxx}p < 0.001 (vs. MWs);

^{**}p < 0.01 (vs. control); ^{***}p < 0.001 (vs. control)

As shown in Fig. 4, exposure to mobile phone radiation induced a significant increase in xanthine oxidase activity after 40 days ($p < 0.01$) and 60 days ($p < 0.001$) when compared to the control group and the Mel group. This XO activity increase in irradiated animals was prevented by melatonin application after 40 days ($p < 0.05$) and 60 days ($p < 0.01$) (Fig. 4).

Alkaline DNase activity was increased in the testicular tissue of rats exposed to MWs in comparison to the control group and the Mel group

after 20 days ($p < 0.001$), 40 days ($p < 0.01$) and 60 days ($p < 0.001$) (Fig. 5). Melatonin administration to animals exposed to MWs did not influence the activity of alkaline DNase in the testicular tissue, as shown in Fig. 5.

Increased acid DNase activity was found in the testicular tissue of rats exposed to MWs in comparison to the control group and the Mel group after 40 days ($p < 0.001$) and 60 days ($p < 0.001$) (Fig. 6). Melatonin administration to irradiated animals

(the MWs+ Mel group) led to a statistically significant decrease of acid DNase activity when compared to the animals exposed to MWs after 20 days ($p < 0.05$), 40 days ($p < 0.001$) and 60 days ($p < 0.001$) (Fig. 6). Melatonin administration led to a reduction in acid DNase activity when compared to the control group after 20 days ($p < 0.01$), 40 days ($p < 0.001$) and 60 days ($p < 0.001$) (Fig. 6).

Discussion

A number of recent studies have suggested a possible link between cell phone use and male infertility [21, 22]. An *in vitro* pilot study conducted by Agarwal et al. [21] showed significant decreases in sperm motility and viability, increases in ROS levels, and decreases in total antioxidant capacity (TAC) score in sperm specimens exposed to MWs in comparison to control specimens. Khaki et al. [23] reported significant structural changes in rat testis boundary tissue after exposure to MW radiation. Those data suggest the existence of germinal epithelium dysfunction under conditions of exposure to MWs.

Microwaves and Oxidative Stress in Testicular Tissue

The high rates of cell division during spermatogenesis and the metabolic processes within Leydig cells during steroidogenesis demand high mitochondrial activity, due to high energy consumption and, therefore, high levels of oxidative metabolism in the germinal epithelium. The high energy consumption causes high sensitivity to the effects of oxidative stress in this tissue, particularly the germinal cells, due to imbalances between pro-oxidant and anti-oxidant factors within the tissue. However, the poor vascularization of the testes keeps oxygen tensions in this tissue at a very low level, and the competition for this vital element within the testes is extremely intense. The low oxygen tension that characterizes this tissue may be an important component of the mechanisms by which the testes protect themselves from free radical-mediated damage [24]. Despite these characteristics of the testicular micro-environment, the tissue remains vulnerable to oxidative stress due to the presence of highly unsaturated fatty acids and potent ROS generating systems [24, 25].

The majority of ROS molecules are detoxified very quickly by mitochondrial and cellular antioxidant defenses. However, MWs can cause disturbances of the sensitive equilibrium between pro-oxidant and anti-oxidant factors within cells and therefore cause oxidative stress-related cell injury and apoptosis [4, 8–11].

The results obtained in the present study indicate significant increases in oxidative stress intensity in the testicular tissues of rats exposed to MW rats. Significant increases in lipid peroxidation, quantified as the MDA level, was first noted after 20 days of exposure to MW radiation (Fig. 1). Kumar et al. [11] also reported MDA increases in the testicular tissue of rats exposed to MWs.

In the present study, increases in protein carbonyl content occurred after 40 days of exposure to MWs (Fig. 2). This indicates that it follows the initial increase of ROS molecules and MDA levels. As a result of oxidative modification, proteins are being degraded by the proteasome complex, due to conformational changes and impaired function [26].

Catalase is considered an anti-oxidative enzyme. Both the sperm and testes of rats contain little catalase activity [27], therefore they are very sensitive to oxidative stress-related injury. The results of the current study show that MW exposure led to significant decreases in catalase activity (Fig. 3). Ozguner et al. [28, 29] also reported reduced catalase activity caused by exposure to 900 MHz microwaves in the retina and kidney. However, Kesari and Behari [11] recently reported increased catalase activity in testicular tissue after 35 days of exposure to MWs. Yet, in this case, enzyme activity might depend on the specific energy absorption rate (SAR), the duration of exposure (4 h/day in the current experiment vs. 2 h/day in the Kesari and Behari study) and the experimental set up.

The current study also showed significant increases in xanthine oxidase (XO) activity due to exposure to MWs (Fig. 4). XO is traditionally considered a pro-oxidative enzyme. XO has previously been reported to be increased in brain tissue after seven days of exposure to a 900 MHz electromagnetic field [5]. In an earlier publication, the current authors also reported an XO increase in brain tissue after 40 and 60 days of exposure to MWs [7]. As far as the authors are aware, changes in XO activity in testicular tissue exposed to MWs have not previously been reported.

Due to both decreases in catalase activity and increases in XO activity in the testes, a weak spot within the cellular anti-oxidative defense mechanism has been created, making the cells more vulnerable to oxidative injury.

The Role of Melatonin in Protecting Against Oxidative Damage Caused by MW Exposure

Melatonin acts as a potent anti-oxidant and therefore contributes to protection against oxidative stress and apoptosis. In the present study,

treating animals exposed to MWs with melatonin led to significant decreases in lipid peroxidation (Fig. 1) and xanthine oxidase activity (Fig. 4) in the testicular tissue. Ozguner et al. [28] reported similar findings regarding melatonin's effect on lipid peroxidation (MDA levels) and catalase activity in renal tissue exposed to MWs. In the present study, melatonin caused significant decreases in XO activity after 40 days of exposure to MWs; at the same time when significant increase of XO activity in irradiated group was observed. Therefore, we propose that protective effects of melatonin are probably partially caused by its effect on XO activity especially after prolonged exposition to MWs.

Microwaves and DNase Activity in Tests – the Role of Melatonin

In the present study, alkaline and acid DNase activity in the testicular tissue was significantly elevated by exposure to MWs (Fig. 5, 6). According to other authors, alkaline and acid DNase are found to increase in a wide range of apoptosis-related disorders, such as malignant and benign tumors or inflammatory diseases [30]. The current authors assume that this elevation is caused by post-apoptotic DNA destruction, which follows apoptosis in tissue exposed to MWs. Therefore, apoptosis levels must also be elevated due to MW exposure. De Iuliis et al. [9] reported increased

DNA fragmentation and oxidative DNA damage bio-marker 8-OH-dG in isolated human spermatozoa exposed to MWs. The percentage of apoptosis among sperm cells has also been reported to be increased in rats exposed to MWs [11], which is consistent with the results of the present study.

In the current study, administering melatonin caused a decrease in alkaline DNase activity, but not to a significant degree; acid DNase activity, however, was significantly reduced (Fig. 5, 6), which suggests that the use of melatonin reduces apoptosis and allows normal spermatogenesis even in cases of MW-induced testicular tissue injury.

According to the results of the present study, melatonin caused a significant decrease in oxidative stress intensity, reflected in the decrease in MDA levels. In addition to this, melatonin caused a reversal of the effects of MW radiation on XO activity in the testicular tissue, lowering XO-induced oxidative stress. On the other hand, melatonin showed no significant ability to reverse the effects of MW exposure on catalase levels in testicular tissue. However, melatonin caused a significant decrease in acid DNase activity, reflecting reduced amounts of apoptosis in the testicular tissue exposed to MWs. In conclusion, melatonin exerts potent protective effects in the testes of rats exposed to microwaves by decreasing oxidative stress intensity and DNA fragmentation.

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