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Original Investigation

# Melatonin Modulates NMDA-Receptor 2B/Calpain-1/ Caspase-12 Pathways in Rat Brain After Long Time Exposure to GSM Radiation

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## ABSTRACT

**AIM:** To investigate the potential protective effects of melatonin on the chronic radiation emitted by third generation mobile phones on the brain.

**MATERIAL and METHODS:** A total of 24 male Wistar albino rats were divided into four equal groups. Throughout a 90-day experiment, no application was performed on the control group. The second group was exposed to 2100 MHz radiation for 30 minutes. Subcutaneous melatonin was injected into the third group. Subcutaneous melatonin injection was applied 40 minutes before radiation and then the fourth group was exposed to radiation for 30 minutes. At the end of the experiment, brain (cerebrum and cerebellum) tissues were taken from the subjects. Histochemical, immunohistochemical, ultrastructural and western blot analyses were applied. In addition to brain weight, Purkinje cells' number, immunohistochemical H Score analyses and the results of the Western blot were examined statistically.

**RESULTS:** With the application of radiation, neuronal edema, relatively-decreased numbers of neurons on hippocampal CA1 and CA3 regions, displacement of the Purkinje neurons and dark neurons findings were observed as a result of histochemical stainings. Radiation also activated the NMDA-receptor 2B/Calpain-1/Caspase-12 pathway, NMDA-receptor 2B and Calpain-1 with the findings being supported by western blot analyses. Pre-increased protein synthesis before apoptosis was identified by electron microscopy.

**CONCLUSION:** Mobile phone radiation caused certain (ultra) structural changes on the brain and activated the NMDA-receptor 2B/ Calpain-1/Caspase-12 pathway; in addition, melatonin was found to be effective, but insufficient in demonstrating the protective effects.

**KEYWORDS:** Radiation, Melatonin, Brain, NMDA-Receptor 2B, Calpain-1, Rat

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## INTRODUCTION

During the last century, the rate of exposure to electromagnetic radiation (EMR) has increased unexpectedly (12). Extremely low frequency microwaves from power lines and public transportation systems, high frequency microwaves from mobile phones, base stations, microwave ovens, satellites, radio/television/computers and strong frequency microwaves from magnetic resonance imaging in medical diagnosis are the most common sources of electromagnetic fields (EMFs) today (17,19).

It is a known fact that EMFs constitute a danger to public health (30). The biological effects of EMR can generally be grouped as thermal and non-thermal (30), and it shows these effects on tissues and their physiological effects (42). There is a possible link between EMF exposure and various types of cancer, such as brain, breast and kidney cancer, and other disorders, such as cardiovascular diseases and leukemia according to epidemiological research; EMFs show their characteristic biological effects on the central nervous, endocrine, immune and reproduction systems (5).

Mobile phones are one of the most important sources of EMFs and their use is increasing day by day (5,24). The radiofrequency (RF) range of emitted EMFs from mobile phones reaches 1.9-2.1 GHz through third generation (3G) mobile phones (33). The rapid increase in the use of mobile phones over the past few years has led to an increase in potential health problems (31). Behavioral changes in children, learning, memory and sleep problems, the development of brain tumors, cardiovascular diseases, inner ear damage and reduced sperm quality associated with male infertility are health problems caused by exposure to EMR from mobile phones; especially with long-term use (30).

It is generally accepted that emitted radiation from mobile phones is absorbed by the brain because of use close to the head (18,42). According to a number of studies, emitted radiation between the range of 800 and 1000 MHz can penetrate the cranium and reach about 40% of the deep brain (4-5 cm) (18). In addition, these studies have shown that emitted EMR from mobile phones shows its effects on the brain in various ways (24), such as cerebral blood flow, breakdown of blood-brain barrier permeability, imbalance between oxidant-antioxidant systems and neurotransmitter oscillation, nerve cell damage, DNA mutations, tumor development, behavioural changes, and changes in gene expression through cortex, hippocampus and cerebellum (7,23,24). Radiofrequency (RF) radiation also affects intracellular signaling pathways through changes in calcium ($Ca^{2+}$) permeability across cell membranes. Changes in $Ca^{2+}$ regulation cause tumorigenesis, neural degeneration and cognitive deficits in the brain (23). Neuronal damage due to EMR is especially seen in the cortex, hippocampus and basal ganglia according to the animal studies (29).

The nervous system is vulnerable to reactive oxygen species (ROS) in particular, because of its high metabolic rate, so the possible generation of oxidative stress and ROS cause the initation or progression of neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (5). The emitted EMR from mobile phones causes an increase in neurodegenerative diseases, including Alzheimer's disease (17). It has also been reported that EMR has a negative impact on learning and memory (19,30).

Glutamate is the major excitatory neurotransmitter of the central nervous system and as the cellular substrate for neuronal development, neurotoxicity, and neuronal plasticity, is also related to learning and memory (32). It is also the potential origin of neurotoxicity depending on its abnormal reduction or excessive increase (11). Among the glutamate receptors, the N-methyl D-aspartate receptor (NMDA-R) plays an essential role in learning/memory, and also in dementia-origin diseases, such as Alzheimer's disease (32). These types of receptors locate especially in the hippocampus, thalamus and also the cortex of the cerebrum/cerebellum (11). NMDA-R stimulation causes an increase in the intracellular $Ca^{2+}$ concentration (43).

Calpains determining the fate of proteins have also been linked to the modulation of memory and are calcium-dependent enzymes (48), becoming overactive when connected to rising cytosolic $Ca^{2+}$ levels (50). Activation of calpain is an accepted assumption for the endoplasmic-reticulum (ER) stress-mediated Alzheimer's pathogenesis (50), and as a result of activation of caspase-12 mediated caspase-3 activation occurs causing neuronal apoptosis (22,49).

Antioxidant treatments can be helpful in preventing or reducing some radiation damage (41). The chief secretory product of the pineal gland, melatonin (N-acetyl-5-methoxytryptamine), illustrates nerve protective effects through many modelling central nervous system diseases (6). Besides its therapeutic effect of detoxifying free radicals, melatonin has been shown to modulate apoptosis caused by EMF-induced oxidative stress through cation channels such as the voltage-gated $Ca^{2+}$ channels in neurons (20), and melatonin also inhibits either glutamate or NMDA-induced excitation (8). In addition, it attenuates calpain upregulation and caspase-3 activation decreasing neuronal death (37).

In our study, we aim to investigate the efficacy of melatonin against potential damage that would appear in brain (cerebrum and cerebellum) tissue due to the administration of long-term exposure to 2100-MHz GSM radiation by assessing Hematoxylin-Eosine/Cresyl violet histochemically, NMDA-Receptor 2B/Calpain-1/Caspase-12 signalling molecules immunohistochemically and with western blotting analyses (for NMDA-receptor 2B and Calpain-1), and also through transmission electron microscope (TEM) ultrastructurally.

## MATERIAL and METHODS

Animal experiments were premeditated and executed in accordance with ethical norms approved by the Institutional Animal Ethics Committee Guidelines and ethical approval was obtained by G.U. ET-12.002 numbered decision.

### Radiation Exposure

A vector signal generator (Rohde & Schwartz, SMBV100A, Germany) was used to create radio-frequency (RF) radiation in all of the experimental sessions. Vector signal generator

was used to stimulate the emission of mobile phones. The frequency, modulation and power exposed to animals were similar with the radiation from mobile phones. An ETS Lindgren horn antenna (ETS Lindgren, Model 3164-03, USA) was used to emit RF radiation from the vector generator. The rats were housed in a Polymethyl methacrylate plastic cage (15x20x20 cm) placed symmetrically along the perpendicular axis, 11 cm below the mid-line of the horn antenna. Exposure performed approximately 11 cm away from antenna and because of this exposure could be defined as near field exposure and we could say that situation is similar with our exposure from mobile phones. The cage was aerated to avoid the possibility of any increase in temperature inside the cage during the all experimental durations. A cage was placed in the near field of the antenna to obtain sufficient field intensity. Applied RF radiation was measured with an EMR 300 (Narda, Germany) with an electric field probe type 8.3. For RF fields, the root mean square value of electric field ($E_{RMS}$) was found to be 17.25 V/m. The RF environmental background level was around 0.21 V/m. The SAR was calculated using the following equation: SAR = $\sigma/\rho[E_{RMS}^2]$ [W/kg] where (9) $E_{RMS}$ is the root mean square value of the electric field (V/m), σ is the mean electrical conductivity of the tissues in Siemens/meter (S/m) and ρ is the mass density (kg/m$^3$) (15,16). Conductivity (0.87 S/m) and mass density (1105 kg/m$^3$) were derived for the equivalent tissue using dielectric properties and the mass densities of these tissues. RF exposure in the experiment resulted in a whole body average SAR of 0.23 W/kg with an $E_{RMS}$ field of 17.25 V/m. This SAR value could be accepted as a non-thermal SAR level for RF radiation, because it is known that 4 W/kg SAR leads to a 1°C increase in the temperature of the exposed tissue. The body temperature of the rats was recorded by rectal measurements prior to and after exposure sessions. Neither exposure resulted in any rectal temperature increase.

### Chemicals

Melatonin (N-acetyl-5-methoxytryptamine) (98.0% purity, Lot: #011M1321V) was purchased from Sigma-Aldrich (St. Louis, USA). Melatonin (100mg) was dissolved in 10% pure ethanol (pH=7.4). All other chemicals were of analytical grade and were obtained from standard commercial suppliers.

### Animals

Twenty four 200-250 gr weight Wistar Albino male rats (Gazi Universty Medical School Experimental Animal Breeding and Experimental Research Center, Ankara, Turkey) were used in this research. They were housed in clean, sterile, polypropylene cages under standard vivarium conditions (12 hours light/dark cycle) with access to water and standard rat chow (Korkutelim Yem Ltd., Antalya, Turkey). The animals were housed six per cage in an air-conditioned animal room at 22 ± 3°C and 55 ± 10% humidity. The animals were acclimatized to laboratory conditions for four weeks prior to the commencement of the experiments.

### Experimental Design

Twenty four male Wistar albino rats were divided into four equal groups. Throughout the 90-day experiment, no application was performed on the control group. The second group was exposed to 2100 MHz radiation for 30 minutes/each day for 90 days. Subcutaneous melatonin (10 mg/kg/day) was injected into the third group each day for 90 days. Subcutaneous melatonin injection was applied 40 minutes before radiation and then the fourth group was exposed to radiation for 30 minutes each day for 90 days. All applications were conducted at 4.00 pm. The dose and application time of melatonin was performed in accordance wtih the study by Akbulut et al. (1). At the end of the 90-day experimental period, tissue samples were collected under ketamine (45 mg/kg) and xylazine (5 mg/kg) anesthesia. Brain (cerebrum and cerebellum) tissues from the control and experimental groups of rats were taken immediately, washed briefly in physiological saline with some stored at -80°C and some placed in neutral formaldehyde and glutaraldehyde for fixation.

### Histomorphological Analysis

Hematoxylin-Eosin and Cresyl violet stainings were performed on 4 µm thick sections. Slides were examined with a Photo-light microscope (DM4000B Image Analyze System, Leica, Microsystems, Heidelberg GmbH, Heidelberg, Germany) and a Leica DFC280 plus camera.

### Immunohistochemical Analysis

Brain tissue samples obtained from the study groups were fixed in 10% neutral formaldehyde for about 72 hours. They were dehydrated in an increasing series of ethanol, and parafin embedded for conventional histological diagnosis. Cross sections (4 µm) were mounted on polylysin-coated slides (Menzer-Glaser, Braunschweig, Germany), deparaffinized with xylene and rehydrated. The slides were kept in a microwave oven in EDTA Buffer (LabVision, Fremont, CA, USA) (pH 10.0) for heat-induced antigen retrieval through microwave irradiation, so as to increase the sensitivity of immunohistochemical detection. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (Fisher Scientific, Melrose Park, IL, USA) (diluted with phosphate buffered saline [PBS]) for 15 minutes and washed three times in PBS solution. The epitopes were stabilized by application of serum blocking solution (Cat: 859043, Lot: 1598424A, Invitrogen) for 15 minutes. The slides were incubated with primary antibodies of NMDA-Receptor 2B (Mouse Monoclonal, ab93610, Lot-GR36240-24, Abcam) (diluted with PBS at a rate of 1/300), Calpain-I (Rabbit Monoclonal, ab92356, Lot-YIO42105C, Abcam) (diluted with PBS at a rate of 1/150) and Caspase-12 (Rabbit Polyclonal, ab62484, Lot-GR1434018, Abcam) (diluted with PBS at a rate of 1/500) for 90 minutes at room temperature. The slides were washed three times in PBS solution. After this, the biotinylated secondary antibody (Cat: 859043, Lot: 1598424A, Invitrogen) was applied for 10 minutes. The slides were again washed three times in PBS solution. Thereafter, streptavidin peroxidase (Cat: 859043, Lot: 1598424A, Invitrogen) was applied to the slides for 10 minutes, after washing with PBS, DAB (Cat: 00-2020, Lot: 1454233A, Invitrogen) was used as a chromogen. Afterwards, all the slides were counterstained with Harris's hematoxylin. The slides were then examined with the Photo-light microscope (DM4000B Image Analyze System, Leica, Germany) and the Leica DFC280 plus camera.

The number of immune positive cells were measured manually using a QWin software programme in consecutive areas for serial cutaways taken. The following semi-quantitative scoring system was used to assess the immunolabeling intensity: (0) no staining; (1) weak; (2) moderate to weak; (3) moderate; (4) moderate to strong; and (5) strong labeling. Two independent observers, who were blind to the treatment protocol, performed the immunolabeling score evaluations independently. The HSCORE was calculated using the following equation: HSCORE= ∑Pi (i+1); where i is the intensity of NMDA-Receptor 2B; Calpain-I and Caspase-12 labeled with values of 0,1, 2, 3, 4, 5; and Pi being the percentage of labeled cells for each intensity, varying from 0% to 100%. The results are expressed as the mean ± SD.

**Western Blot Analysis**

Brain tissue samples (cerebrum and cerebellum) were washed three times with PBS and then lysed with RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.25% Na-deoxycholate, 2% Triton-X100, 1 mM PMSF, 2 µM leupeptin). The protein concentration of each lysate was measured by a BCA protein assay Kit (Pierce, Rockford, Illinois, USA). Western blot analysis was performed using a modified version of our previous protocols (34). In brief, equal amounts of protein (20 µg per lane) from each sample were denaturated, electrophoresed, and transferred onto nitrocellulose membranes (Millipore, Bedford, Massachusetts, USA), then the mebranes were blocked with 5% bovine serum albumin in Tris-buffered saline Tween-20 (TBST; 0.05% (w/v) Tween 20, 150 mm sodium chloride, 20 mm Tris-HCl, pH 7.5) solution for one hour at room temperature. The blots were incubated with specific primary antibodies against NMDA-Receptor 2B (Mouse Monoclonal, ab93610, Lot: GR36240-24, Abcam) (diluted at a rate of 1/1000), Calpain-I (Rabbit Monoclonal, ab92356, Lot: YIO42105C, Abcam) (diluted at a rate of 1/1000) and Beta actin (ACTB, CST #4970; 1:1000) (Cell Signalling Technology, Danvers, MA, USA) overnight at 4 °C. The membranes were then incubated with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (CST, #7074; 1:2000) (Cell Signalling Technology, Danvers, MA, USA) for one hour at room temperature. The expression levels of the target proteins were normalized against a loading control, ACTB. All the blots were visualized and captured by a Kodak Gel Logic 2200 PRO Imaging System (Carestream, Health, Rochester, NY, USA). Protein band intensities were calculated using Image J 1.50i Software (NIH, USA), relative to ACTB.

**Transmission Electron Microscopy Analysis**

A portion of cerebrum and cerebellum (about 1 $mm^3$) from the control and experimental groups of rats were fixed in 4% glutaraldehyde in 200 mM sodium phosphate buffer (pH 7.4) for three hours at 4°C. Tissue samples were washed with the same buffer, post-fixed in 1% osmium tetroxide and 200 mM sodium phosphate buffer (pH 7.4) for one hour at 4°C. The samples were again washed with the same buffer for three hours at 4°C, dehydrated with graded series of ethanol and embedded in Araldite. Thin sections were cut with Leica EMUC7 ultramicrotome using a diamond knife (Leica EMUC7, Hernalser, Germany), mounted on a copper grid and stained with 2% uranyl acetate and lead citrate. The grids were examined under a Carl Zeiss EVO LS 10 TEM-SEM microscope (Germany).

**Statistical Analysis**

In addition to brain weight, the number of Purkinje cells, immunohistochemical H Score analyses and the results of the western blot were examined statistically. Data analysis was performed using a Statistical Package for Social Sciences (SPSS) version 19.0 software (SPSS Inc., Chicago, IL, USA). The results are shown as mean and standard deviation. The groups were compared using Kruskal-Wallis and ANOVA tests. A value of $p<0.001$, $p<0.01$, $p<0.05$ was considered to be statistically significant.

Statistical analysis of the western blot study was performed using the One-way analysis of variance (ANOVA) with a Bonferroni post hoc test or Student's t-test, using SigmaStat v 3.5 software (SYSTAT Software Inc, USA). The results are expressed as mean values ± SEM and p values <0.05 are considered significant values.

## RESULTS

**Histomorphological Analysis**

*Hematoxylin-Eosin Staining Results*

The histological appearance of the cerebrum prefrontal cortex, cerebrum hippocampal CA1 (Cornu Ammonis 1) and CA3 (Cornu Ammonis 3) regions and cerebellum tissues were seen in normal structure. In the radiation group, certain regions of the prefrontal cortex were similar to the control group, although some pyramidal neurons were seen in a dark colour and with edema in some regions. The number of neurons on the hippocampal CA1 and CA3 regions were relative-decreased and these neurons did not show regular placement. In addition, neuronal edema and dark neurons, especially in the CA3 region, were evident. Displacement (the organisation of Purkinje neurons changed toward the granular layer) and a decreased number of the Purkinje neurons were seen in the cerebellum tissue. In the radiation group, the number of Purkinje neurons was found to be lower statistically compared to the control group and the melatonin groups ($p<0.05$) (Figure 1). In the melatonin group, the general appearance of the cerebrum and cerebellum was the same as the control group. In the radiation and melatonin group, the number of dark neurons were relative-decreased. The general structure of the prefrontal cortex and the CA3 regions were observed to be similar to the radiation group, but the appearance of the CA1 region and cerebellum were observed to be similar to the control group (Figure 2, 3). In the radiation and melatonin group, the number of Purkinje neurons was found to be statistically higher compared to the radiation group ($p<0.05$) (Figure 1).

No significiant differences were recognized statistically in the weights of the cerebrum and cerebellum through all the groups ($p>0.05$ and $p>0.05$) (Figure 1).

*Cresyl Violet Staining Results*

Dark neurons were noticeably distinguishable in the radiation

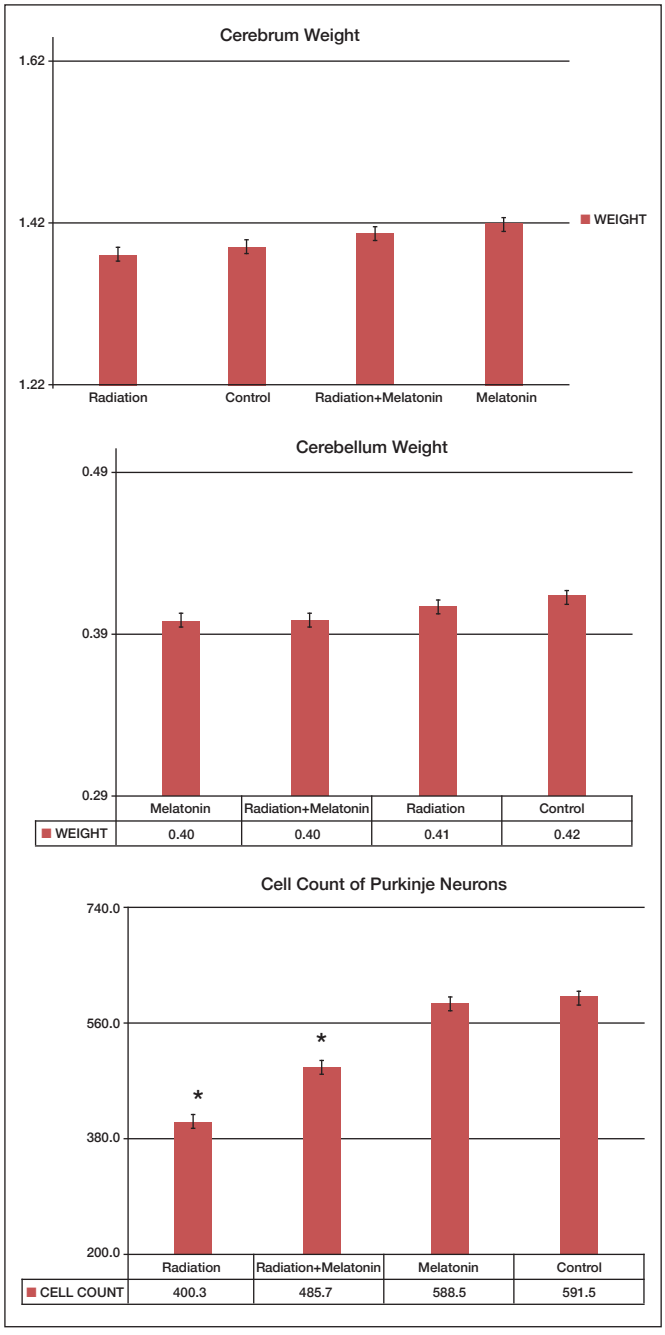


**Figure 1:** The descriptive statistical graphics with the error bars of cerebrum/cerebellum weights and cell count of Purkinje neurons results for all groups. Statistically significant groups (*).

group compared with the control and melatonin groups in the cerebrum prefrontal cortex, hippocampal CA1 and CA3 regions and in cerebellum tissues. It was seen that the number of dark neurons were relatively decreased in the radiation and melatonin group.

According to literature reviews it was thought that dark neurons in the radiation group associated with albumin extravasation because radiation exposure causes deterioration in the Blood Brain Barrier (BBB) (Figure 4).

### Immunohistochemical Analysis

*NMDA-Receptor 2B Primary Antibody Immunstaining Results*

Weak immunreactivity was seen in the control and melatonin groups for three regions of the cerebrum and the cerebellum. Wide cytoplasmic immunreactivity was recognized in the radiation group. In this group, the density of NMDA-Receptor 2B immunreactivity was found to be higher statistically compared to the control group and the melatonin groups for the cerebrum and cerebellum ($p<0.05$). Additionally, in the radiation and melatonin group it was noted that the immunreactivity was less than for the radiation group for the cerebrum and cerebellum. This finding is supported statistically ($p<0.05$) (Figures 5, 6).

*Calpain-1 Primary Antibody Immunstaining Results*

Weak cytoplasmic immunreactivity was seen in the control and melatonin groups for three regions of the cerebrum and also for the cerebellum. In the radiation group strong cytoplasmic immunreactivity was recognized in the cerebrum prefrontal cortex and the cerebellum, although strong nuclear and perinuclear involvement were seen in the CA1 and CA3 regions. In this group, the density of Calpain-1 immunreactivity was found to be higher statistically compared to the control and melatonin groups for the cerebrum and cerebellum ($p<0.05$). In the radiation and melatonin group it was observed that immunreactivity was less than for the radiation group for the cerebrum and the cerebellum. This finding is supported statistically ($p<0.05$) (Figures 5, 7).

*Caspase-12 Primary Antibody Immunstaining Results*

In certain neurons, weak cytoplasmic immunreactivity was seen in the control and melatonin groups for three regions of the cerebrum and also for Purkinje neurons of the cerebellum. In the radiation group, strong cytoplasmic and for some cells strong nuclear immunreactivity were recognized in the cerebrum prefrontal cortex, hippocampus CA1 and Purkinje neurons of the cerebellum. In addition to these findings, a number of neurons showed no immunreactivity in the hippocampal CA3 region. In this group, the density of Caspase-12 immunreactivity was found to be statistically higher compared to the control and melatonin groups for the cerebrum and the cerebellum ($p<0.05$). In the radiation and melatonin group, most cells showed no immunreactivity through the prefrontal cortex and Purkinje neurons, although weak to moderate cytoplasmic and nuclear immunreactivity were seen in some neurons. In this group, less immunreactivity was recognized compared to the radiation group in the hippocampal CA1 and CA3 regions. In addition, the density of Caspase-12 immunreactivity was found to be statistically lower than for the radiation group for the cerebrum and the cerebellum in this group ($p<0.05$) (Figures 5, 8).

### Western Blot Analysis Results

The effects of chronic radiation emitted by third generation mobile phones and melatonin used for protective purposes on the cerebrum and the cerebellum were determined using Western blot analysis. When compared with the control group, radiation exposure induced approximately a 1.5-fold to 2-fold

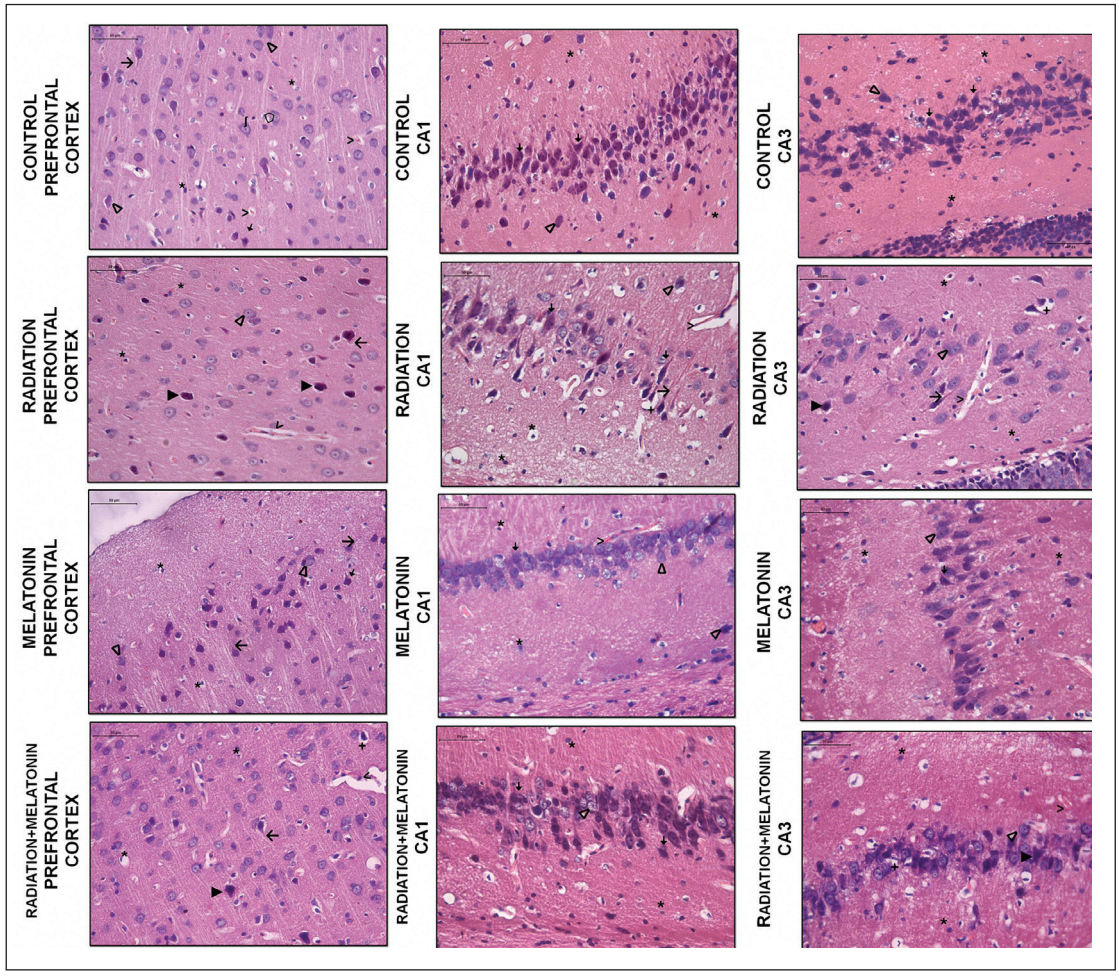


**Figure 2:** Neurons (**Δ**), nerve cell extensions (→), neuroglia cells ( ∗ ), dense staining neurons (↓), blood vessels (>), euchromatic nucleus (⌂), nucleolus ( ∫ ), dark neurons ( ► ) and neuronal edema (+) structures were seen for all groups on cerebrum sections (Hematoxylin-Eosin x400).

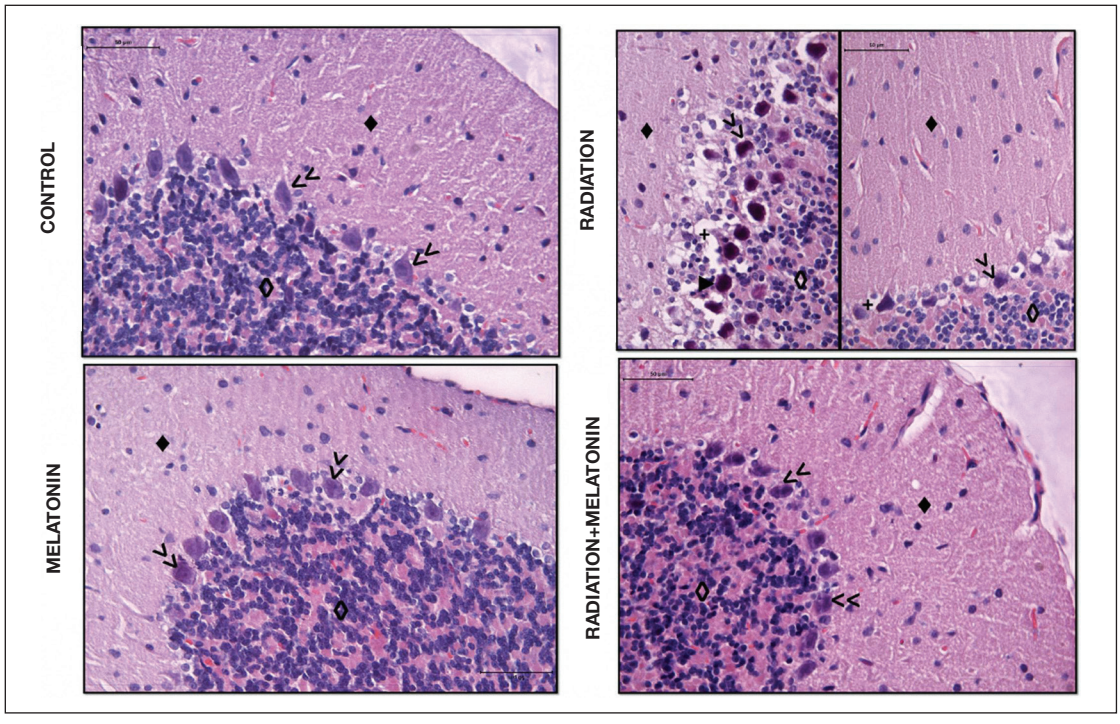


**Figure 3:** Molecular layer (♦), granular layer (◊) and Purkinje neurons (>>) structures were seen for all groups on cerebellum sections (Hematoxylin-Eosin x400).

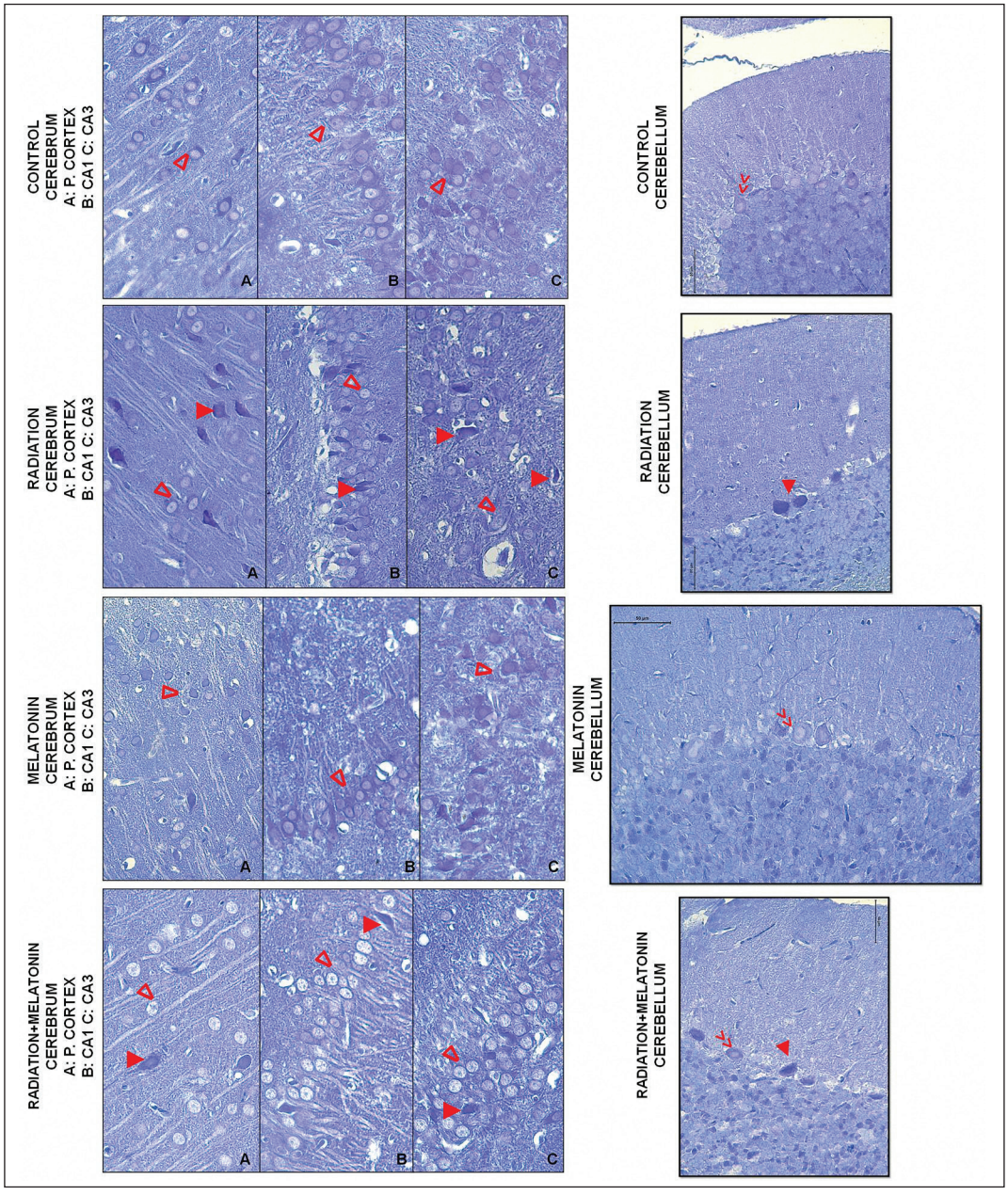


**Figure 4:** Neurons (**Δ**), Purkinje neurons (>>) and dark neurons (►) structures were seen for all groups on brain sections (Cresyl violet x400).

increase in NMDA-Receptor 2B levels in the cerebrum and the cerebellum, respectively ($p<0.05$). While Calpain-1 levels were increased 2.3-fold in the radiation group in the cerebrum, this increase was about 3.8-fold in the cerebellum, compared to the control group ($p<0.05$). On the other hand, Calpain-1 and NMDAR-2B levels were significantly downregulated in the radiation and melatonin group in both the cerebrum and the cerebellum, compared with the radiation group ($p<0.05$). Downregulation of Calpain-1 levels, with the radiation and melatonin group being markedly significiant (2.6-fold) in the cerebellum, compared to the radiation group (Figures 9, 10).

### Transmission Electron Microscopy (TEM) Analysis Results

The ultrastructural appearance was normal with the nucleus and nucleolus, free ribosomes, rough (granular) endoplasmic reticulum tubules, mitochondrion and myelinated nerve fibers in the surrounding tissue in the prefrontal cortical neurons and Purkinje neurons through the control group. In the radiation group, the nucleus and cytoplasm were seen to be dense in shape in some of the prefrontal cortical neurons additional to the normal appearance of neurons. Separation of myelinated nerve fibers in the surrounding tissue were distinguished for these cells. In this group, the nucleus of the Purkinje neurons was seen to be undulating in shape with unclear nucleolus, as well having as shrinkage mitochondrion. In the melatonin group, the cytoplasm of the prefrontal cortical neurons and Purkinje neurons were seen to be dense in shape compared to the control group. In addition, it was noted that rough endoplasmic reticulum tubules dilated, the number of primary lisosomes increased, the structure of myelinated nerve

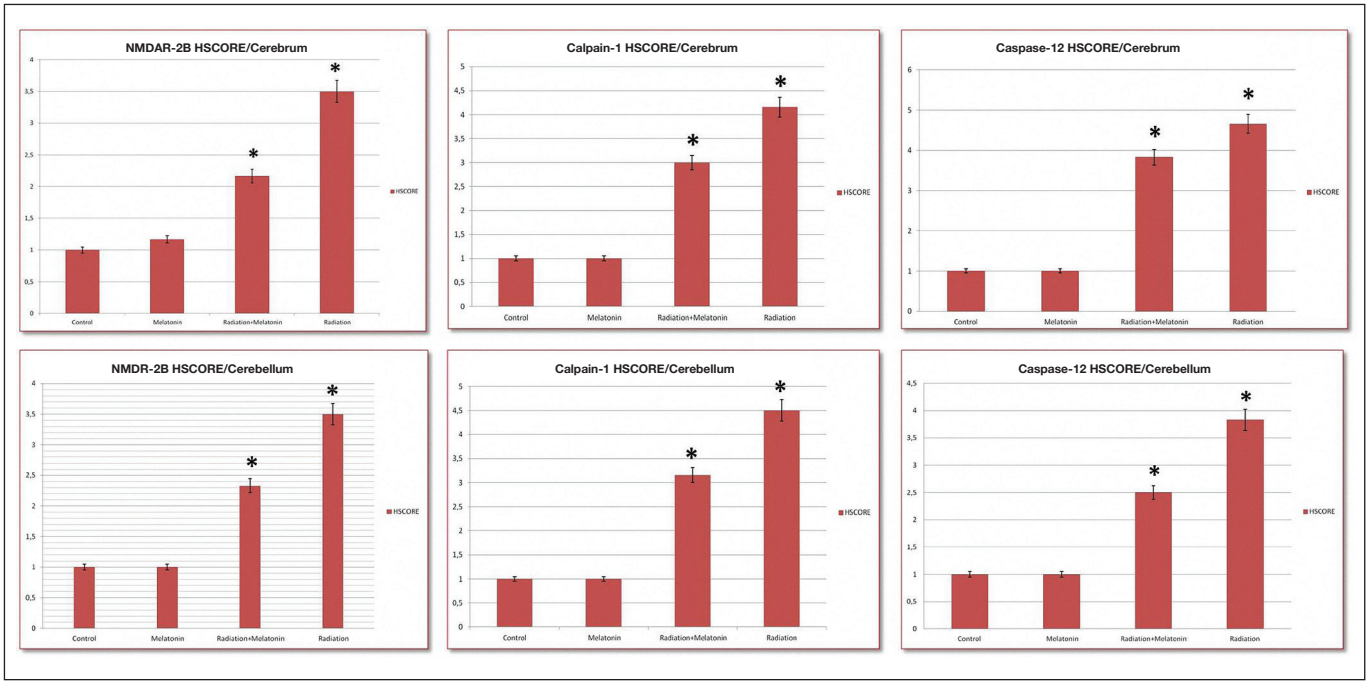


**Figure 5:** The descriptive statistical graphics with the error bars of cerebrum/cerebellum HSCOREs for NMDA-Receptor 2B, Calpain-1 and Caspase-12 immunstainings results in all groups. Statistically significant groups (*).

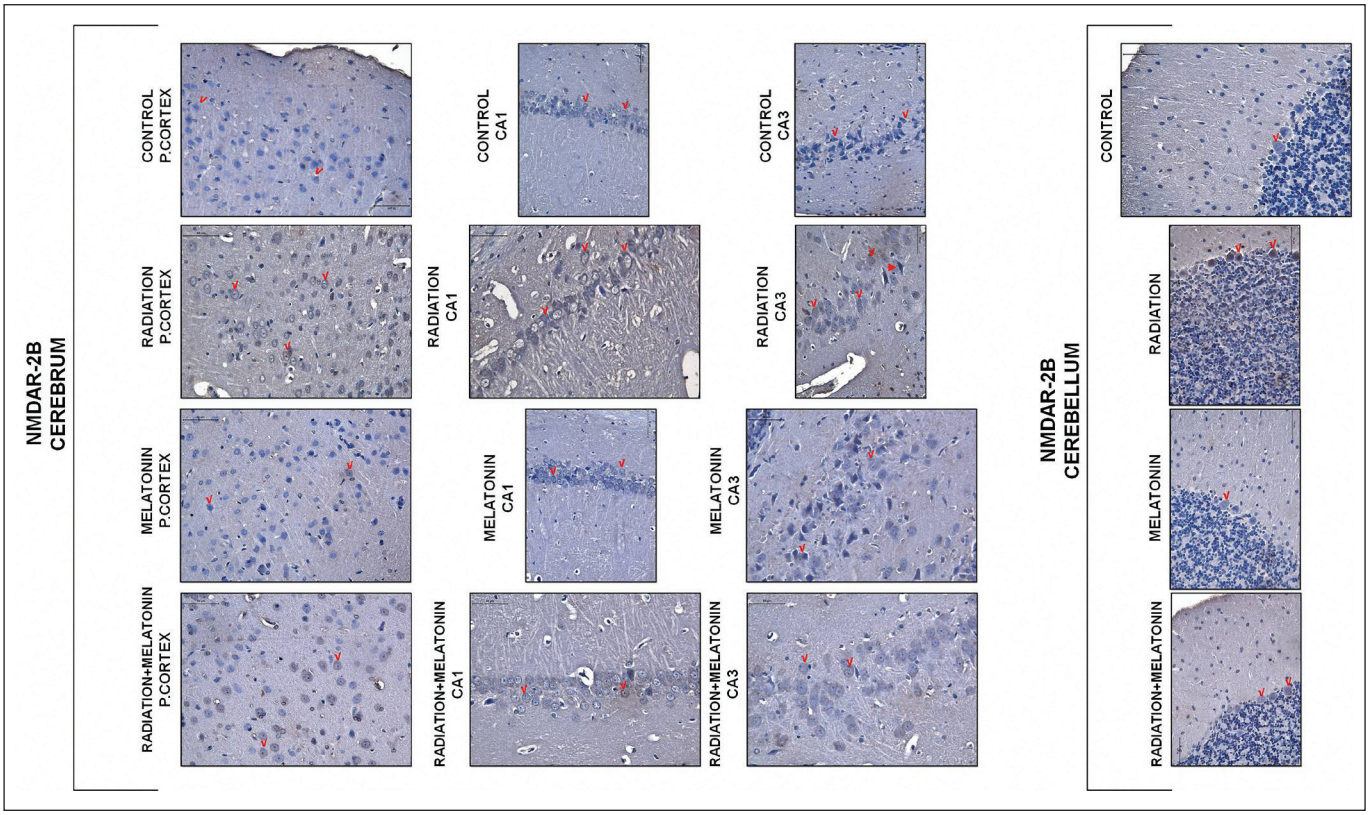


**Figure 6:** Neurons with NMDA-Receptor 2B immunreactivity (√) and dark neurons (►) were seen for all groups on brain sections (NMDA-Receptor 2B Primary Antibody x400).

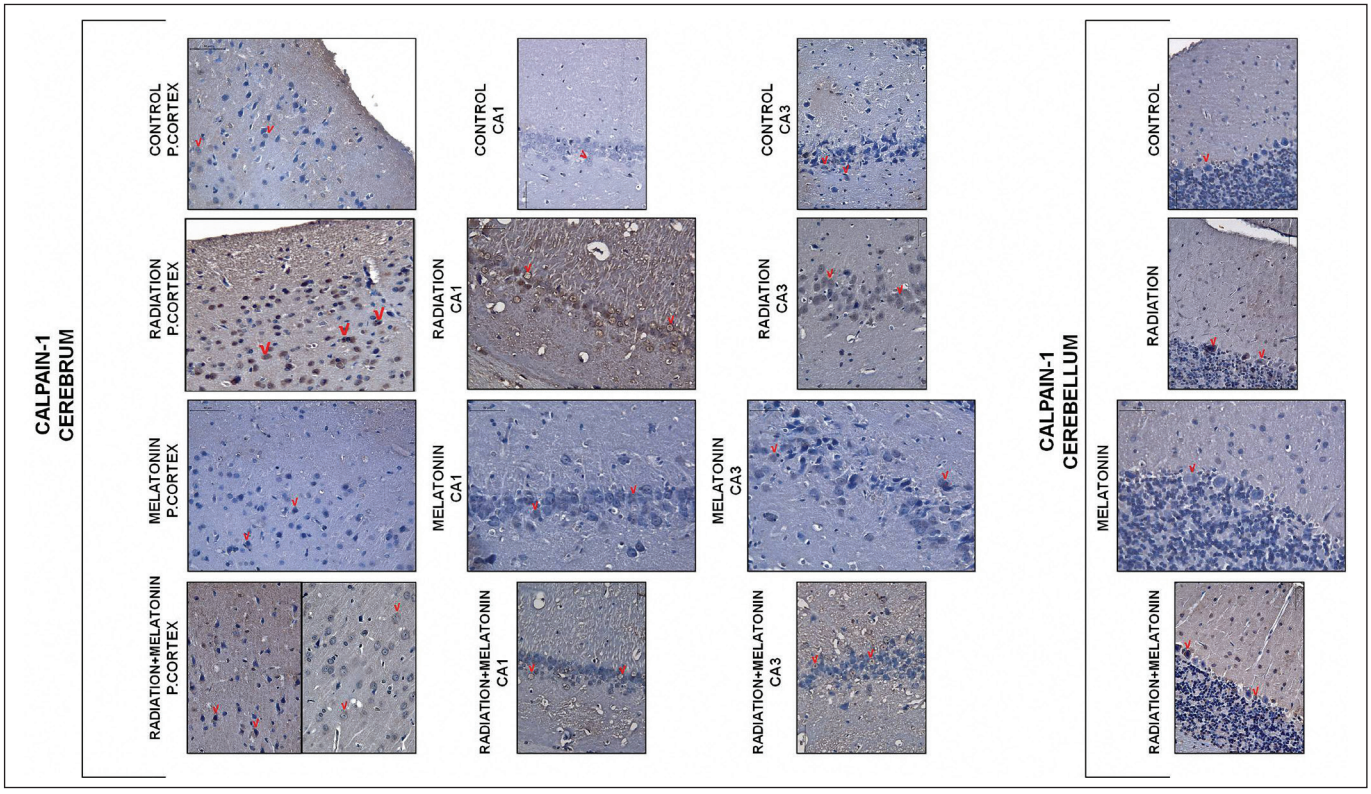


**Figure 7:** Neurons with Calpain-1 immunreactivity (√) were seen for all groups on brain sections (Calpain-1 Primary Antibody x400).

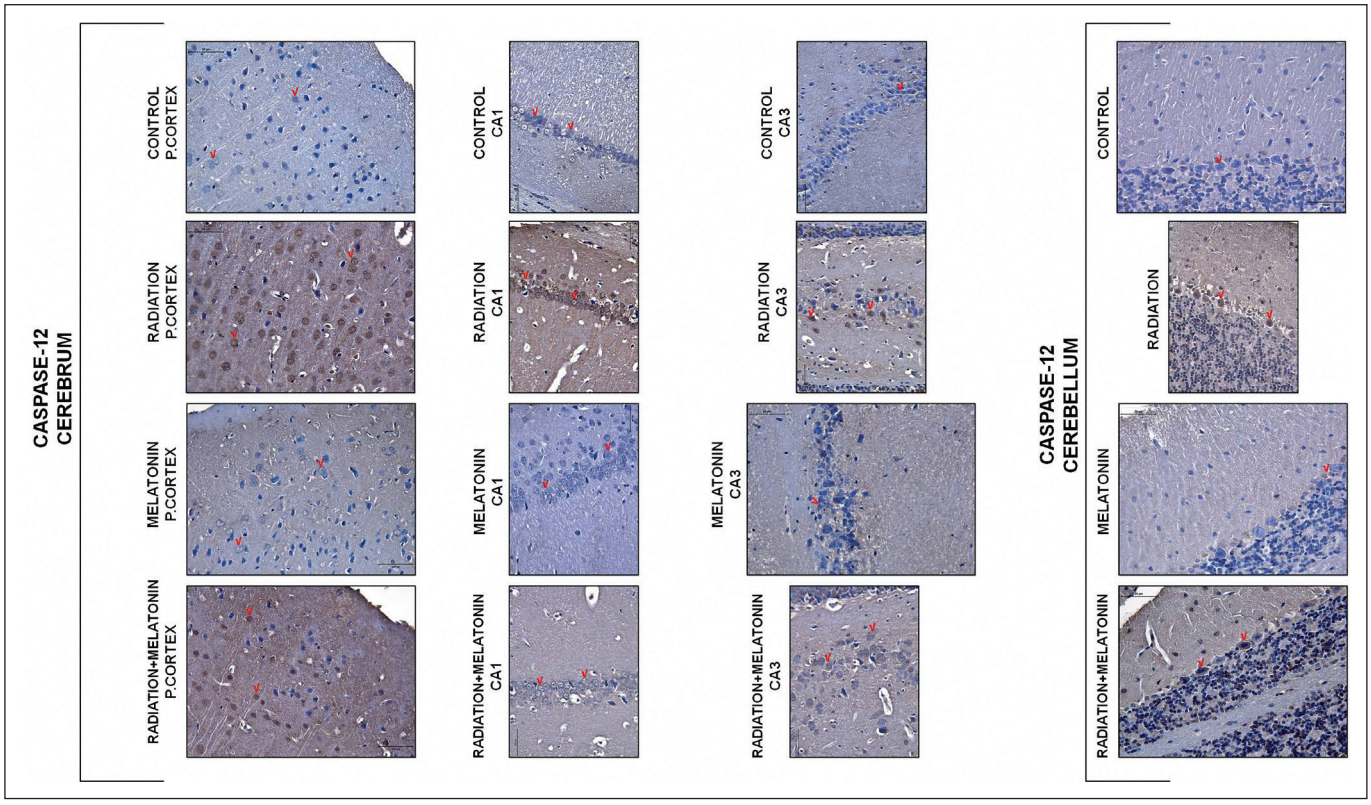


**Figure 8:** Neurons with Caspase-12 immunreactivity (√) were seen for all groups on brain sections (Caspase-12 Primary Antibody x400).

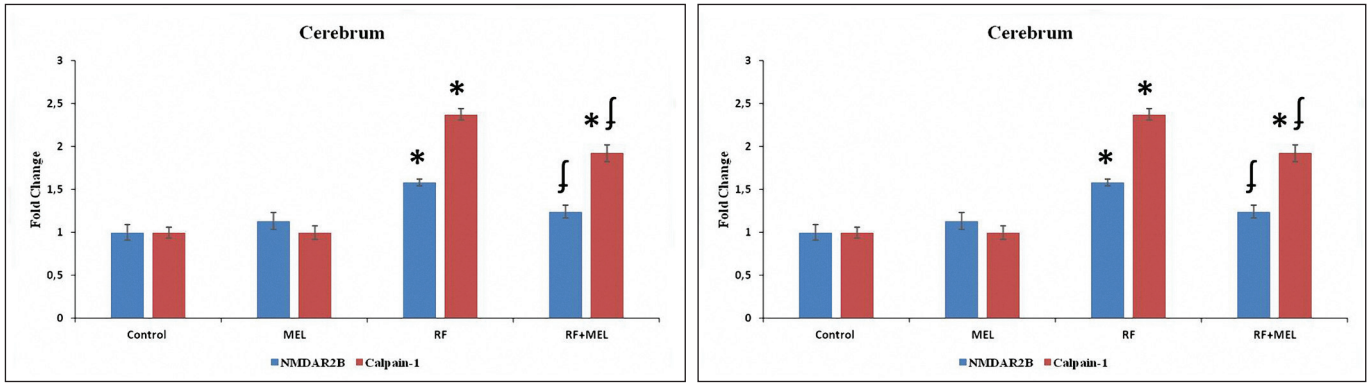


**Figure 9:** The descriptive statistical graphics with the error bars of cerebrum/cerebellum Western blot analysis for NMDA-Receptor 2B and Calpain-1 results for all groups. Statistically significiant groups comparison to the control group (*) and Statistically significiant groups comparison to the radiation group (ʃ).

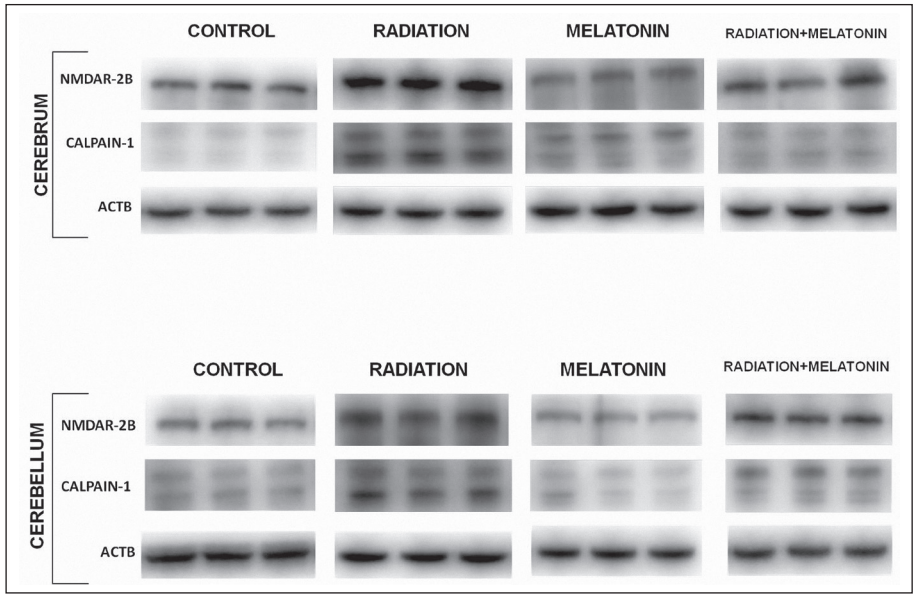


**Figure 10:** Western blot analyses for NMDA-Receptor 2B and Calpain-1 primary antibodies in all groups.

fibers partially disrupted and that nuclear chromatin was chromatolysis shaped in appearance through these cells. In the radiation and melatonin group, the ultrastructure of the prefrontal cortical neurons was seen to be similar to the control group, but the appearance of the Purkinje neuron was seen to be similar to that of the radiation group (Figure 11).

## ■ DISCUSSION

It has been reported that exposure to EMFs of mobile phones causes an increase in neurodegenerative diseases, including Alzheimer's disease (17), and have a negative impact on learning and memory (30). Celikozlu et al. found using Hematoxylin-Eosin staining that 900 MHz mobile phone radiation especially affects the frontal cortex, hippocampus, basal ganglia and cerebellum regions of the brain (5). We researched the prefrontal cortex, hippocampus and cerebellum regions of the brain for 2100 MHz mobile phone radiation exposure in our study.

A decrease in the number of hippocampal CA neurons statistically were found in the research of Bas et al. at the end of an experiment of 900 MHz/1 hour EMF exposure by Hematoxylin-Eosin staining (2). Additionally, we also found a relative-decrease in the number of hippocampal neurons, in particular for the CA1 region in the radiation group. A decrease in the number of Purkinje neurons were found with a study of 900 MHz radiation exposure for 28 days, but did not find any change in brain weight according to Sonmez et al. (42). Additionally, in our study, a decrease in the number of Purkinje neurons were found with 2100 MHz radiation exposure for 90 days but we did not find any change in brain weight.

In some research studies, exposure to EMFs was found to have caused an increase in brain temperature of 1°C, thereby causing deterioration of the BBB and affecting permeability against macromolecules (44). Dark neurons and albumin-positive areas were distinguished in cortical, hippocampal regions and basal ganglia with cresyl violet stainings according to researchers (36). These findings were also seen in 0.12; 1.2;

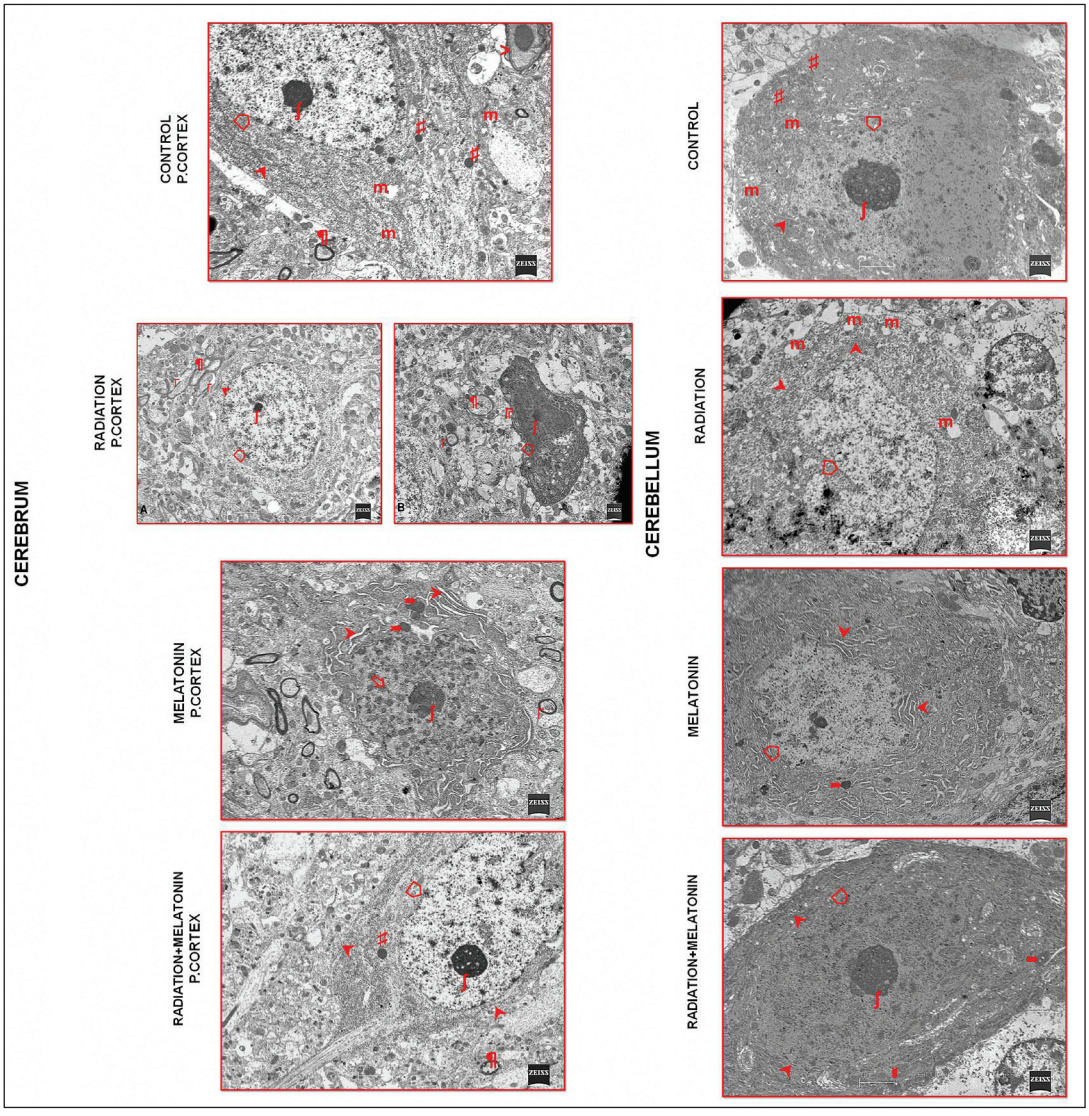


**Figure 11:** Nucleus (⌂), nucleolus (ʃ), myelinated nerve fibers (¶), mitochondrion (m), free ribosomes (#), RER tubules ( ), blood vessels (>), separation of myelinated nerve fibers ( ┌), degenerative neuron (╔), chromatolysis shaped-nuclear chromatin (⇨) and primary lysosome (➡) structures were seen in prefrontal cortical neurons for cerebrum and Purkinje neurons for cerebellum by ultrastructural examinations (Uranyl Acetate and Lead Citrate Staining).

12 and 120 Mw/kg radiation exposure for 2 hours/7 days in a separate study (25). In our 2100 MHz/30 minutes/90 days radiation group, dark neurons were noticeably distinguishable in the cerebrum prefrontal cortex, the hippocampal CA1 and CA3 regions and in cerebellum tissues.

According to one study, 50 Hz/90 days EMF exposure causes abnormal NMDA-Receptor activation in rat hippocampus and, as a result, causes changes to $Ca^{2+}$ signaling (21). Simpkins et al. found in their study conducted with cultured hippocampal neurons, that usage of the suppressor calpains causes a decrease in NMDA-receptor 2B subunit levels, instead of 2A (40). Guttmann et al. indicate that NMDA-Receptors play an important role in memory, learning and excitotoxicity and that calcium-mediated NMDA-Receptor activates calpains leading to the destruction of substrates (13). In another study, a $Ca^{2+}$ entry to an NMDA-Receptor causes activation of $Ca^{2+}$-dependent protease calpains (51). In research by Salamino et al., low frequency magnetic field exposure changed intracellular $Ca^{2+}$ ions availability and in this way, affected cell response (35). Other researchers found that 2.45 GHz microwave frequency/2 hours/day for 45 days causes a decrease in pineal melatonin levels and an increase in brain caspase-3 and $Ca^{2+}$ levels (18). According a study by O'Connor et al., 900 MHz radiofrequency exposure does not cause to any change to basal $Ca^{2+}$ levels of rat hippocampal neurons (26). In our study we found that 2100 MHz radiation exposure causes an increase in NMDA-Receptor 2B immunreactivity in long term radiation exposure for brain tissue and this finding is supported by Western blot analyses. In addition, increased levels of ($Ca^{2+}$- stimulated) Calpain-1 were found by immunohistochemical and Western blot analyses in the radiation group, suggesting that intracellular $Ca^{2+}$ levels may be affected by 2100 MHz mobile phone radiation exposure. According to a study by Tremper-Wells and Vallano, calpains may locate in the nucleus although it is known that calpains are cytosolic enzymes and some calpains are localized or translocated to the cell nucleus, such as Schwann cells treated with growth factors, lens epithelial cells treated with

ionomycin and hippocampus in the case of ischemia (47). We found nuclear and perinuclear involvement of calpain-1, in particular in CA1 and CA3 regions for the radiation group in our study.

Tan et al. indicate that calpains cause caspase-12 and JNK activation in Endoplasmic Reticulum (ER)-stress induced apoptosis (46). In addition, ER stress causes calpain/caspase-12 activation in liver satellit cells according to another study (14). Cadena et al. researched the role of glucose on brain functions in their study and, as a result, they found that usage of the ER-stress inhibitor protected the culture from caspase-12 and neuronal death (3). In our study we found that 2100 MHz radiation exposure causes an increase in caspase-12 immunreactivity with long term radiation exposure for brain tissue. This finding is supported by Western blot analyses. According to a study by Fujita et al., ER stress induces the processing of caspase-12 located in the ER, but processing of caspase-12 at the N-terminal region causes the translocation of processed caspase-12 into nuclei. As a result, cell death occurs by ER stress. The processing of caspase-12 is at the N-terminal region, either by caspases or by calpains (10). Similar to this study, Shimoke et al. found translocated caspase-12 in nuclei in cortical and hippocampal neurons in the case of an experimental blood clot model (38). We particularly found nuclear immunreactivity of caspase-12 in the radiation group in our study.

Researchers indicate that melatonin, which is secreted by the pineal gland, is an effective and non-toxic protective agent for the destructive effects of ionizing radiation in tissue (39). According to our study, in the radiation and melatonin group, the ultrastructure of prefrontal cortical neurons was observed to be similar to the control group, but the appearance of the Purkinje neuron was seen as similar to the radiation group. Similar to this, in the radiation and melatonin group, the number of dark neurons were relative-decreased. The general structure of the prefrontal cortex and CA3 regions were seen as similar to the radiation group, but the appearance of a CA1 region and cerebellum were seen as similar to the control group. Cazevieille et al. indicate that melatonin protects rat cortical neurons from free radicals occuring as a result of NMDA-excitotoxicity or hypoxia (4). In the radiation and melatonin group it can be seen that the NMDA-Receptor 2B immunreactivity is less than the radiation group for the cerebrum and cerebellum in our study. This data is supported by western blot analyses. According a study by Suwanjang et al., melatonin shows protective effects on SH-SY5Y neuroblastome cells by decreasing the calpain and caspase-3 activation on oxidative stress conditions (45). In the radiation and melatonin group it was observed that immunreactivity was less than the radiation group for the cerebrum and cerebellum in our study. We performed our study with 10 mg/kg dose at 4.00 pm according to relating studies (1). Additionally, it has known that the optimal injection time for melatonin 4.00-6.00 pm or according to another view, about 2 hours before light off (27,28), but also we are in the opinion that similar studies can be planned on this field by using different doses of melatonin to compare the possible effects of various doses.

## CONCLUSION

In summary, long-term exposure (90 days) of 2100 MHz GSM radiation activated the Endoplasmic Reticulum-stress *via* the NMDA-Receptor 2B/Calpain-1/Caspase-12 pathway in the brain. Dark neurons are noticeably distinguishable with radiation as a result of deterioration of BBB and radiation caused neuronal degeneration in the cerebellum. Additionally, a number of (ultra) structural changes were seen in the cerebrum and cerebellum as a result of radiation. Melatonin was found to be effective but insufficient in demonstrating these protective effects.

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