

Research report

Neuroprotective efficacy of luteolin on a 900-MHz electromagnetic field-induced cerebellar alteration in adult male rat

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HIGHLIGHTS

- Increased superoxide dismutase (SOD) level was detected following exposure to EMF.
- Electromagnetic field (EMF) caused a significant loss of the granular cells.
- Exposure to EMF caused a significant decrease in the Purkinje cell number.
- Luteolin significantly decreased SOD activity in the cerebella exposed to EMF.
- Luteolin caused a significant increase in the granular and Purkinje cell number.

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ABSTRACT

The adverse health consequences of exposure to electromagnetic field emitted from cell phone has recently raised public concerns worldwide. Also, the Global System for Mobile Communications (GSM) standard that operates in 900 MHz frequency is the most popular. Therefore, we aimed to investigate the adverse effect of exposure to 900 MHz EMF (1 h/day) on the cerebella of 12-week-old rats. We also evaluated the protective activity of luteolin (20 µg/kg/day) against possible biological change in the cerebellar tissues exposed to EMF. Twenty-four male *wistar albino* rats were randomly assigned into four group of six rats: Control, EMF, EMF + luteolin, luteolin. Serological and biochemical analyses, as well as histopathological examination were performed on all cerebellar samples. We found that SOD (superoxide dismutase) level was significantly increased in the EMF group compared to the control group ($p < 0.05$). To the contrary, decreased SOD activity was detected in the EMF + luteolin group compared to control group ($p < 0.05$). The total number of Purkinje and granular cells was significantly decreased in the EMF group compared to the control group ($p < 0.05$). In the EMF + luteolin group, the total number of Purkinje and granular cells was significantly higher than the EMF group ($p < 0.05$). Histopathological evaluation also showed destructive damage to the architectures of cerebellar tissues. Our results suggest that exposure to EMF may cause cellular damage to the rat cerebellum. Further, the improvement of cerebellar damage may have resulted from antioxidant efficacy of luteolin by alleviating oxidative stress.

1. Introduction

Long-term use of cell phone has recently increased concerns regarding the negative effect of electromagnetic field (EMF) on the bio-system. Deleterious health effect of EMF on body organs is attributed to various factors such as body mass index, bone density, body weight, water, and electrolyte levels (Tabrah et al., 1998; Vesselinova 2015).

Body organisms are equipped with the antioxidant enzyme system

that scavenges free radicals normally produced during metabolism. The oxidative effect of EMF radiation is one of the topics that researchers have been addressing recently (Yahyazadeh et al., 2018). EMF in interaction with biomolecules can cause damage to vital organs (Ulubay et al., 2015; Yahyazadeh and Altunkaynak, 2019b; Kim et al., 2019). This is possibly due to overproduction of reactive oxygen species (ROS) posed by EMF. Therefore, exposure to EMF may induce oxidative damages to main cellular structures such as carbohydrate, protein, lipids,

Abbreviations: CNS, Central Nervous System; EMF, Electromagnetic Field; ROS, Reactive Oxygen Species; SOD, Superoxide Dismutase

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and enzymes, leading to impairment of cellular function and even cell death (Hanukoglu, 2006; Hasegawa et al., 1997). Also, ROS can damage single or double strand breaks, base, or sugar lesions in DNA (Fujii and Tsunoda, 2011). Kivrak et al. (2017) reported that EMF impaired the antioxidant status in CNS and caused a significant decrease in neuron number. Şahin et al. (2015) also documented the adverse impact of EMF on hippocampal pyramidal neurons in rat. Cerebellum like other parts of the CNS may be impacted by EMF-induced oxidative stress (Akbari et al., 2014).

The blood-brain barrier that protects the CNS from environmental toxic substances is the first line of defence mechanism. Permeability of this barrier plays a crucial role in maintenance of neural environment as stable as possible. The findings regarding the adverse effect of EMF on the blood-brain barrier is inconclusive. Experimental studies have documented the change in the permeability of the blood-brain barrier following exposure to EMF, resulting in leakage of undesirable molecules into the CNS (Sirav and Seyhan 2011; Salford et al., 2008). This may change the homeostasis of CNS and cause neural apoptosis, pyramidal neuron loss, alteration in the function of myelin and electrical activity of neurons (Kim et al., 2019; Şahin et al., 2015; Ertlav et al., 2018). Natural antioxidants can strengthen endogenous antioxidant defense in the living organisms.

Luteolin (3', 4', 5,7-tetrahydroxyflavone), a common flavonoid, is a major antioxidant compound (Yin et al., 2017). The reason that luteolin is considered as a neuroprotective substance is due to its properties such as antioxidant and anti-inflammatory activities (Ashaari et al., 2018). Luteolin also can strongly scavenge free radicals, then contribute to the alleviation of oxidative damage to biosystem (yahyazadeh and Altunkaynak, 2019a).

Global System for Mobile Communications (GSM) standard that operates in 900 MHz frequency is the most popular. Therefore, 900 MHz wave frequency was used in the present study. Despite numerous studies, there are insufficient information about the health effect of EMF exposure on cerebellum, as well as an approach that minimizes the serious biological damages caused by EMF. We decided to investigate the effect of EMF on the rat cerebellum and the antioxidant activity of luteolin against possible damage. Morphometrical, biochemical and histopathological examinations provided a different perspective to our study. Fig. 1

2. Results

2.1. Biochemical results

Biochemical data are given in Fig. 2. We found a significant increase in SOD level in the EMF group compared to the control group ($p < 0.01$). To the contrary, there was no significant difference in SOD level between the EMF + luteolin or luteolin groups and the control group. SOD activity was significantly decreased in the EMF + luteolin group ($p < 0.05$) and luteolin ($p < 0.001$) group compared to EMF group.

2.2. Stereological results

Stereological results are given in Figs. 3–8. The physical disector was used to estimate the number of granular cells and Purkinje cells. We also calculated the mean volume of cerebellum, grey matter, and white matter, as well as the volume fraction ratio of grey matter to white matter by means of the Cavalieri method.

Fig. 3 shows the total number of Purkinje cells in all group. We found a significant decrease in the Purkinje cell number in the EMF group compared to the control group ($p < 0.01$). By contrast, there was no significant difference between the EMF + luteolin or luteolin groups and the control group. Also, the Purkinje cell number was significantly increased in the EMF + luteolin ($p < 0.05$) and luteolin ($p < 0.01$) groups compared to EMF group.

Fig. 4 exhibits the granular cell number in all group. The total number of granular cells was significantly decreased in the EMF group compared to the control group ($p < 0.01$). We also found a significant increase in the EMF + luteolin ($p < 0.05$) group and the luteolin ($p < 0.001$) group compared to the EMF group. Significant difference was not observed in the granular cell number between the EMF + luteolin or luteolin groups and the control group.

Volumetric results are summarized in Figs. 5–8. The mean volume of the grey matter was decreased in the EMF group compared to the control group, but the differences were not significant. We also found no significant difference in the mean volume of cerebella and white matters, as well as the volume fraction ratio of grey matter to white matter between the EMF group and the control group.

2.3. Histopathological results

The architecture appearances of cerebellar tissues were normal in the control group (Fig. 9a and b). By contrast, there was histopathological change in the cerebellar structures of the EMF group (Fig. 9c-f). We observed degenerated Purkinje and granular cells, as well as few regions where Purkinje cells were absent in the EMF group. We found no structural alteration in the luteolin group compared to the control group (Fig. 10a and b). In the EMF + luteolin group, architectural change was diminished than the EMF group; however, we observed degenerated Purkinje and granular cells were detected (Fig. 10c-f).

3. Discussion

The long-term use of cell phone emitting EMF radiation may impact the brain and cerebellum more than the other vital organs due to its proximity to the CNS during phone conversation (Altun et al., 2017). Findings concerning the adverse effect that EMF on biosystem is inconclusive. Muscat et al. (2000) suggested no potential risk on the CNS exposed to EMF radiation. Finnie et al. (2006) also documented that EMF exposure for 60 min/day for 19 days was not associated with risk of vascular permeability in the foetal mouse brain. To the contrary, numerous studies have reported the toxic effect of EMF on the CNS (Yahyazadeh and Altunkaynak, 2019b; Kim et al., 2019). However, investigations regarding the toxic effect of EMF on the cerebellum and the reduction of its potential risks are limited.

Our quantitative data were obtained using the unbiased stereological methods, which have recently been developed as reliable practical approaches. To the best of our knowledge, this is the first study to survey protective effect of luteolin on the rat cerebella following exposure to EMF.

SOD, one of the main endogen antioxidant enzymes, can be considered as an indicator to determine the severity of EMF-induced oxidative stress. Our biochemical findings showed increased SOD level in the EMF group compared to the control group. This was possibly due to potential effect of oxidative stress caused by EMF. Earlier reports that EMF via excessive ROS formation significantly increased the SOD level in the rat cerebella are congruent with our findings (Altun et al., 2017). We also found that administration of luteolin significantly reduced SOD level in the EMF + luteolin group than the EMF group; luteolin therefore served as a powerful antioxidant scavenger against EMF-induced ROS.

We found no volumetric changes in the cerebella exposed to EMF radiation, whereas there was a relationship between EMF exposure and the subsequent number of granular cells and Purkinje cells. Our finding that EMF caused a significant decrease in the number of Purkinje cells and granular cell is parallel to the previous studies. Altun et al. (2017) reported that EMF radiation for 60 min/day for 15 days decreased the number of Purkinje and granular cells in the rat cerebella. Others have also documented a significant decrease in the number of Purkinje cell in the rat cerebella exposed to 900 MHz EMF once daily during 21-day experimental period (Kivrak et al., 2017). Long duration exposure to

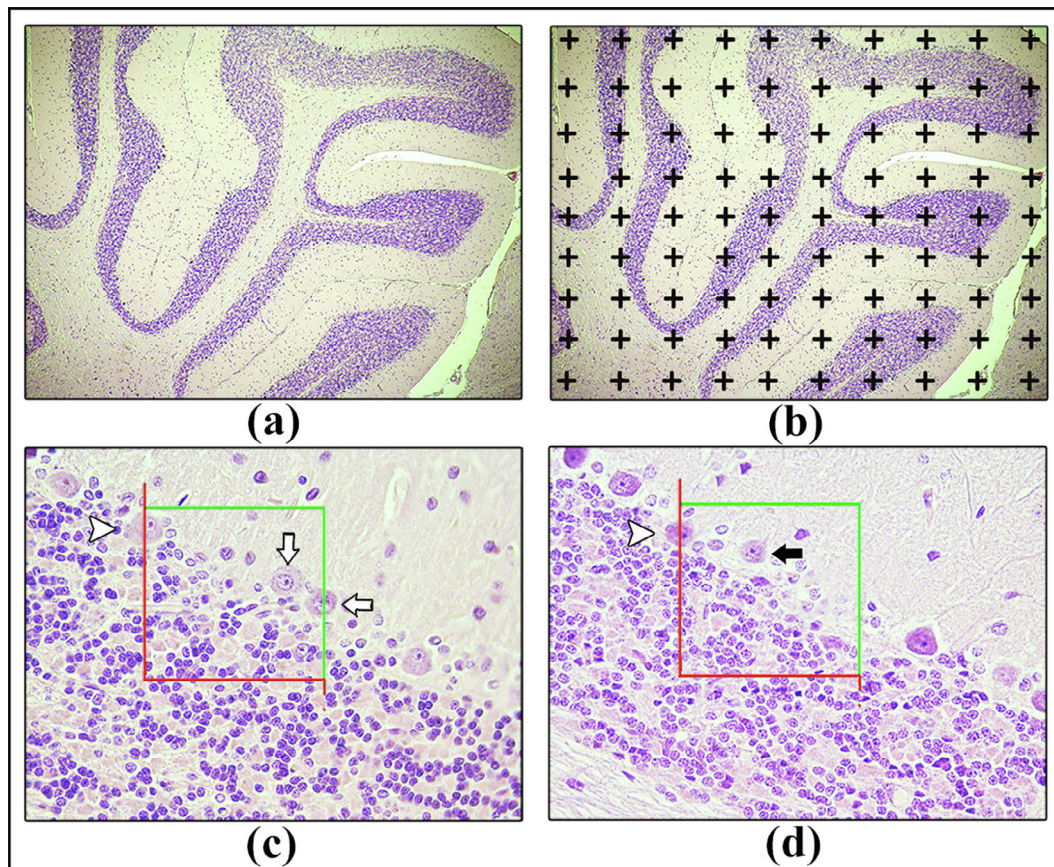


Fig. 1. The stereological procedures for the Cavalieri (a and b) and the physical disector (c and d) methods. Red line, exclusion line; inclusion line, green line; micrograph (c), reference section of ; micrograph (d), look-up section; white arrow, neurons hitting the inclusion lines or located inside the counting frame in the reference sections; black arrow, neurons located inside the counting frame in the look-up sections; arrowhead, neurons hitting the exclusion lines in the reference and look-up sections. The neurons are counted as disector particles that are observed in the reference section but not in the look-up section.

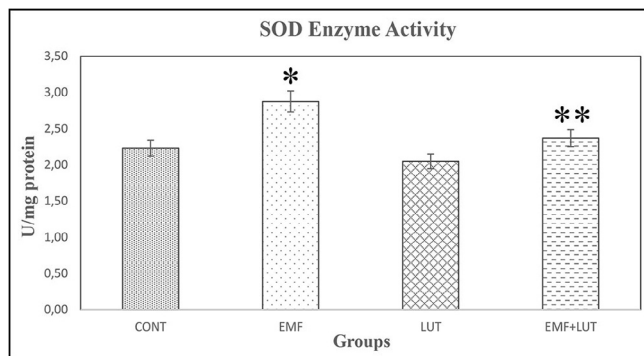


Fig. 2. The antioxidant activity of superoxide dismutase in the control, EMF, luteolin and EMF + luteolin groups. *, there is significant difference between the EMF and control groups; **, there is significant difference between the EMF + luteolin and EMF groups.

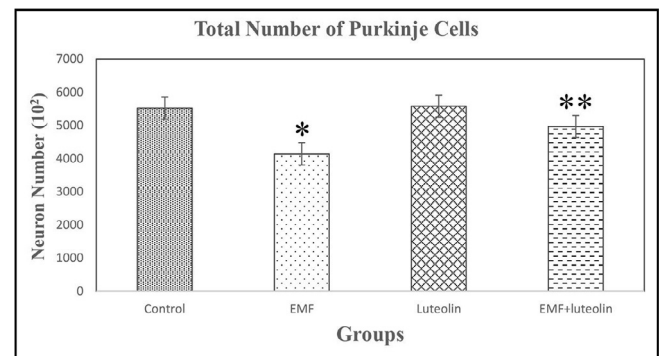


Fig. 3. The total number of cerebellar Purkinje cells in the control, EMF, luteolin and EMF + luteolin groups. *, there is significant difference between the EMF and control groups; **, there is significant difference between the EMF + luteolin and EMF groups.

EMF can also cause damage to Purkinje cells in the rat cerebella (Sonmez et al., 2010). From our point of view, the most common cause of this reduction may have resulted from impairment in oxidative balance caused by EMF. Oxidative stress due to excessive ROS formation triggers a series of complex reactions that contribute to unexpected disorders (Yahyazadeh et al., 2018). Another possible cause is the change in the plasma membrane potential due to EMF, leading to a modification in membrane permeability (Hamada et al., 2011). This may provide the underlying impairment in cell functions and architectures. The cerebellum participates in important functions such as language, motor control, and cognitive-sensory functions (Marques

et al., 2012; Steinlin, 2008; Schmähmann et al., 2007). Structural change in cerebellar tissue following exposure to EMF may also alter cerebellar function. However, the studies regarding functional effect of cerebellar damage caused by EMF is limited. Haghani et al. (2013) reported that EMF caused the change in electrophysiological properties of Purkinje neurons such as decrease in neuronal excitability. They also observed the change in spike frequency and afterhyperpolarization amplitude, as well as first spike latency. Preece et al. (1999) documented that exposure to EMF emitting from cell phone affected cognitive function. Another study reported that any disorder in cerebellar tissues can lead to psychiatric and neurological diseases (Schmähmann

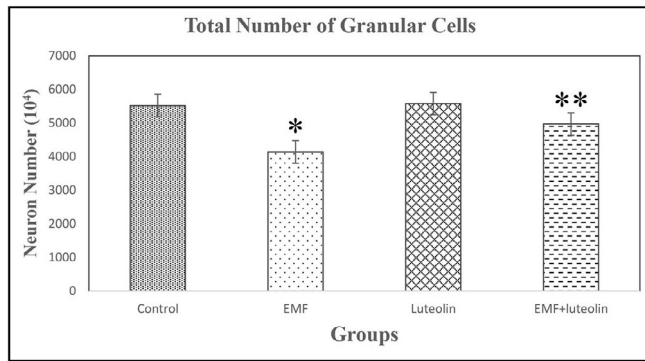


Fig. 4. The total number of cerebellar granular cells in the control, EMF, luteolin and EMF + luteolin groups. *, there is significant difference between the EMF and control groups; **, there is significant difference between the EMF + luteolin and EMF groups.

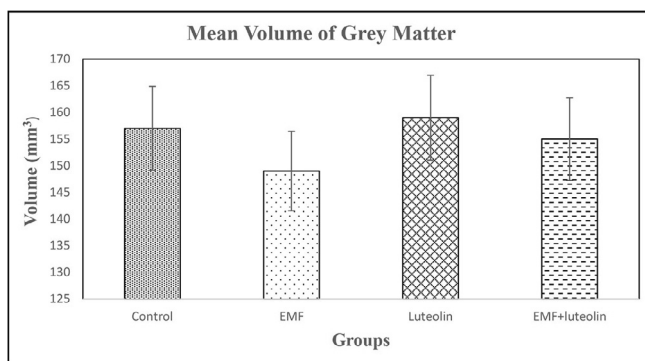


Fig. 5. The mean volume of cerebellar grey matters in the control, EMF, luteolin and EMF + luteolin groups.

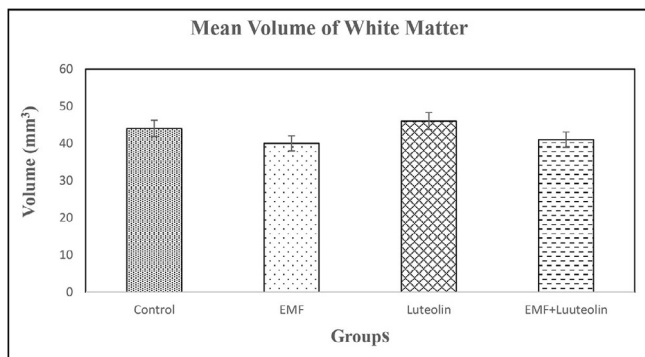


Fig. 6. The mean volume of cerebellar white matters in the control, EMF, luteolin and EMF + luteolin groups.

2004; Patel et al., 2002). Basal ganglia in interaction with cerebellum plays a key role in error-related cognitive control. Brunamonti et al. (2014) reported that damage to cerebellar tissue could cause impairment in the processing of errors. In the EMF + luteolin group than the EMF group, the increased number of granular and Purkinje cells may have attributed to antioxidant activity of luteolin.

We observed that EMF caused the marked histopathological damage to the architectures of cerebellar tissues in the EMF group. Our findings are consistent with earlier report that have determined a correlation between EMF exposure and the subsequent architectural damages to the cerebella (Sonmez et al., 2010). Aslan et al. (2017) investigated the effect of 900 MHz EMF on adolescence male rats; they documented the abnormal cerebellum morphology in the rats. Another study reported by Odacı et al. (2016) exhibited pyknotic neurons and Purkinje cell loss

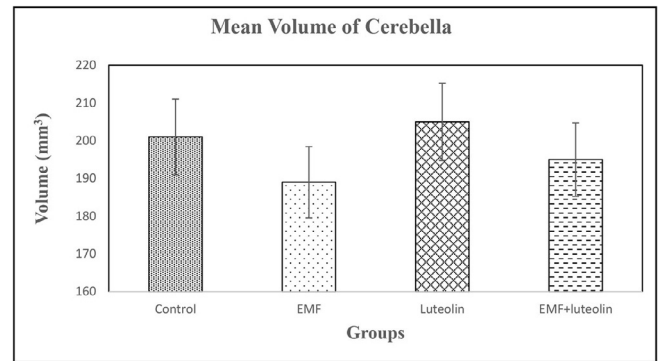


Fig. 7. The mean volume of cerebella in the control, EMF, luteolin and EMF + luteolin groups.

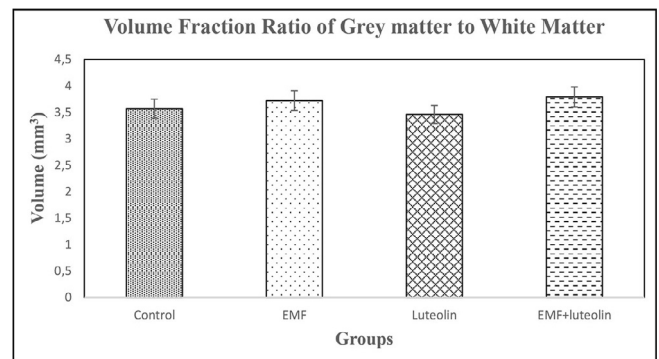


Fig. 8. The volume fraction ratio of grey matter to white matter in the control, EMF, luteolin and EMF + luteolin groups.

in the female rat offspring cerebella during maternal exposure to EMF. Given the difference in EMF dosage and exposure duration, as well as animal age, changes in the histologic structures of the cerebellum may vary. To the contrary, in the EMF + luteolin group compared to the EMF group, luteolin through antioxidant activity improved damage to the cerebellar structures caused by EMF.

The limitation of the present study is that the dose-dependent effect of luteolin was not investigated. Therefore, additional doses of luteolin should be surveyed in future studies. The reason for this recommendation is not only to obtain more conclusive data but also to direct us to use the more appropriate dosage.

3.1. Conclusion

Our findings that indicated the deleterious effects of exposure to 900 MHz EMF radiation on the cerebella is consistent with the previous reports (Kopani et al., 2017; Rağbetli et al., 2010). However, we speculate that the most notable change due to EMF is a decrease in the granular cell and Purkinje cell numbers. Further, administration of luteolin after period of 28 days exerts a neuroprotective efficacy against such cerebellar damages caused by EMF.

4. Materials and methods

4.1. Animals and experimental procedure

Ethical approval of the present study was granted by Laboratory Animal Ethics Committee of Ondokuz Mayıs University. Twenty-four adult male *Wistar albino* rats (200 g in weight, 12 weeks old) were used in this study. All animals were obtained from the Experimental Animal Research and Application Centre of Medicine Faculty of Ondokuz Mayıs University (Samsun, Turkey). The rats were housed in plastic cages

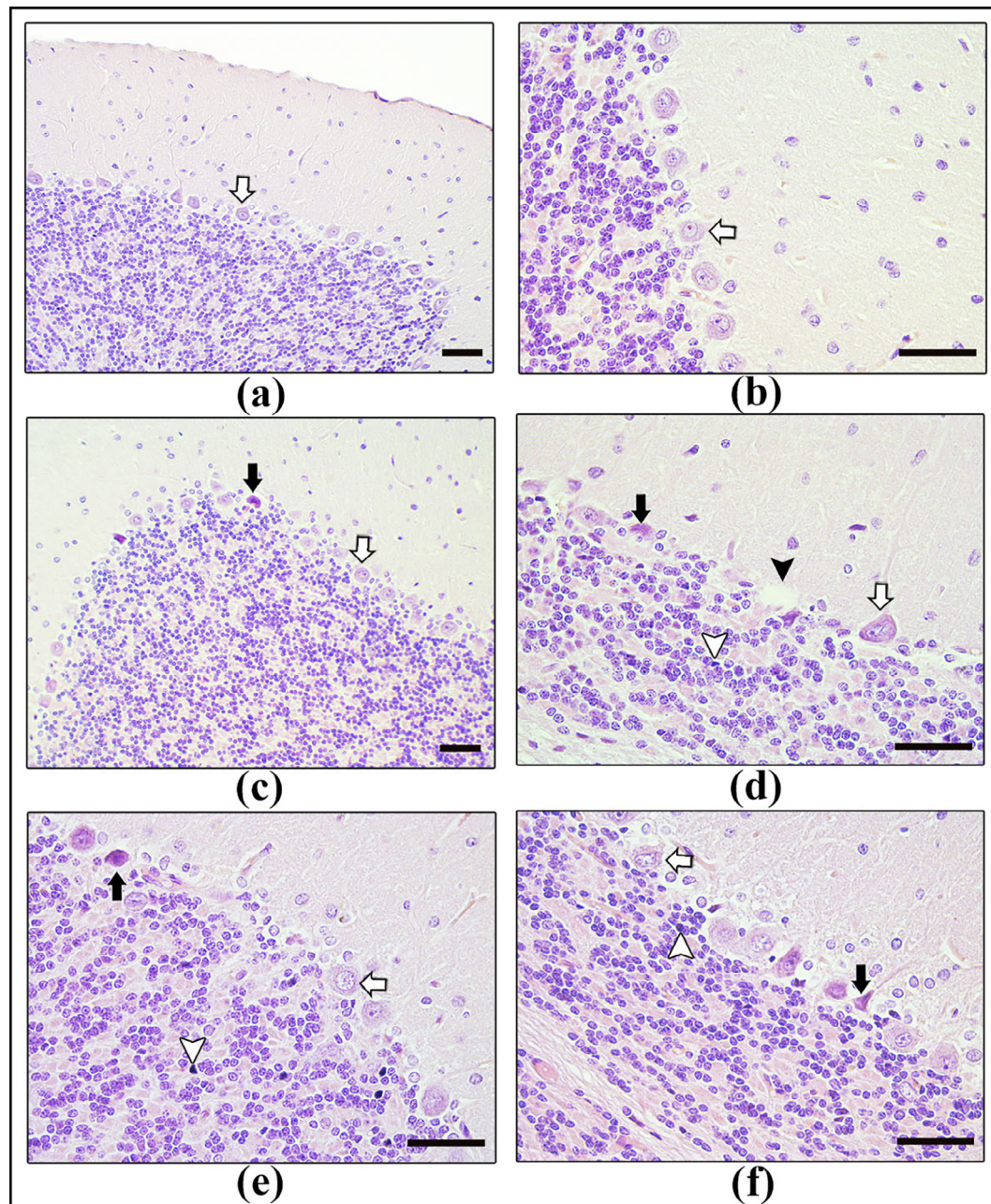


Fig. 9. Light micrographs of cerebella in the control (a and b) and EMF (c-f) groups. White arrow, healthy Purkinje cells; black arrow, degenerated Purkinje cells; white arrowhead, degenerated granular cells; black arrowhead, absence of Purkinje cell. Scale bars = 25 μ m.

under 12:12 h day/night cycle in a temperature of 22 ± 2 °C and humidity of $50 \pm 5\%$ with *ad libitum* access to food and water. All applications were performed during the 28 day experimental period of. The rats were randomly assigned into four equal groups (n = 6 per group). Main study groups are given below:

1. Control group: Rat were not exposed to EMF and any substance.
2. Electromagnetic field (EMF) group: Rats were only exposed to 900 MHz EMF for 1 h/day for 28 days.
3. Luteolin group: Rats received intraperitoneal (i.p.) injection of luteolin (20 μ g/kg/day; Sigma-Aldrich, St. Louis, MO, USA) for 28 days (Zhang et al. 2016).
4. Exposure to electromagnetic field + luteolin (EMF + luteolin) group: Rats were exposed to 900 MHz EMF for 1 h/day, and they received i.p. injection of luteolin (20 μ g/kg/day; Sigma-Aldrich, St. Louis, MO, USA) for 28 days.

At the end of the 28th day, cardiac perfusion was performed on all rats under anesthesia by the injection of a 5:1 ratio of ketamine/xylazine (ketamine: 0.5 ml; 60 mg/kg body weight, Sigma Chemical Comp, St. Louis, MO, USA)(xylazine: 0.1 ml; 5 mg/kg Sigma Chemical Comp, St. Louis, MO, USA). The rat cerebella then were removed immediately and cut into two equal parts. Half of the parts was used for stereological and histopathological examinations, while others were stored at -80 °C for biochemical analysis.

4.2. Histology

All cerebellar samples were fixed in 10% formalin solution, processed through graded alcohols and xylene series, then embedded in paraffin. Cerebellar paraffin blocks were cut into 7 μ m thick sections based on the systematic random sampling technique using a rotary microtome. Consecutive pair sections were superimposed on glass sides,

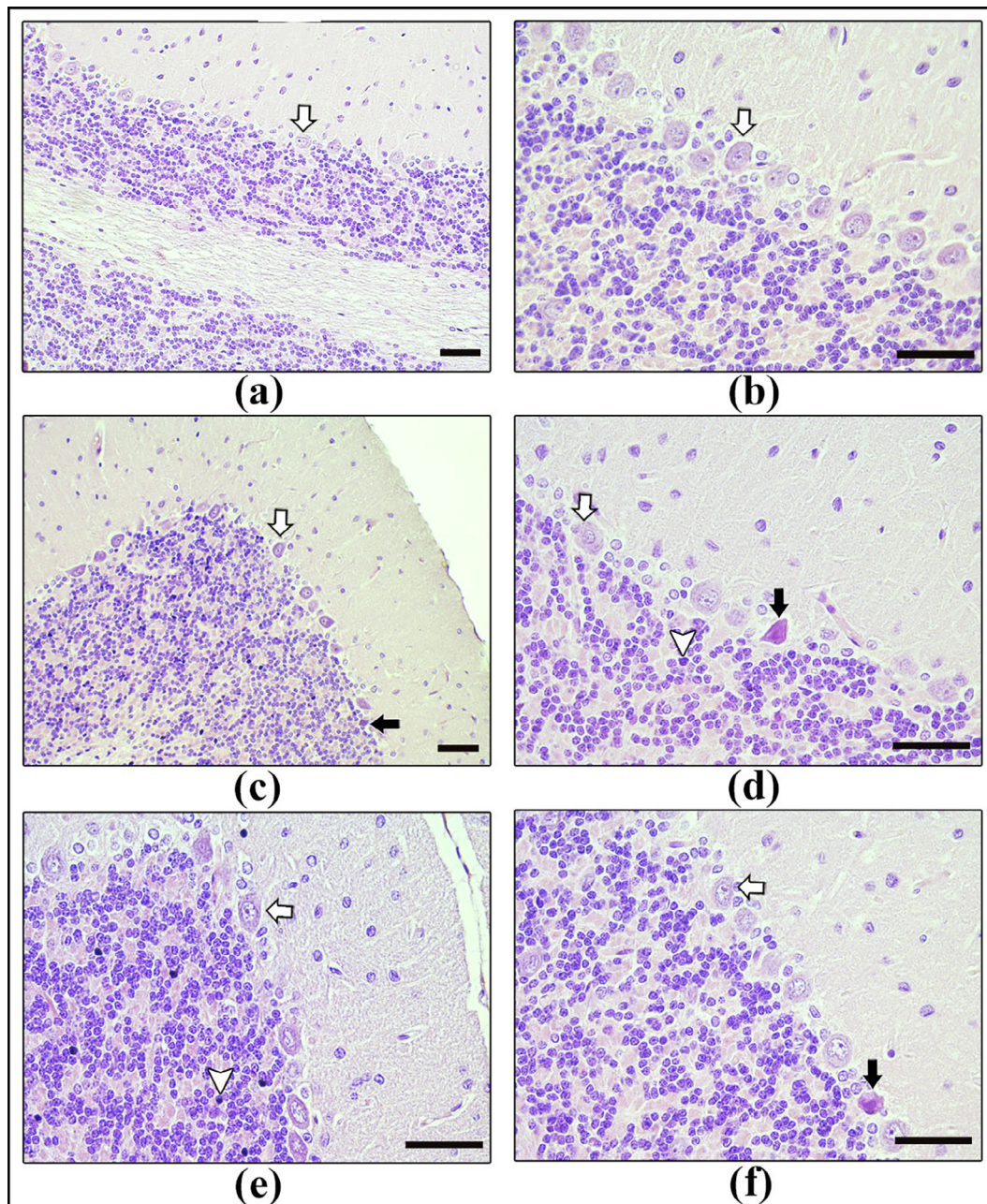


Fig. 10. Light micrographs of cerebella in the luteolin (a and b) and EMF + luteolin (c-f) groups. White arrow, healthy Purkinje cells; black arrow, degenerated Purkinje cells, white arrowhead, degenerated granular cells. Scale bars = 25 μ m.

then stained with hematoxylin-eosin. We took photographs from the sections using a Leica DM2500 LED microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) and a HD digital camera (Leica Microsystems Ltd., CH 9435, Heerbrugg, Switzerland) for morphometrical analysis and histopathological examination.

4.3. Electromagnetic field exposure system

EMF exposure was performed using a generator, EMF meter and plastic cage according to the procedure of our previous study (Yahyazadeh and Altunkaynak, 2019b). The EMF Generator produced 900 MHz continuous electromagnetic wave (peak specific absorption rate (SAR): 2 W/kg; average power density: 1 ± 0.4 mW/cm²). This generator was manufactured by the Electromagnetic Compatibility Laboratory of Suleyman Demirel University (Koyu et al., 2005). The localized SAR values were calculated as described by Sirav and Seyhan

(2011). Additionally, an EMF meter measured the power density of EMF (Holaday Industry Inc., Adapazarı, Turkey). Briefly, the monopole antenna of the exposure system was placed perpendicular to the center of the round plastic cage in order to an even distribution of the electric field (Koyu et al., 2005). In order to alleviate stress on the rats, 1 cm diameter air holes were devised on the lid of the plastic cage. Rats were arranged adjacent to each other in the plastic cage at a 1 cm distance with their heads in the antenna direction. Subsequently, the animals were exposed to 900 MHz EMF for 1 h daily during the experiment period.

4.4. Biochemistry

Superoxide dismutase (SOD) as a main antioxidant enzyme in cellular defense system protects the CNS against oxidative stress. The stored cerebellar samples were taken out of the freezer and

homogenized in phosphate buffer (pH 7.4) at 5000 rpm for 3 min on ice (IKA, Germany). Supernatants then were collected from tissue homogenates by centrifugation at 5000 rpm for 25 min at 4 °C. SOD activity was determined based on generation of superoxide anion radical using xanthine and xanthine oxidase (Sun et al., 1988). The superoxide radical generates a color by reducing NBT (nitroblue tetrazolium) to a blue colored formazan. Briefly, 2.5 ml supernatant was added to 2.45 ml assay reagent, then mixed well using vortex. The mixture was homogenized by adding 0.05 ml of xanthine oxidase, then incubated at 25 °C for 20 min to allow the reaction to take place. Subsequently, the reaction was stopped by adding 1 ml CuCl₂ to the tubes. Finally, SOD activity was calculated using the absorbance of the formazan via a UV–vis spectrophotometer (Shimadzu UV-MINI 1240; Shimadzu; Istanbul, Turkey) at 560 nm. We also used distilled water as blank control solution. Protein assays also were performed using the method of Lowry et al. (1951). SOD level was expressed as units per milligram protein.

4.5. Stereology

4.5.1. Estimation of the mean volume of cerebella

The Cavalieri and point-counting methods were conducted to estimate the mean cerebellum volumes (Fig. 1) (Altunkaynak et al., 2019). The optimal grid size was designed based on a pilot study to provide valuable data (Yahyazadeh et al., 2017). We placed randomly a tested point grid on images photographed from the cerebella, then counted the point number hitting the interested regions. The area value was estimated using the following formula:

$$\text{Area}(A) = a(p) \times \sum P$$

where, “a(p)” is the point interval area, and “ΣP” is the total number of points hitting the regions of interest. Finally, the mean volumes of the interest regions of cerebella were calculated as:

$$\text{Volume}(V) = t \times \Sigma A$$

where, “t” is the total thickness of each section and interval, and “ΣA” is the total area of the regions of interest.

4.5.2. Estimation of total number of neurons

The physical disector counting technique was used for unbiased estimation of the neuron number in the cerebella (Altunkaynak et al., 2016). The sampling and counting strategy were determined using a pilot study. Disector pairs were randomly chosen using the systematic random sampling; the first section of pair was reference and the other was look up. ImageJ also was utilized to count the neuron number according to the physical disector rules; the neuron that appears in the reference section, but not in the look-up section was accepted as a disector particles. An unbiased two-dimensional counting frame was randomly placed on each sampled fields of the pairs. The numerical density of neurons was estimated as:

$$N_v = \frac{\sum Q^-}{\sum V \text{ disector}}$$

where, “ΣQ” is the total number of neurons counted in the disector pairs, and “ΣV disector” is the total volumes of the disector frames. Finally, the total number of cerebellar neurons was calculated as:

$$TN(\text{total cerebellar neuron}) = N_v \times V_{\text{ref}}$$

where, “V_{ref}” is the mean volume of cerebellum, and N_v is the numerical density of cerebellar neurons.

Coefficient of error (CE) and coefficient of variation (CV) values was revealed that the neuron number in each animal and each group is sufficient (Gundersen and Jensen 1987).

4.6. Statistical analysis

Statistical analysis was performed using IBM version 25.0 SPSS software (SPSS Inc., Chicago, IL, USA). Since data were normally distributed, One-Way ANOVA and the Tukey's post hoc test were used to analyze the data. The results were expressed as mean ± SEM. P values less than 0.05 were considered statistically significant.

CRediT authorship contribution statement

Ahmad Yahyazadeh: Conceptualization, Methodology, Software, Visualization, Investigation, Data curation, Writing - original draft. **Berrin Zuhall Altunkaynak:** Conceptualization, Methodology, Validation, Visualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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