

Investigation of the neuroprotective effects of thymoquinone on rat spinal cord exposed to 900 MHz electromagnetic field

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ABSTRACT

Exposure to electromagnetic field in long-term use of cell phones has increased concerns about serious health problems. Our aim was to survey the possible effects of electromagnetic field radiation (60 min/day for 28 days) on the spinal cords of 12 weeks old rats. Further, we investigated whether the administration of thymoquinone (10 mg/kg/day) would protect the spinal cord tissue against the adverse effects of electromagnetic field or not. Twenty-four adult male *Wistar albino* rats were assigned randomly into four groups: control, electromagnetic field, thymoquinone and electromagnetic field + thymoquinone. The cervical spinal cords of all rats was evaluated using the stereological, biochemical and histological methods. The number of motor neurons were reduced in the electromagnetic field group compared to the control group ($p < 0.05$). Superoxide dismutase level was higher in the electromagnetic field group compared to the control group ($p < 0.05$). In the electromagnetic field + thymoquinone group, we found an increase in the number of motor neurons and decrease in superoxide dismutase activity compared to the electromagnetic field group ($p < 0.05$). Our histological findings also exhibit the remarkable architectural alterations. We speculated that electromagnetic field radiation induced the morphological and biochemical damage to the spinal cord tissue of rats. Administration of antioxidant, thymoquinone, also ameliorated such complications caused by electromagnetic field.

1. Introduction

Cell phone use is growing day by day, and people in the modern world are constantly exposed to electromagnetic field. Epidemiological, clinical and experimental studies have reported the various types of biological damages in the living organism exposed to electromagnetic field.

There is a natural oxidative balance between formation of free radical and antioxidant defence in organism body, which eliminates the produced free radicals (Yahyazadeh et al., 2018). Disruption of oxidative balance leads to excessive formation of reactive oxygen species and finally oxidative stress. Electromagnetic field, which is considered as one of the environmental factors, may participate in disturbance of oxidative balance (Yahyazadeh et al., 2018; Lee et al., 2004). Generated oxidative stress can interact with the biological structures such as DNA, protein, lipid, carbohydrate and enzyme, finally leading to cellular damage and the programmed cell death (apoptosis) (Yahyazadeh and Altunkaynak, 2019; Hanukoglu, 2006; Moustafa et al., 2004). Numerous experimental researches have documented the oxidative effects of electromagnetic field on morphology and biochemistry of vital

organs (Yahyazadeh and Altunkaynak, 2019). The spinal cord as a component of central nerve system can act as an antenna, which contributes to electromagnetic field energy leakage (Balaguru et al., 2012). Kesari et al. (2019) reported an increase in reactive oxygen species by electromagnetic field radiation that was associated with neurotoxicity. Also, it has been proved that the electromagnetic field can induce the oxidative damages to spinal cord (İkinci et al., 2016).

The blood-brain barrier, which restricts the passage of soluble substances and pathogens, play a substantial role in the health of central nerve system. In a study of Sirav and seyhan (2009), blood-brain barrier permeability was impacted following exposure to 900 MHz electromagnetic field in rats; therefore, disruption of this defensive barrier may be one of the major reasons for neuronal damage in the central nerve system (Fredriksson et al., 1988). Numerous studies have been reported the negative impact of electromagnetic field on hippocampus, cerebellum, brain and spinal cord. A study of Altun et al. (2017) documented that exposure to electromagnetic field can cause the significant difference in the number of cerebellar Purkinje cell and hippocampal pyramidal cells as well as granular cells. In nerve system, electromagnetic field also impacts the regulation of synaptic plasticity,

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neurotransmitter release, signal transduction, neuronal life, neuronal functions such as learning and memory (Fritze et al., 1997; Koivisto et al., 2000; Brillaud et al., 2007; Manikonda et al., 2007; Maskey et al., 2010). Besides, there is a correlation between electromagnetic field and brain cancer risk (Prasad et al., 2017).

Thymoquinone, as important component of black seed (*Nigella sativa*), have been widely used in treatment of various diseases worldwide. There are a lot of scientific studies regarding the health benefits of thymoquinone. Thymoquinone that acts a potent antioxidant against many types of reactive oxygen such as the superoxide anion and the hydroxy radical. Antioxidant, thymoquinone, also alleviates intracellular reactive oxygen species and, therefore, supports the preservation of the cellular structures (Ismail et al., 2016). Ashraf et al. (2011) reported the other antioxidant features of thymoquinone was known as neuroprotective, anti-diabetic, anti-carcinogenic, anti-viral and anti-allergic effects. In the spinal cord injury, ameliorative influence of thymoquinone may be due to attenuating the oxidative stress and inflammatory response as well as apoptosis (Chen et al., 2018).

However, despite numerous publications, little research has been conducted on the biological effects of electromagnetic field and antioxidant activity of thymoquinone regarding spinal cord. We therefore aimed to survey the adverse effect of 900 MHz electromagnetic field on the morphology and biochemistry of the cervical spinal cord; also, possible protective effect of thymoquinone against electromagnetic field radiation was examined.

2. Materials and methods

2.1. Experimental procedure and animals

Twenty-four adult male *Wistar albino* rats (average body weight 200 g, 12-week-old) were used. All rats were obtained from the Experimental Animal Research and Application Centre of Medicine Faculty of Ondokuz Mayıs University (Samsun, Turkey). Rats were randomly chosen and housed in plastic cages under 12:12 h day/night cycle at $50 \pm 5\%$ humidity and $22 \pm 2^\circ\text{C}$. All rats were accessed to food and water ad libitum. The applications were carried out during the 28-day experimental period. All rats were randomly divided into four equal groups ($n = 6$ per group) as follow: control group, not treated with thymoquinone substance and exposed to 900 MHz electromagnetic field; electromagnetic field group, only exposed to 900 MHz electromagnetic field for 60 min/day during the experiment period; thymoquinone group, only treated orally with 10 mg/kg/day thymoquinone that was dissolved in 1 ml corn oil (Sigma-Aldrich, St. Louis, MO, USA) during the experiment period (Aras et al., 2015); electromagnetic field + thymoquinone group, exposed to 900 MHz electromagnetic field for 60 min/day and treated orally with 10 mg/kg/day thymoquinone (Sigma-Aldrich, St. Louis, MO, USA) during the experiment period.

At the end of the experiment, all animals were perfused under anaesthesia by the injection of 5/1 ratio of ketamine (60 mg/kg body weight, Sigma Chemical Comp, St. Louis, MO, USA) /xylazine (5 mg/kg body weight, Sigma Chemical Comp, St. Louis, MO, USA). Afterwards, the cervical spinal cord samples were harvested and divided into two unequal parts. While cervical spinal cord samples at the C6-C7 level were used to stereological and histopathological examination, the rest of spinal tissues were stored at -80°C for biochemical evaluation.

2.2. Electromagnetic field exposure system

All our procedures of electromagnetic field exposure were carried out according to the study of Ulubay et al. (2015). A 900 MHz continuous wave electromagnetic energy generator was used with an average power density of $1 \pm 0.4\text{ mW/cm}^2$ and a peak specific absorption rate (SAR) of 2 W/kg (Koyu et al., 2005). Generator was manufactured by the Electromagnetic Compatibility Laboratory of

Suleyman Demirel University. The localized SAR values were calculated as described by Sirav and Seyhan (2011). Moreover, electromagnetic field meter was utilized to determine the power density (Holaday Industry Inc., Adapazarı, Turkey). Air holes were devised in the plastic cage to attenuate stress on the animals (Bilgili et al., 2013). To distribute an equal electric field, monopole antenna of the exposure system was inserted perpendicular in the centre of the round plastic cage (Koyu et al., 2005). Animals were placed adjacent to each other so that rat's heads were in the antenna direction and animals were exposed to 900 MHz EMF.

2.3. Histological studies

All samples were fixed in 10% formalin solution and processed according to histologic procedure including the dehydration and clearing as well as embedding. Sections from each spinal cord blocks. Spinal cord samples were cut at $7\text{ }\mu\text{m}$ thickness and 1/140 interval using a rotary microtome in a transverse plane. Subsequently, consecutive sections provided using the systematic random sampling technique were superimposed on glass slides and stained with cresyl violet (Altunkaynak et al., 2019; Yahyazadeh et al., 2017). Finally, images were captured 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 stereological and histological study.

2.4. Stereological investigation

The number of motor neurons and mean volume of interested regions of spinal cord were estimated by means of the efficient and unbiased stereological methods, physical disector and Cavalieri methods. Application of stereological study was conducted on the consecutive disector pairs (Fig. 1).

2.4.1. Estimation of mean volume of spinal cord

The mean volume of spinal cord was estimated using the Cavalieri and point-counting methods (Ulubay et al., 2015) (Fig. 1a and b). Based on pilot study, we designed the point density of the point-counting grid to specify whether coefficient of error was significant or not. After scanning the photographs from serial sections, sum of the points hitting on the region of interest were counted. Finally, the area of central canal, white and grey matters was calculated in each spinal cord section using the formula as follow:

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

Where, "a(p)" is the area of point interval, and "ΣP" is the number of points hitting on the interested regions in each section.

$$\text{Volume}(V_1) = t \times A$$

Where, "V₁" is the mean volume of interested regions in each section, "t" is the total thickness of each section and interval, and "A" is the area of interested regions in each section. Finally, the mean volume of interested regions of spinal cord was calculated using the formula below:

$$\text{Total } V = V_1 + V_2 + \dots V_n$$

2.4.2. Estimation of the number of motor neurons

The physical disector method was utilized to estimate the number of motor neurons (Altunkaynak et al., 2019) (Fig. 1c and d). Based on pilot study, the sampling and counting strategy of our study was determined. The disector pairs were obtained from the spinal cord blocks, which the first section of pair was reference and the other was look up. Consecutive sections were also chosen using the systematic random sampling. Reference and look up sections were photographed from the same region of interest and then transferred to another computer. The number of particle profiles were estimated using the ImageJ program.

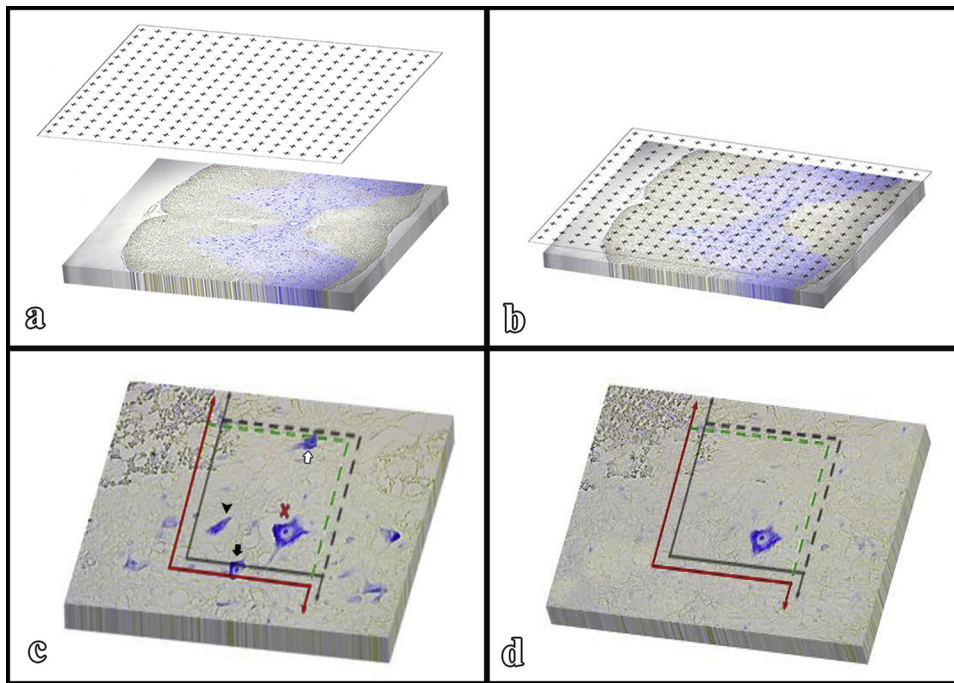


Fig. 1. Representative micrographs for the stereological procedures of the Cavalieri principle (a and b) and the physical disector (c and d). Micrograph (c), reference section; micrograph (d), look-up section; X, motor neurons inside the counting frames in the both reference and look-up sections; arrowhead, motor neuron inside the reference section; black arrow, motor neuron hitting the continuous line; white arrow, motor neuron hitting the non-continuous line; arrowhead and white arrow; motor neurons acceptable for counting that are detected in the reference section but not in the look-up section.

Afterwards, an unbiased counting frame was superimposed on the same counting field of each pair section. The numerical density of motor neurons was calculated using the following formula (Altunkaynak et al., 2016):

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

Where, “ Q^- ” is the number of motor neurons counted in each animal, and “ $\sum V \text{ disector}$ ” is the total volume of the disector frames in reference sections. Finally, the number of cells was calculated as follow:

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

Where, “ V_{ref} ” is the mean volume of spinal cord, and N_v is the numerical density of motor neurons.

The coefficient of error was evaluated to determine the adequacy of the cell number in each animal (Gundersen and Jensen, 1987; Tunc et al., 2007). Also, the coefficient of variation exhibited a sufficient number of animals in each group. Based on pilot study, we validated coefficient of error of the sampling schedule in the spinal cord (coefficient of error $\leq 10\%$).

2.5. Biochemical assay

Spinal cord samples were homogenized in phosphate buffer (pH 7.4) at 5000 rpm for 2 min on ice (IKA, Germany). To prepare supernatants, tissue homogenates were centrifuged at 5000 rpm for 30 min at 4°C. The level of superoxide dismutase enzyme was studied using the method of Sun et al. (1988) via measuring the generation of superoxide radicals produced by xanthine and xanthine oxidase. Spectrophotometric analysis was finally performed at 560 nm on a UV-vis spectrophotometer (Shimadzu UV-MINI 1240; Shimadzu; Istanbul, Turkey). Protein assays were also proceeded with the method adapted from Lowry et al. (1951). Superoxide dismutase activity was expressed as units per milligram protein.

2.6. Statistical analyses

IBM version 25.0 SPSS software (SPSS Inc., Chicago, IL, USA) was used to statistical analysis. To analyse the data, one-way ANOVA and

the Tukey's *post hoc* test were performed. The results were expressed as mean $\pm$ SEM. P value lower than 0.05 was significant.

3. Results

3.1. Stereological results

The motor neurone number and mean volume of interested regions of spinal cord were estimated by the physical disector and Cavalieri methods, respectively. Stereological data are given in Figs. 2 and 3, and Fig. 5.

3.1.1. Volumetric results

Volumetric results are given in Figs. 2 and 3. We found no significant difference in the mean volumes of spinal cords between the control group and the electromagnetic field or electromagnetic field + thymoquinone groups. The mean volumes of grey and white matters were also not significantly different in the electromagnetic field and electromagnetic field + thymoquinone groups compared to the control group.

3.1.2. The number of motor neurons

We found a significant decrease in the number of motor neurons in the electromagnetic field group compared to the control group ($p < 0.00$). The number of motor neurons was significantly higher in the electromagnetic field + thymoquinone ($p < 0.04$) group and the thymoquinone ($p < 0.02$) group compared to the electromagnetic field group. By contrast, there was no significant difference between the thymoquinone group and control or the electromagnetic field + thymoquinone groups (Fig. 5).

3.2. Biochemical results

Biochemical data are given in Fig. 4. Superoxide dismutase level was increased significantly in the electromagnetic field group compared to the control group ($p < 0.00$). We found no significant difference between the thymoquinone group and the control or electromagnetic field + thymoquinone groups. Superoxide dismutase activity was decreased significantly in the thymoquinone group ($p < 0.00$) and the

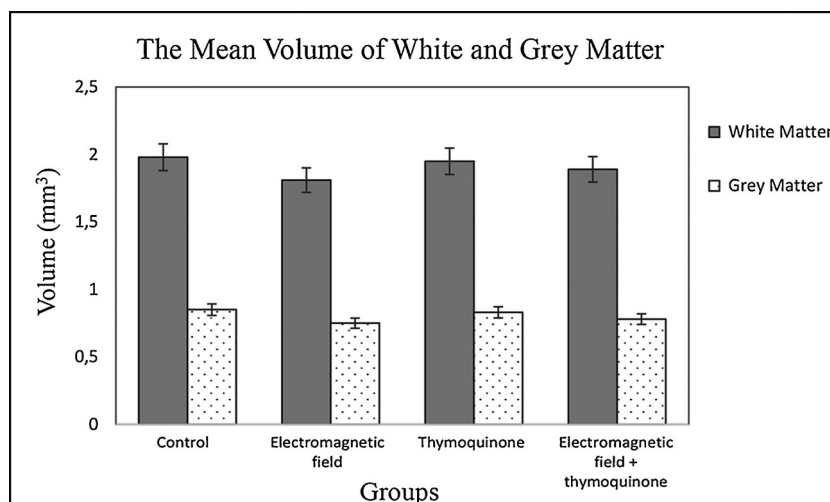


Fig. 2. The representative graph of white and grey matter volume in the spinal cords of all groups.

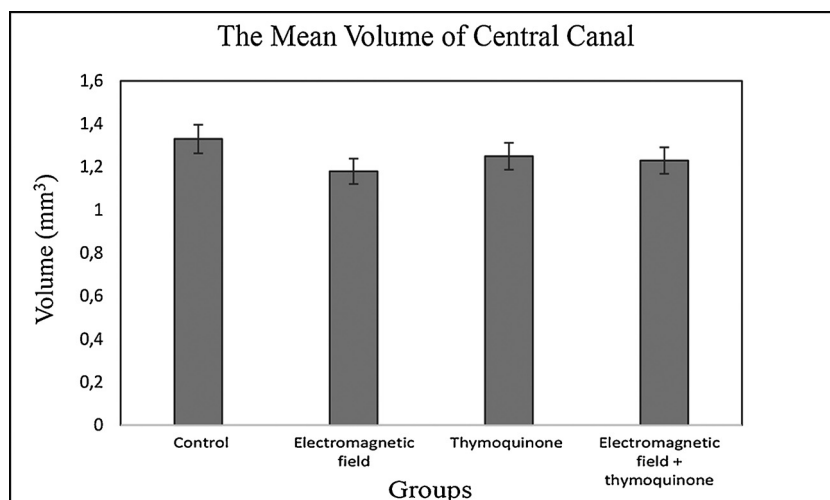


Fig. 3. The representative graph of central canal volume ($\times 10^{-3}$) in the spinal cords of all groups.

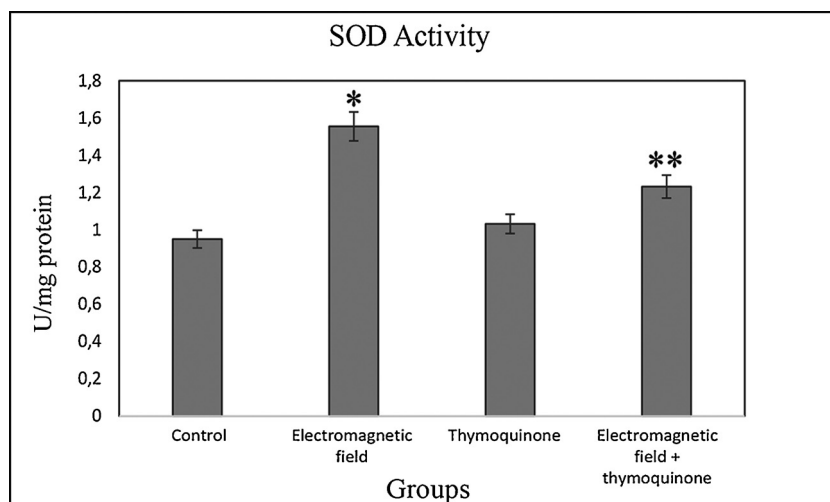


Fig. 4. The representative graph of superoxide dismutase activity in the spinal cord tissues of all groups. *, significantly different from the control group; **, significantly different from the electromagnetic field group.

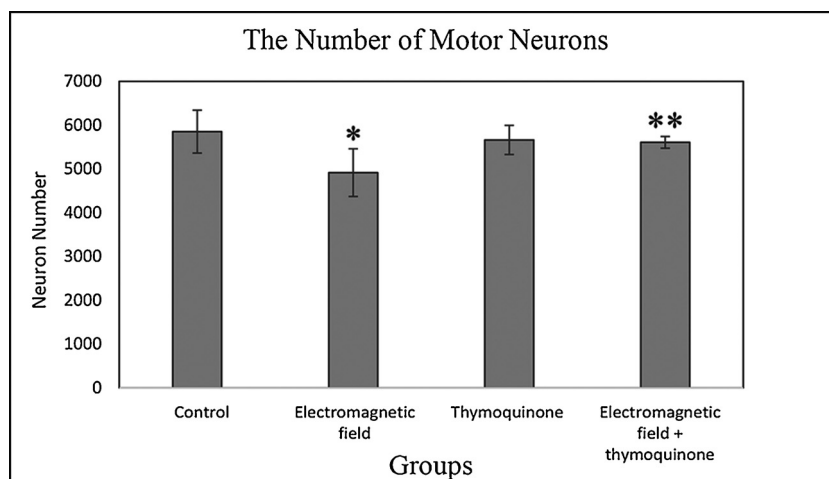


Fig. 5. The representative graph of the motor neuron number in the spinal cords of all groups. *, significantly different from the control group; **, significantly different from the electromagnetic field group.

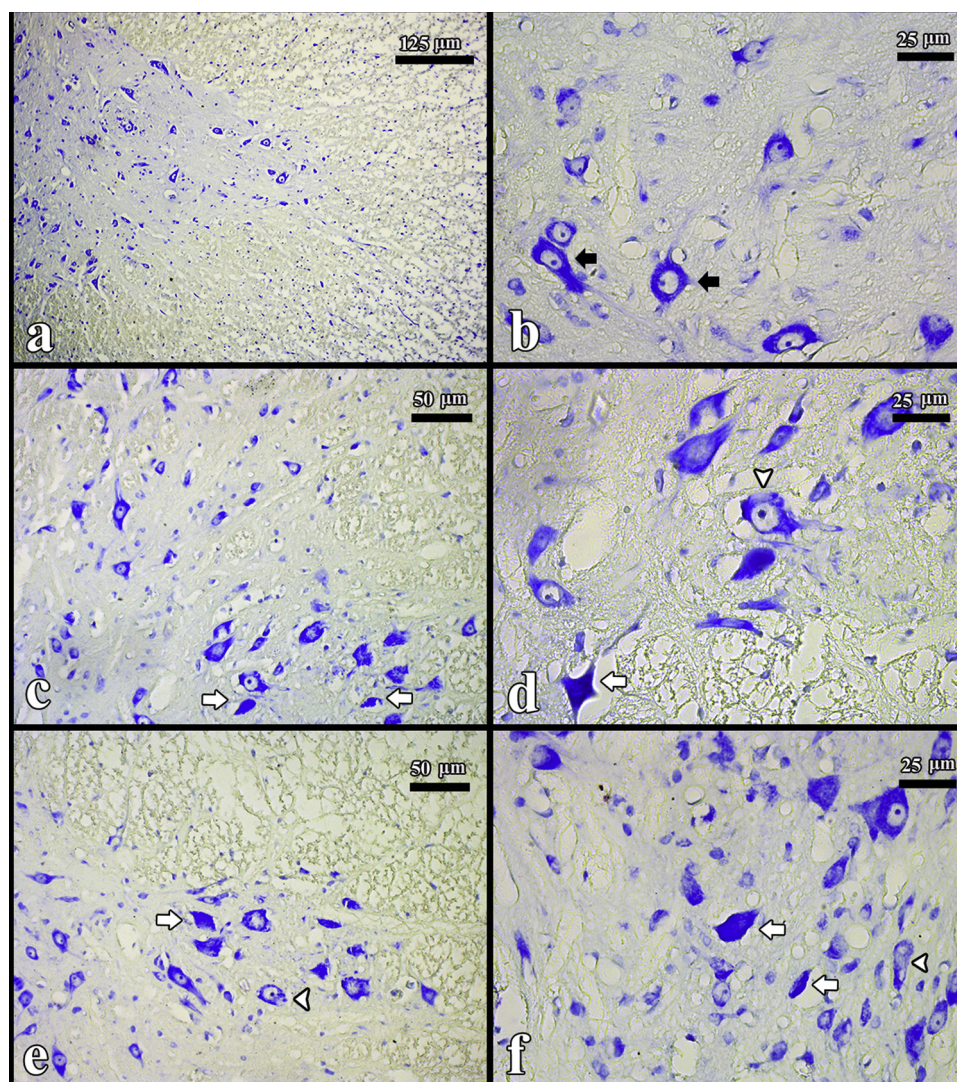


Fig. 6. Representative micrographs of spinal cords of the control (a and b) and the electromagnetic field (c–f) groups. Black arrows, healthy motor neurons; arrowhead, motor neurons with sparse Nissl substances; white arrow, degenerated and shrunken motor neurons.

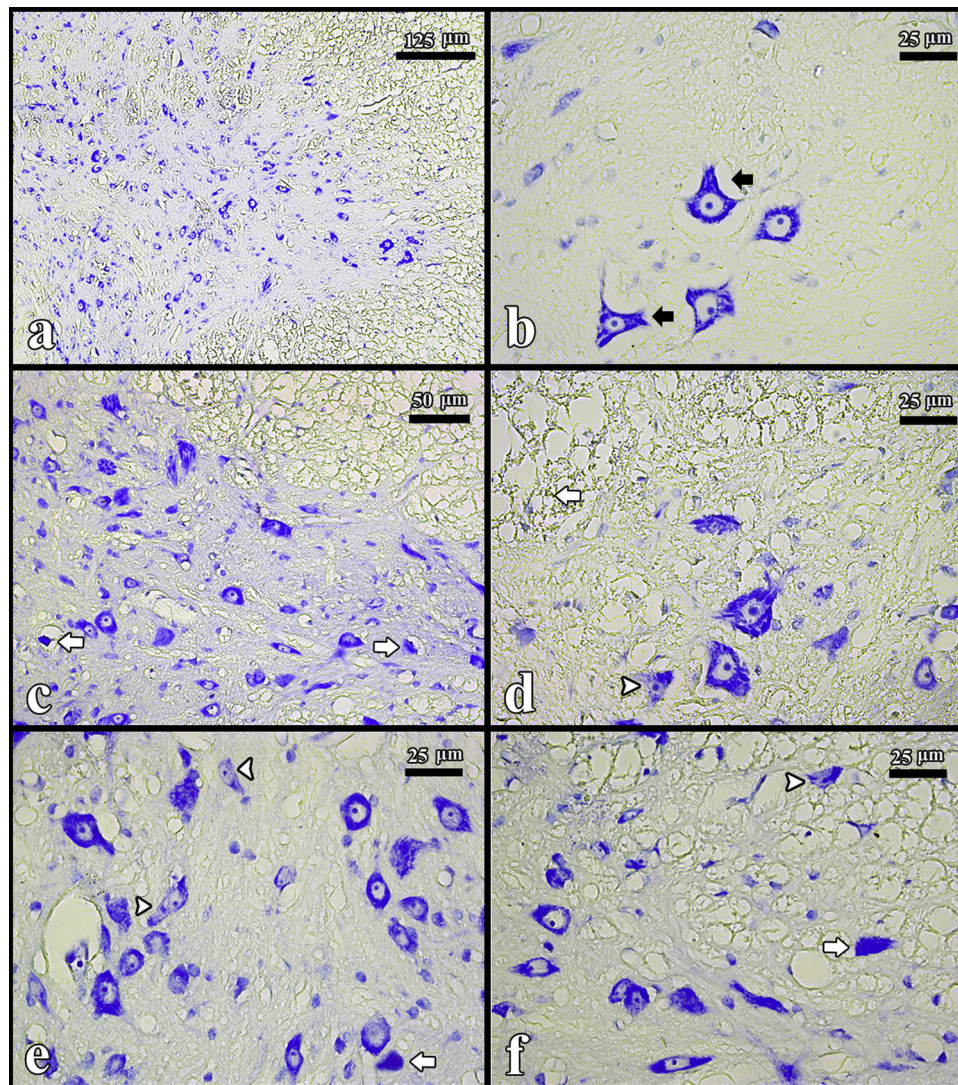


Fig. 7. Representative micrographs of spinal cords of the thymoquinone (a and b) and the electromagnetic field + thymoquinone (c–f) groups. Black arrows, healthy motor neurons; arrowhead, motor neurons with sparse Nissl substances; white arrow, degenerated and shrunken motor neurons.

electromagnetic field + thymoquinone ($p < 0.03$) group compared to the electromagnetic field group.

3.3. Histological and histopathological results

The intact motor neurons with prominent nuclei and nucleoli as well as Nissl substances was observed in the control group (Fig. 6a and b). To the contrary, structural alterations of motor neurons were obvious in the electromagnetic field group (Fig. 6c–f). We found regions where the motor neurons were degenerated and shrunken. Also, motor neurons with sparse Nissl substances were also detected somewhere. We observed no significant difference between the control and the thymoquinone groups (Fig. 7a and b). Motor neurons were in normal appearance in the thymoquinone group. Anomalies were also observed in the electromagnetic field + thymoquinone group, but these were less compared to those of the electromagnetic field group (Fig. 7c and f). We found some degenerated and shrunken motor neurons as well.

4. Discussion

Electromagnetic field exposure is one of the most important environmental agents that affect the living organisms, and many people are also consciously exposed to electromagnetic field radiation every

day (Mokarram et al., 2017; Fragopoulou et al., 2010). Researches regarding the biological effect of electromagnetic field radiation show controversial results. Some literatures suggest that exposure to electromagnetic field had no adverse effect on the blood-brain barrier and brain tumour risk (Muscat et al., 2000; Finnie et al., 2006). By contrast, numerous studies have also documented the detrimental effects of electromagnetic field radiation on central nerve system systems (Altun et al., 2017). Since spinal cord is a nervous tissue that carries messages between the peripheral nerve system and brain, any disorder in the structure of spinal cord possibly disturbs the entire central nerve system (Balaguru et al., 2012).

Studies that clarify the adverse effect of electromagnetic field radiation on spinal cord are very limited. To the best of our knowledge, our work was the first to survey the protective effect of thymoquinone on spinal cord following exposure to electromagnetic field by the stereological method.

In our study, the mean volume of central canal, grey and white matter as well as the number of motor neurons in the C6–C7 level of spinal cord were estimated. Based on unbiased stereological methods, the precise and accurate data were obtained (Altunkaynak et al., 2019). Our findings display that electromagnetic field impacts no significant volumetric changes matter in the electromagnetic field groups compared to the control group. We also found in the electromagnetic field

group; the total number of motor neurons was significantly less compared to the control group. This reduction was possibly due to oxidative stress that damages the motor neurons. Our finding is consistent with earlier study that 900 MHz electromagnetic field caused significant decrease in the both glia cells and neuron number in the rat spinal cord (Kerimoğlu et al., 2016). It is evident that physiological endogenous electric fields play a fundamental role in the function of the cell and subsequently the organ (Panagopoulos, 2013). As a result, interaction between physiological endogenous electromagnetic field and electromagnetic field emitted from the devices may alter the biological functions. Electromagnetic field radiation, however, through the formation of excessive levels of reactive oxygen species may disturbs the normal functioning of the central nerve system and causes the neurological damage (Yahyazadeh et al., 2018; Ertılav et al., 2018). On the other hand, spinal cord tissue is susceptible to reactive oxygen species possibly because the weak antioxidant capacity, lipid-rich content as well as high oxygen consumption (Hulbert et al., 2007). Accordingly, neuronal degradation may occur in reactive oxygen species-mediated peroxidation causing the oxidative damage (Salim, 2017). Reactive oxygen species also stimulates the apoptosis as the main manifestation of spinal cord damage (Uchida et al., 2012). Sahin et al. (2016) documented that exposure to electromagnetic field (6 h/day for 10 days) led to the oxidative damage to DNA in neurons.

In many literatures, the use of antioxidants has been recommended to alleviate the oxidative stress posed by electromagnetic field. Our findings reveal that there is a correlation between thymoquinone administration and architectural and biochemical alterations caused by electromagnetic field. We found that administration of antioxidant, thymoquinone, diminished the damage to motor neurons, resulting in the increased motor neuron number (Chen et al., 2018).

We found that exposure to electromagnetic field induced a significant increase in superoxide dismutase level in the electromagnetic field group compared to that of the control group. Our finding is consistent with the previous report that antioxidant enzyme activity of spine tissue was impaired due to the harmful effect of electromagnetic field radiation (İkinci et al., 2016). As a member of antioxidative defence system, antioxidant enzymes play a crucial role in alleviating of oxidative damage (Altun et al., 2017). Superoxide dismutase, an antioxidative enzyme, can enhance the breakdown of free radicals within cells. Increased superoxide dismutase level is in fact an antioxidative response to spinal cord disorder arisen from electromagnetic field; consequently, elimination of reactive oxygen species occurs in motor neurons. Kerimoğlu et al. (2016) suggested that the superoxide dismutase level was significantly altered in the lumbar spinal cords exposed to electromagnetic field. In present study, the reduced superoxide dismutase level in the electromagnetic field + thymoquinone group may have emanated from the protective effect of thymoquinone compared to the electromagnetic field group.

Our histological examination exhibited that the histological structures of spinal cords in the control group were in normal appearance. Marked structural alterations in the electromagnetic field group, including the degenerated and shrunken motor neurons, possibly derived from the destructive influence of electromagnetic field inducing the oxidative stress. Our histopathological results that is similar to the study of Kerimoğlu et al. (2016) that they revealed the damage to spinal cord neurons exposed to 900 MHz electromagnetic field. Transmission electron microscopic examination by İkinci et al. (2016) also exhibited the obvious loss of myelin sheath integrity and axon cytoplasm in rat spinal cord due to electromagnetic field. In the electromagnetic field + thymoquinone group, improvement of the structural deterioration possibly was due to the protective efficacy of thymoquinone. Findings concerning the antioxidant activity of thymoquinone is inconclusive. It has been reported that thymoquinone (10 mg/kg/day) had no ameliorative effect against histopathological damages to spinal cord (Üstün et al., 2014). To the contrary, neuroprotective effect of thymoquinone has been much reported in literatures (Gökce et al.,

2016).

5. Conclusion

In conclusion, our findings exhibit that 900 electromagnetic field radiation leads to the harmful effects on the cervical spinal cords of *wistar albino* rats; the loss of motor neurons was also proved by the stereological examination. Further, increased superoxide dismutase enzyme activity was indicative of the destructive changes resulted from reactive oxygen species. Administration of thymoquinone alleviated the hazardous influence of electromagnetic field by improving the antioxidant status. Besides, further studies should be carried out regarding antioxidant features of thymoquinone in association with neurotoxicity.

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Ethical statements

Our ethical approval was granted by Laboratory Animal Ethics Committee of Ondokuz Mayıs University.

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