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Received: 10 July 2020 | Revised: 10 August 2020 | Accepted: 13 August 2020

DOI: 10.1111/and.13834

ORIGINAL ARTICLE

First International Journal of Andrology
aNDROLOGIa
WILEY

The protective effect of melatonin on radiofrequency electromagnetic fields of mobile phone-induced testicular damage in an experimental mouse model

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Funding information
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Abstract

Radiofrequency electromagnetic radiation (RF-EMR) from mobile devices has undesirable effects on the male reproductive organs. Melatonin with antioxidant potential can help to prevent these damages. Therefore, the aim of this study was to evaluate the protective effect of melatonin on testicular damage induced by RF-EMR of mobile phone. In this experimental study, 32 adult male BALB/c mice were divided randomly into four groups: control, melatonin (2 mg/kg, for 30 consecutive days, intraperitoneally), RF-EMR (900 MHz, 100 to 300 MT, 54 to 160 W/m) (4 hr per day, whole body) and melatonin + RF-EMR groups. One day after the last prescription were evaluated oxidative stress parameters, testosterone level and histopathological assays of the testis. EMR of mobile phone led to the induction of oxidative stress, testicular tissue damage and decreased testosterone. Treatment with melatonin improved oxidative stress parameters such as MDA and GSH, and testis injury score, increased the thickness of the germinal epithelial thickness and diameter of the seminiferous tubule, and decreased testosterone hormone in the EMR-exposed mice, and these differences were significant($p < .05$). Data showed that melatonin with its antioxidant property can decrease oxidative damage induced by RF-EMR of mobile phones on testis tissue.

KEYWORDS
cell phone, electromagnetic radiation, melatonin, oxidative stress, testicular toxicity

1 | INTRODUCTION

Today, the use of wireless devices such as Internet routers and the global system for mobile communication (GSM) are growing rapidly and people use them almost every day. That is why we have to worry about its safety (Valberg, Deventer, & v., & Repacholi, M. H., 2007). On the other hand, approximately 48.5 million couples suffer from infertility, and 20 to 30 per cent of them are due to male infertility without any known cause (Agarwal, Mulgund, Hamada, & Chyatte, 2015). Some studies confirmed that the nonthermal mechanism is the cause of the destructive effect of radiofrequency electromagnetic radiation (RF-EMR) (Nizhelska, Marynchenko, & Piasetskyi, 2020), such as reactive oxygen species (ROS), which leads to lipid peroxidation and induction of oxidative stress (Oyewopo, Olaniyi, Oyewopo, & Jimoh, 2017) and apoptosis (Li et al., 2020). Malondialdehyde (MDA) is a final lipid peroxidation product that has toxic effects on tissues such as the kidney, liver, blood, heart, brain and spermatozoa after exposure to EMR (Adebayo, Adeeyo, Ogundiran, & Olabisi, 2019; Berköz et al., 2018). Gonads are sensitive to environmental stresses such as EMR and heat (Alkis, Akdag,

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Dasdag, Yegin, & Akpolat, 2019). Many studies show that EMR exposure can affect on the quality of semen and reduce sperm count, change sperm motility and histological structure of seminiferous tubule, finally leading to infertility in human and animal model (Al-Bayyari, 2017; Gautam et al., 2019). Also, EMR reduces blood testosterone through oxidative stress and its effect on Leydig cells (Lin et al., 2018).

Melatonin (N-acetyl-5-methoxytryptamine) (MEL) is a neurohormone that is synthesised and released from the pineal gland during the dark period and acts as an antioxidant (Hacışevki & Baba, 2018). Melatonin is known as a free radical scavenger (Reina & Martínez, 2018) that has the protective potential for many environmental stresses (Santosh & Prianka, 2020). Not only melatonin is a free radical scavenger, but also easily cross from the cell membrane with having MT1 and MT2 receptors and its lipophilic and hydrophobic properties (Fernando & Rombauts, 2014). Several studies have examined the protective effect of melatonin on testicular damage caused by EMR induced by microwave or wireless devices (Oksay et al., 2014; Sokolovic et al., 2015). Due to its antioxidant property, melatonin has an inhibitory effect on pre-meiotic spermatogenesis arrest in germ cells and improves the reparative pathway of DNA (Pandey & Giri, 2018). Damage to the reproductive system is very dangerous because it can affect fertility. Based on the above, the effect of melatonin on testicular damage caused by EMR of cell phones has not been investigated but was used a generator-amplifier for the generation of electromagnetic field (Pandey & Giri, 2018). So, the aim of this study was to investigate the protective effect of melatonin on testicular damage caused by EMR of mobile phones.

2 | MATERIALS AND METHODS

2.1 | Experimental design

In this experimental study, 32 male BALB/c mice (8/group) with 25–30 g weight were purchased from Mazandaran University of Medical Sciences Research Center. One week before the beginning of the study, all of them were housed under laboratory conditions and were provided with food and water; the period of darkness/brightness was 12:12 hr with 55% ± 5% humidity and 22–24°C temperature. All the experimental methods were performed in accordance with the Institutional Animal Ethics Committee of the Mazandaran University of Medical Sciences, Mazandaran, I.R. Iran **(ID: IR.MAZUMS.REC.1398.879)**. After adaptation to the laboratory environment, the mice were randomly divided into four groups (8 mice/group):

Group I: normal saline (Control), mice received normal saline intraperitoneally as the same volume of other groups.

Group II: melatonin (MEL), mice received melatonin at 2 mg/kg dosage intraperitoneally for 30 continuous days.

Group III: electromagnetic radiation (EMR), mice were exposed with electromagnetic radiation of mobile phones (900 MHz, 100 to 300 MT, 54 to 160 W/m) for 30 continuous days, 4 hr per day.

Group IV: electromagnetic radiation and melatonin (MEL + EMR), mice received 2mg/ Kg of melatonin daily, 1h before exposure to the EMR of mobile phone.

The dose of melatonin and radiation was determined based on previous studies (Djordjevic et al., 2015; Sokolovic et al., 2015). The experiment was repeated twice during the study period.

The irradiation system includes a plastic cage (40 × 40 × 30 cm) and a mobile device (having a SAR value of 1.3 watts/kg for the head area and SAR equivalent to 1.43 kb/kg for the whole body). A continuing wave signal with MHZ 900 was emitted from the cell phones. The cell phone was placed on animal cages during experiments. At this time, the mice were able to move freely in their cages during the radiation period. During irradiation, the cell phone was in conversation mode and a pre-recorded voice was playing in the environment. The phone was fully charged before it was started. The control group had the same condition, but the mobile phone was in airplane mode. A summary of the experimental design is represented in Figure 1.

2.2 | Sample collection

One day after the experiment period (on day 31), the mice were anaesthetised with xylazine 5 mg/kg and ketamine 50 mg/kg. The blood sample for analysing testosterone was collected from the heart and transferred to a serum separator tube, and then was centrifuged at 402 *g* for 15 min and stored in −40°C freezer. Afterwards, animals were sacrificed by displacement of the neck, shaved inguinal area, removed both testicles from the scrotum and recorded their weight. One of the testes was transferred in liquid nitrogen and then was frozen in −80°C for histochemical assay, and another testis was fixed in 10% formalin for histological assessment.

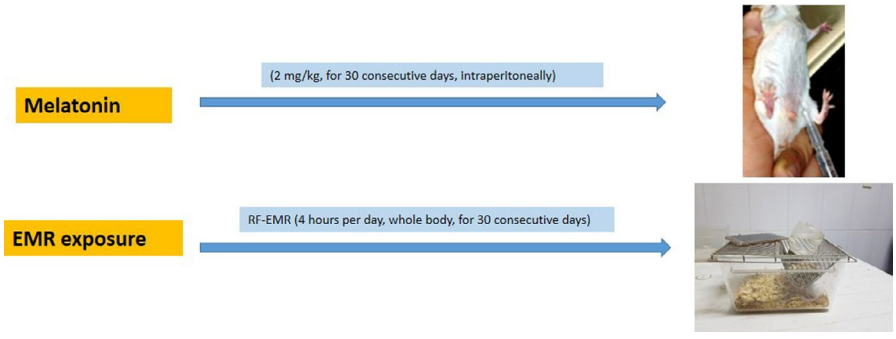


FIGURE 1 Study design diagram for the effect of melatonin on testicular damage induced by RF-EMR of cell phone

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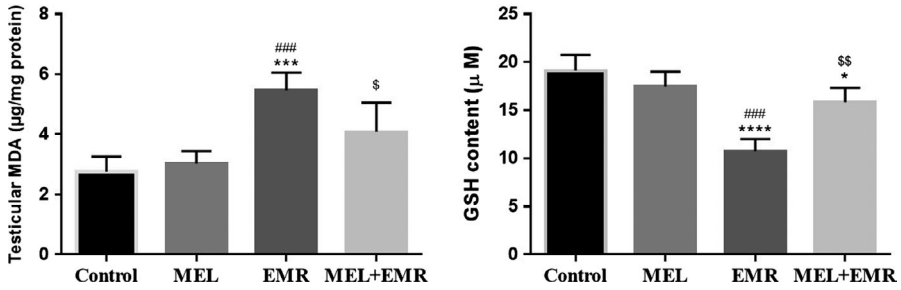


FIGURE 2 Effect of melatonin on MDA and GSH levels in testis damage induced by EMR in mice. All values were expressed as mean ± standard deviation. * is significant against the control group, # is significant against the melatonin group, and $ is significant against the group exposed to electromagnetic radiation (*, $p < .05$; **, $p < .01$; ***, $p < .001$). MEL: melatonin; EMR: electromagnetic radiation; MDA, malondialdehyde; GSH, glutathione

2.3 | Analysis of MDA

To analyse the oxidative stress, two of the oxidative stress markers, MDA and GSH, were evaluated in testicular tissue. The concentration of lipid peroxidation in the testis was measured by spectrophotometry using thiobarbituric acid (TBA). To begin, 0.25 ml of phosphoric acid (0.05 mol) was mixed with 0.2 ml sample, and then, 0.3 ml of 0.2% thiobarbituric acid (TBA) was added. Samples were placed in a boiling water bath for 30 min. Then, the sample tubes were immediately transferred to the ice bath, and afterwards, 0.4 ml of n-butanol was added to each sample. The resulting mixture was centrifuged (3,500 rpm for 10 min), and the MDA was measured based on reaction with thiobarbituric acid. The ʿreated MDA was calculated for each sample in the supernatant (490 nm) using an ELISA reader (Tecan, Rainbow Thermo, Austria). The content of MDA was expressed as nmol/mg protein. Tetramer propane (TEP) was used as a standard in this test (Karimi, Hosseinimehr, Mohammadi, Khalatbary, & Amiri, 2018).

2.4 | Analysis of GSH

Glutathione (GSH) content in the testis tissue was measured using a spectrophotometer (UV-1601 PC, Shimadzu, Japan) and 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) as an index at 412 nm, and was expressed as (μM) (Karimi et al., 2018).

2.5 | Testosterone assessment

The level of serum testosterone was determined with a diagnostic kit with its company instructions (Mouse Testosterone ELISA Kit, Bioassay, Cat. No. E0260MO). A quantitative diagnosis was performed in serum using the photometric method.

2.6 | Histopathology assay

For histological analysis, samples were fixed for 24 hr, processed in alcohol series, immersed in paraffin and clarified in xylene. Then, sections with 5-micron thickness were stained with haematoxylin and eosin. The histological structure of the testis was analysed blindly by a histologist with an optical microscope. For semi-quantitative analysis, tissue micrographs were analysed using a scoring system. Spermatogenic cell lineages, Leydig and Sertoli cells, were investigated. Seminiferous tubules that had round cross-section scored with Johnson's score system. In this system, each tubule takes a score from 1(very poor) to 10 (great) (Naeimi et al., 2017).

2.7 | Histomorphometric assay

For quantitative analysis, the average diameter of seminiferous tubules (from the basal membrane to basal membrane) and germinal epithelial thickness of seminiferous tubules (from the basal membrane to the lumen) were measured. Seminiferous tubules were evaluated in the 10 seminiferous tubules of each section and 10 sections from each group. We used a calibrated OLIESIA Soft Imaging System GmbH System (version 3.2 japans) with × 40 magnifications.

2.8 | Statistical analysis

Data were analysed by Prism software (GraphPad, version 6.07, USA). All of the data normally distributed are presented as the mean ± standard deviation ($M \pm SD$). One-way analysis of variance and Tukey post-test were used. p-values < .05 were considered to be statistically significant.

3 | RESULTS

3.1 | The effect of melatonin on oxidative stress markers in EMR-exposed mice

MDA as a final product of lipid peroxidation is shown in Figure 2 that was higher in the EMR group, and this increase was significant compared to the control and MEL alone groups ($p < .001$). Treatment with melatonin could decrease MDA in EMR-exposed mice (MEL + EMR), and it was significant compared with the EMR alone group ($p < .05$).

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GSH is one of the endogenous antioxidants that protect the body from oxidative stress and is shown in Figure 1. GSH was decreased in the EMR-exposed mice compared to the control ($p < .0001$) and MEL ($p < .001$) groups, and it was significant. MEL treatment increased GSH level in EMR-exposed mice compared with the EMR alone group ($p < .01$).

3.2 | The effect of melatonin on histomorphometry of testis in EMR-exposed mice

Seminiferous tubule diameter and germinal epithelial thickness as parameters that show sperm production are shown in Table 1. EMR decreased epithelial thickness compared to the control and MEL groups ($p < .01$). MEL treatment could improve epithelial thickness compared to the EMR group, and it was significant ($p < .05$).

As expected, the average diameter in the EMR-exposed mice decreased compared to MEL and the control groups ($p < .001$). MEL treatment could improve the seminiferous tubule diameter in EMR-exposed mice. This difference was statistically significant ($p < .05$).

3.3 | The effect of melatonin on serum testosterone in EMR-exposed mice

The level of serum testosterone is shown in Figure 3. Testosterone plays a key role in the development of the male reproductive system that EMR decreased it compared with the control ($p < .0001$) and MEL groups ($p < .01$). Also, treatment with MEL increased testosterone concentration compared to the EMR alone group ($p < .05$), but this improvement did not reach to the control group ($p < .01$). The MEL alone group could decrease testosterone levels compared to the control group ($p < .05$).

3.4 | The effect of melatonin on histopathological changes in EMR-exposed mice

The photomicrography of the testicular structure in all groups is shown in Figure 4. Normal histomorphologic features such as spermatogonia, primary spermatocytes and spermatids with normal seminiferous tubules can be seen in the control group (Figure 4a). Testis tissue in the MEL group (Figure 4b) was almost similar to the control group.

Testis samples in EMR-exposed mice revealed many histological changes such as degeneration, disorganisation and desquamation of spermatogenesis cell lineages in the ST, dilatation of seminiferous lumen, congestion, oedema in the interstitial tissue and vacuolisation of cytoplasm in the spermatogonia and Sertoli cells (Figure 4c). MEL treatment relatively improved the histological structure of testis in EMR-exposed mice (Figure 4d).

3.5 | The effect of melatonin on testis injury score in EMR-exposed mice

The mean of the testis injury score is shown in Figure 5. Johnson's score helps to estimate the extent of testicular tissue damage, and semi-quantitative data are obtained from histological micrographs. EMR decreased testicular injury score (5.8 ± 0.84) compared to the control (9 ± 0.71) ($p < .0001$) and MEL groups (8.2 ± 0.45) ($p < .001$). But MEL treatment in the EMR-exposed mice could decrease the score of testicular injury (7.6 ± 0.55) compared to the EMR alone group $p < .01$), although the score in the MEL + EMR group is lower than Control ($p < .05$).

4 | DISCUSSION

Many recent studies showed a possible relationship between mobile phone radiation and testicular damage that led to male infertility (Gautam et al., 2019). In this study, we showed that EMR exposure increased oxidative stress, reduced testosterone and altered testicular tissue structure. Also, melatonin was able to improve these changes.

We found that mice were exposed to EMR for four hours per day and for a month; in the histological structure of the severely damaged testis, a sharp decrease in spermatozoa was seen in the lumen of the seminiferous tubule. Wdowiak et al. showed exposure to EMR of cell phones increases the percentage of abnormal sperm cells, and the severity of this damage was related to the duration of exposure to the radiation that was evolved by the phone call (Wdowiak, Wdowiak, & Wiktor, 2007). They also stated EMR of mobile phones decreases sperm count, but motility and morphology of spermatozoa are normal. But most of these injuries were seen in the group that

TABLE 1 Effect of melatonin on epithelial thickness and seminiferous tubule diameter in testis damage induced by EMR in mice

Groups	Control	MEL	EMR	MEL + EMR
Epithelial thickness (μM)	91/3 ± 19/6	88 ± 11/2 **	49/7 ± 7/1 ##	75/5 ± 5/1 $
Seminiferous Tubule (μM)	347/3 ± 41/9	324/7 ± 29/2	180 ± 56/3 *** ###	236/2 ± 16/9 $ #

Note: All values were expressed as mean ± standard deviation. * is significant against the control group, # is significant against the melatonin group, and $ is significant against the group exposed to electromagnetic radiation (*, $p < .05$; **, $p < .01$). MEL: melatonin; EMR: electromagnetic radiation.

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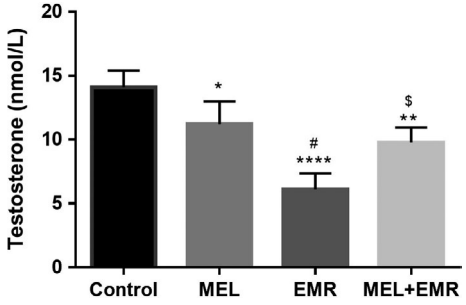


FIGURE 3 Effect of melatonin on serum testosterone levels in testicular damage induced by EMR in mice. All values were expressed as mean ± standard deviation. * is significant against the control group, # is significant against the melatonin group, and $ is significant against the group exposed to EMR (*, $p < .05$; **, $p < .01$; ***, $p < .001$, ****$p < .0001$). MEL: melatonin; EMR: electromagnetic radiation

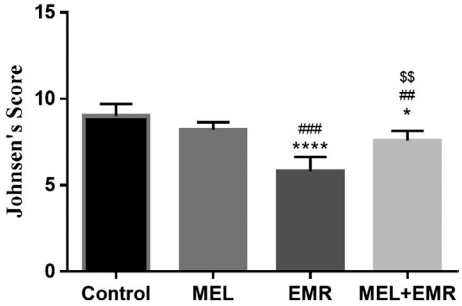


FIGURE 5 Effect of melatonin on Johnson's testicular injury score in EMR-exposed mice. All values were expressed as mean ± standard deviation. * is significant against the control group, # is significant against the melatonin group, and $ is significant against the group exposed to electromagnetic radiation (*, $p < .05$; **, $p < .01$; ***, $p < .001$, ****$p < .0001$). MEL: melatonin; EMR: electromagnetic radiation

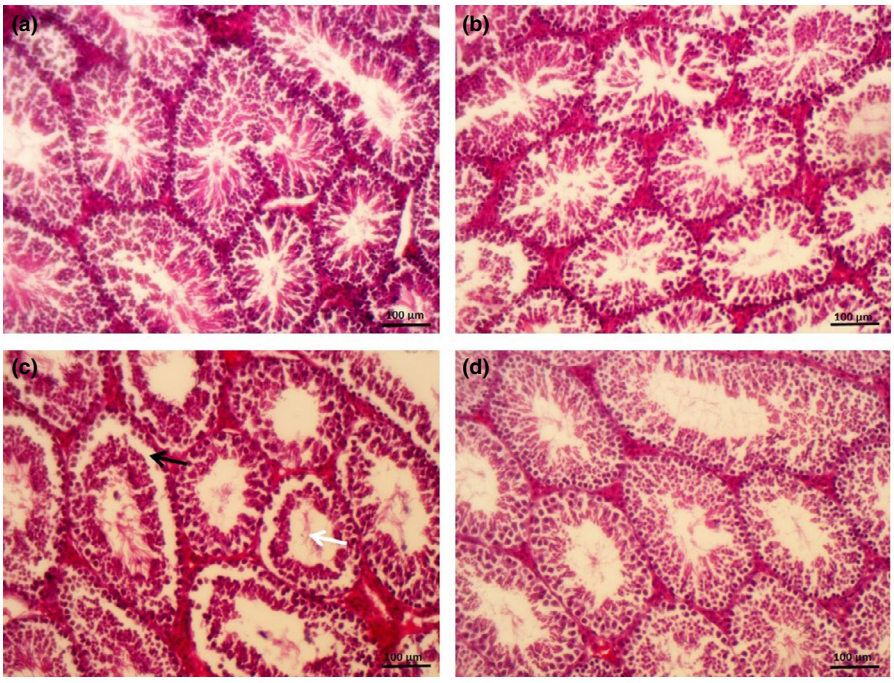


FIGURE 4 Photomicrographs show the effect of EMR and melatonin treatment on the histological futures of testis in the four groups. Control (A), MEL (B), EMR (C) and MEL + EMR (D). Normal structure can be seen in the control and melatonin groups. Desquamation (black arrow) and dilatation of seminiferous lumen (white arrow). H & E staining with a magnification of 20 ×, scale bar = 100µm. MEL: melatonin; EMR: electromagnetic radiation

was exposed to the EMR for 3 hr and was not seen in the group that was exposed for one and two hours. On the other hand, changes in oxidative stress markers were observed in all groups. Kesari et al. reported microwave radiation (MW) changes the reproductive cell cycle rate. They showed microwave radiation (MW) increases apoptosis statistically significant compared to the control group (Kesari, Kumar, & Behari, 2011).

We also analysed oxidative stress parameters. Our data showed that the amount of MDA increased and the amount of GSH decreased. MDA is the final product of lipid peroxidation that can be used as a marker of oxidative stress (Yurekli et al., 2006). Several studies showed that EMR can increase lipid peroxidation and therefore raises MDA (Ceyhan et al., 2012; Oyewopo et al., 2017). In the present study, we obtained the same result too. EMR not only can increase MDA but also can interrupt cell antioxidant capacity. GSH as an endogenous antioxidant acts as an intracellular antioxidant and clears ROS from the cytoplasm (Pompella, Visvikis, Paolicchi, De Tata, & Casini, 2003) and prevents apoptosis (Kıvrak, Yurt, Kaplan, Alkan, & Altun, 2017). Studies showed that EMR can weaken antioxidants such as GSH and can make cells vulnerable to oxidative stress (Kıvrak et al., 2017). In the present study, we concluded that EMR with the increase in ROS and lipid peroxidation can decline antioxidant capacity and therefore decrease GSH. Our result is as same as Pandey et.al. (2018) study, they admit that EMR exposure will decrease GSH 1.5-fold compared to normal conditions. MEL treatment mitigated oxidative stress in EMR-exposed mice. Melatonin is able to maintain biological membrane fluidity against free radicals via reducing oxidative stress (García et al., 2014).

Spermatozoa are very sensitive to oxidative stress because their membrane is full of polyunsaturated fatty acids (Aitken, Wingate,

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De Iuliis, Koppers, & McLaughlin, 2006; Wathes, Abayasekara, & Aitken, 2007), and many studies have shown that exposure to EMR leads to lipid peroxidation and cell damage (Al-Damegh, 2012; Esmekaya, Ozer, & Seyhan, 2011). Although the amount of antioxidants in the cytoplasm of spermatozoa is low (Agarwal, Saleh, & Bedaiwy, 2003), but when ROS is produced, it is cleared by antioxidants in the seminal plasma (Mahfouz, Sharma, Sharma, Sabanegh, & Agarwal, 2009). However, in stress conditions that the production of ROS increases, the antioxidant capacity of seminal plasma cannot clear ROS and methylate keap1 and these conditions are harmful for sperm production (Darbandi et al., 2019). Studies have shown that EMR is emitted from many devices (i.e. cell phone and Internet routers) lead to produce ROS (Erogul et al., 2006). Gautam et al stated EMR of mobile phones with the induction of oxidative stress and increased MDA, leading to changes in sperm membrane structure, morphology and sperm count, and ultimately infertility (Gautam et al., 2019).

Another change made by EMR exposure in this study is the reduction of seminiferous tubule diameter and germinal epithelial thickness. EMR with the increased oxidative stress can induce cell apoptosis and subsequently change some histomorphologic features in the seminiferous tubule structure (Azab, Khalat, Ebrahim, & Albasha, 2018). Ozguner et al. reported the mean germinal epithelial thickness in rats decreases when exposing to 900MHz radiation for 30 min/day for 5 days/week and for 4 weeks (Ozguner et al., 2005). Lee et al. also stated that 60Hz EMR, 24h/day for 8-week exposure, can decrease normal seminiferous tubules and increase spermatogonia apoptosis, DNA damage and germ cell death in the testis compared to the control group (Lee, Ahn, Jung, Kim, & Lee, 2004). But Oyewopo stated EMR of the cell phone with dual-band EGSM900/1,800 MHz for 1 and 2 hr per day shows no significant change in histomorphometric parameters, but 3-hr exposed radiation showed a significant decrease in germinal epithelial thickness and diameter of seminiferous tubule (Oyewopo et al., 2017). In our study, we observed similar results too. Exposure to EMR decreased epithelial thickness and the diameter of the seminiferous tubule in the testis of mice significantly.

Testosterone is an androgen hormone that mostly secretes from Leydig cells. Kaur et al showed that cell phones reduce testosterone through their effect on the pituitary gland and with decreased LH and FSH. Decreased testosterone leads to a reduction in sperm concentration, sperm motility and alteration in sperm morphology in exposed male rats (Kaur & Khera, 2018). Meo et al. showed that EMR can decrease serum testosterone levels after 30 and 60 days of exposure compared to the control group (Meo, Al-Drees, Husain, Khan, & Imran, 2010). Our study shows the same result, although, in Figure 3, melatonin itself can decrease serum testosterone compared to the control group significantly. Li et al explained the reason for the decrease in serum testosterone in a review study (Li & Zhou, 2015). Their study showed melatonin can reduce the secretion of GnRH and LH from the pituitary gland. These two neurohormones are responsible for testosterone production from Leydig cells.

In studies of the rat model, it has been reported that exposure to mobile phones makes negative morphological and histological alternations in the testes (Mugunthan, Shanmugasamy, Anbalagan, Rajanarayanan, & Meenachi, 2016; Salama, Kishimoto, Kanayama, 2010). Cetkin et al have shown that mobile phones, both in speech and in standby condition, lead to degeneration of the germinal epithelial separation from the tunica propria in some seminiferous tubules compared to the control and sham groups (Cetkin et al., 2017). It was also seen a decrease in Leydig cell in the interstitial tissue. In the present study, similar results were seen in the EMR group. The lumen of the seminiferous tubule widened, and the germinal layer destroyed and epithelial thickness decreased significantly compared to the control and melatonin groups.

For converting a qualitative concept to a quantitative one, Johnson's score can be used. Johnson's score is a semi-quantitative scoring system that helps us to have a better concept of testicular injury (Ozguner et al., 2005). Cetkin et al. showed a significant decrease in Johnson's score in both speech and standby groups compared to the control and sham groups (Cetkin et al., 2017). Hanci et al. also showed that 900MHz EMR exposure can significantly decrease Johnson's score compared to the control and sham groups (Hancı, Kerimoğlu, Mercantepe, & Odacı, 2018). In our study, we achieved the same result that EMR exposure with its cell destruction can affect Johnson's score.

Melatonin is a neurohormone released from the pineal gland and protects nuclear and mitochondrial DNA, membrane lipids and cytosolic proteins from oxidative damage. It as well can remove reactive nitrogen species and ROS and help to increase antioxidant enzymes (Oktem, Ozguner, Mollaoglu, Koyu, & Uz, 2005; Ozguner, Bardak, & Comlekci, 2006). EMR not only can reduce antioxidant capacity but also can decrease melatonin production that leads to more oxidative stress and making more ROS (Jarupat, Kawabata, Tokura, & Borkiewicz, 2003; Kumar, Kesari, & Behari, 2011). Meena et al. showed MDA levels significantly increase in MW-exposed mice compared to the control and MEL groups (Meena et al., 2014). Also, they showed that MEL treatment significantly reduces MDA in MW-exposed mice that this result can prove the antioxidant activity of melatonin against EMR oxidative exposure. Furthermore, this study reported that EMR can decrease testosterone levels compared to other groups, and treatment with melatonin can lead to an increase in testosterone levels. Our data showed that treatment with melatonin protects testicular cells to prevent the reduction of testosterone due to EMR exposure. Melatonin also can improve antioxidant enzyme levels too. Oksay et al. reported that EMR exposure can decrease GSH level, and treatment with melatonin can prevent this decrease, and also melatonin increases other antioxidant enzymes such as glutathione peroxidase (Oksay et al., 2014). Pandey et al expressed melatonin inhibits pre-meiotic spermatogenesis arrest in spermatogenic germ cells through its antioxidative activity and improves sperm parameters through DNA reparative pathways in radiofrequency radiation-exposed animals (Pandey & Giri, 2018).

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5 | CONCLUSION

Overall, according to present study results, EMR exposure can harm testicular tissue with oxidative stress induction. This concept showed itself as an increasing MDA level that melatonin treatment can decrease it. Furthermore, melatonin increases one of the intracellular antioxidants named GSH. Melatonin also protected testis from EMR-induced damage and increased testosterone levels that decreased due to EMR exposure, closer to the normal range. However, studies on other animal spices and comparisons with other antioxidants can provide more reliable results.

ACKNOWLEDGEMENTS

This research was supported by Molecular and Cell Biology Research Center, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran (ID: IR.MAZUMS.REC.1398.879).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

AUTHOR CONTRIBUTIONS

F.T.A. performed the experiments, analysed and interpreted the results, and drafted the manuscript. M.S. and M.E.S. performed the animal experiment and recorded data. A.K.M and F.T.A. contributed ideas for experimental design. All authors read and approved the final manuscript.

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How to cite this article: Shokri M, Shamsaei ME, Malekshah AK, Amiri FT. The protective effect of melatonin on radiofrequency electromagnetic fields of mobile phone-induced testicular damage in an experimental mouse model. *Andrologia*. 2020;52:e13834. https://doi.org/10.1111/and.13834

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