Effect of carnosine on ovarian follicle in rats exposed to electromagnetic field

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SUMMARY

The electromagnetic field (EMF) has an effect on various organs, including the female reproductive system. The purpose of this study was to evaluate the impact of carnosine on ovarian follicle number and diameter in rats exposed to a 900 Megahertz (Mhz) electromagnetic field. In this study, six different groups were used. 40 female rats divided into groups were evaluated. The ovaries of the rats were removed at the end of the study. Routine histological procedures were performed on ovarian tissues. Follicle number and diameter of all groups were calculated and evaluated under the light microscope. When primary follicle number and diameters were compared statistically among the groups, there was a remarkably meaningful difference between the EMF group and the control, 20 mg carnosine and EMF+20 mg carnosine groups ($p<0.05$). There were significant irregularities in the structure of the oocyte and the granulosa cells surrounding the oocyte, especially in the EMF-treated groups. However, the structure of the oocyte and the granulosa cells surrounding the oocyte in the EMF+20 mg carnosine group showed a more regular structure compared to the EMF group. In this study, it can be concluded that the number and diameter of ovarian follicles decreased in rats exposed to electromagnetic field and 20 mg of carnosine may prevent damage caused by EMF.

Key words: Electromagnetic field – Ovarian – Follicle number – Follicle diameter – Carnosine

INTRODUCTION

With the advancement of technology, the electromagnetic field (EMF) effect of many devices that facilitate daily life and the widespread use of these devices adversely affect human health (Feychting et al., 2005). People are constantly exposed to harmful environmental agents, including electromagnetic fields from household appliances (e.g., television, Wi-Fi and microwaves), diagnosis equipment (e.g., magnetic resonance imaging, tomography), industrial tools, smartphones and electronic devices. Also, the recent use of 6 billion mobile phones suggests that EMF is perhaps one of the most hazardous

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environmental factors for humans (Davis et al., 2013). Studies on the impacts of mobile phones on human health have reported that EMF, which is often associated with mobile phone use, causes sleep problems (Huber et al., 2002), fatigue, headaches, and loss of concentration (Oftedal et al., 2000). Cell phones are the most common sources of EMF used near the human body. Kilgallon and Simmons (2005) found a significant reduction in sperm motility in men who kept their mobile phone on their hip or waist field, compared to those who kept their mobile phone elsewhere or did not use one. According to this study, it can be thought that the tissues close to the EMF resources are more impressed. In addition, some studies have shown that EMF causes oxidative stress and DNA damage, leading to deterioration of the structure and function of the cell (Odacı and Ozyılmaz, 2015).

In some animal studies, it was concluded that long-term cell phone use may cause a decrease in sperm development and production, thus reducing fertility in men (Kesari et al., 2011). In their study, researchers found that there was a decrease in Leydig cell numbers in male rats (Saygin et al., 2011). Some researchers have noted that exposure to EMF can lead to embryonic developmental disorders or infertility issues. In one study, it was found that exposure to EMF was associated with miscarriage in women (Belyaev et al., 2016). It has been noted that exposure to radiofrequency EMF during and after pregnancy and breastfeeding does not cause developmental abnormalities in offspring (Shirai et al., 2017). In animal studies, it was shown that EMF decreases the number of follicles in the ovaries (Gul et al., 2009), causes DNA damage in the endometrium and ovary (Diem et al., 2005), increases apoptosis and oxidative stress (Nikolova et al., 2005; Oral et al., 2006).

Carnosine is a histidine derivative, a multifunctional dipeptide synthesized endogenously in the body. It is found in high concentrations in tissues such as heart muscle, skeletal muscle, and brain (Yay et al., 2013). Studies have reported that carnosine has a protective effect on cells against oxidative damage, and that carnosine has antioxidant properties by binding metal ions and scavenging free radicals (Bakardjiev and Bauer, 2000; Brownson and Hipkiss, 2000; Kohen et al., 1988). Also, it has been indicated that carnosine has protective effects against ischemia in tissues and organs such as kidney, brain, myocardium, skeletal muscle and spinal cord (Albayrak et al., 2015; Aydin et al., 2015). *In vivo* studies, it has been reported that carnosine reduces ischemia-reperfusion injury in organs such as testis, brain and kidney (Fujii et al., 2005; Dobrotvorskaya et al., 2011).

Although various frequencies have been used on the ovarian follicles of EMF as a result of the literature review, there is no study yet investigating the effects of 900 Megahertz (MHz) EMF with carnosine application (Khoshbakht et al., 2021; Altındag et al., 2017). The Global Mobile Communication System (GSM)-900 communication system, which is generally, it has an operating frequency of 880–960 MHz, as in Europe and Turkey. Therefore, the purpose of the study was to evaluate the effect of carnosine on ovarian follicle number and diameter in rats exposed to a 900 Mhz electromagnetic field.

MATERIALS AND METHODS

Ethical procedures, Work Plan, Animals and Groups

The work protocol was evaluated and confirmed by the Erciyes University Experimental Animals Ethics Committee (Kayseri, Turkey) (approval number: 2013/82). After getting approval from Erciyes University, the procedures were implemented. Forty female Wistar Albino rats weighing 200-250 g, 16 weeks old, were provided by Erciyes University Experimental Animals Research Center (DEKAM, Kayseri, Turkey). Rats were held in a normal 22 ± 2 °C and 50% ± 5% humidity environment for 12 hours on a light/dark cycle and ventilated with an aspirator. Rats were fed a balanced diet and unlimited water. Rats were placed in normal clear polycarbonate cages. All described experimental and surgical procedures were performed at Erciyes University Experimental Animals Research Center (DEKAM, Kayseri, Turkey).

The animals used were divided into 6 groups without any discrimination. The groups were created as follows.

1. Control group (K): Not exposed to any treatment, EMF, or carnosine injection (n = 10).
2. 2 mg Carnosine group (2 mg car): Rats were not exposed to EMF, but only to carnosine injection (n=5). The rats in this group were injected intraperitoneally with carnosine between 11:00 and 12:00 every day.
3. 20 mg Carnosine group (20 mg car): Rats were not exposed to EMF, but only to carnosine injection (n=5). The rats in this group were injected intraperitoneally with carnosine between 11:00 and 12:00 every day.
4. Electromagnetic field group (EMFG): Rats were exposed to EMF 1 h daily over 28 days (900 MHz) (Onger et al., 2016) (n = 10). Rats were exposed to EMF between 11:00-12:00 every day (Turedi et al., 2016). The specific energy absorption rate (SAR) was approximately 0.008 W/kg for the whole body and 2 W W/kg locally for the head. In addition, the positions of the rats were changed daily during the exposure period (Ulubay et al., 2015).
5. EMFG +2 mg carnosine (EMFG+2 mg car): exposed to EMF (900 MHz) 1 h daily over 28 days (Kerimoglu et al., 2016) (n = 5). During the experimental period, 2 mg intraperitoneal carnosine was administered to this group 30 minutes before EMF exposure (Bae et al., 2013).
6. EMFG +20 mg carnosine (EMFG+20 mg car): exposed to EMF (900 MHz) for 1 hour per day for 28 days (Kerimoglu et al., 2016) (n = 5). During the experimental period, 20 mg intraperitoneal carnosine was administered to this group 30 minutes before EMF exposure (Bae et al., 2013).

After the experimental procedure, the rats were sacrificed and the number of ovarian follicle cells was counted by performing histological studies.

Exposure System

In the current study, the same exposure system design was used as in last studies (Bas et al., 2009). The exposure system used and the EMF application are explained in detail (Kerimoglu et al., 2016; Ragbetli et al., 2007). A special EMF exposure system consisting of a dipole antenna and a circular cage was used (Aslan et al., 2017). The electromagnetic power unit producing 900 MHz continuously regulated EMF (2 W peak output power and 1 ± 0.4 mW/cm^2 power volume) was made in TEKNOPARK Conformity Laboratory (Kayseri, Turkey). The average SAR rate was 2 W/kg and the peak SAR was calculated based on the model with force density indicators made using an EMF meter (Kayseri Technopark) (Bas et al., 2009). Rats were exposed to EMF using a dipole antenna (Bas et al., 2009). The heads of the rats were placed in the direction of the dipole antennae. The gap between the rat and antenna was 1 cm when the rat was placed perpendicular to the antenna. The longitudinal axis of the rats was placed perpendicular to one of the antennae (Bas et al., 2009). All reviews were blinded to the unbiased results obtained.

Histological Procedures

At the end of experimental work, right and left ovaries were removed under anesthesia (Ketolar 50 mg, Pfizer, Turkey). For histopathological examination, ovarian tissues obtained from control and experimental groups were fixed in 10% formaldehyde solution. After fixation, the tissues were embedded in paraffin. Hematoxylin Eosin routine histological (Table 1) follow-ups were performed by taking 5 µm thick sections from the paraffin blocks with a microtome. In order to see the general histological structure, the sections were stained with Masson's trichrome (MT) (Table 2) and examined under the Olimpus BX51 microscope and photographed. Evaluation parameters of follicles were evaluated according to Table 3.

Statistical analysis

All statistical analyses were done in SPSS 22 program. After testing the normality (Kolmogorov-Smirnov and Shapiro-Wilk) of the research data, comparisons between groups were made using the OneWay Analysis of Variance (ANOVA) for normally distributed variables, and multiple comparisons were made with the Tukey test in case of difference. In the variables that did not show normal distribution, comparisons

between groups were made using the Kruskal-Wallis Analysis, and in case of difference, multiple comparisons were made with the Mann Whitney U test. A p value of <0.05 was considered statistically meaningful.

Table 1. Light microscopy tissue preparation technique.

Sequence	Action taken	Duration	Sequence	Action taken	Duration
1	Tap water	1 hour	**7**	Absolute Alcohol	1 hour
2	50% Alcohol	1 hour	**8**	Absolute Alcohol	1 hour
3	%70 Alcohol	1 hour	**9**	Xylene	20 minutes
4	%80 Alcohol	1 hour	**10**	Xylene	20 minutes
5	%96 Alcohol	1 hour	**11**	Xylene	20 minutes
6	Absolute Alcohol	1 hour	**12**	Melted paraffin (60 °C)	1 night

Table 2. Masson's trichrome dyeing technique.

Sequence	Transaction Time	Duration	Sequence	Transaction Time	Duration
1	Oven (60°C)	2 hours	**18**	Phosphomolybdic acid	5 min
2	Xylene I	10 min	**19**	Drying	
3	Xylene II	10 min	**20**	Aniline blue	2-5 min
4	Xylene III	10 min	**21**	Distilled water	2-3 min
5	Absolu Alcohol I	5 min	**22**	%1 lyacetic acid	2 min
6	Absolu Alcohol II	5 min	**23**	%50 Alcohol	5 min
7	%96 Alcohol	5 min	**24**	%70 Alcohol	5 min
8	%80 Alcohol	5 min	**25**	%80 Alcohol	5 min
9	%70 Alcohol	5 min	**26**	%96 Alcohol	5 min
10	%50 Alcohol	5 min	**27**	Absolu Alcohol I	1 min
11	Stream	2 min	**28**	Absolu Alcohol II	2 min
12	Hematoxylin	5-8 min	**29**	Absolu Alcohol III	2 min
13	Stream	5 min	**30**	Xylene I	20 min
14	%1 acid alcohol	1-2 sec	**31**	Xylene II	20 min
15	Stream	1 min	**32**	Xylene III	20 min
16	Asit fuksin	5 min	**33**	Closure (Canada Balsam)	
17	Distilled water	2-3 min			

Table 3. Evaluation parameters of ovarian follicles.

Follicles to be counted	**Primordial follicle:** oocyte with a prominent nucleus and a squamous granulosa cell layer around it
	Primary follicle: oocyte with a prominent nucleus and a layer of cubic granulosa cells around it
	Preantral follicle: oocyte with a prominent nucleus and several layers of granulosa cells around it
	Secondary follicle: oocyte with a prominent nucleus, several layers of granulosa cells around it, and antrum within granulosa cells
	Tertiary follicle: oocyte with prominent nucleus observed pushed aside in the antrum

RESULTS

The statistical results of primary, preantral, secondary and tertiary follicle numbers of the experimental and control groups were given in Table 4. When the mean primary follicle numbers were compared statistically, it was determined that there was an important dissimilarity among the control group and both the 20 mg carnosine group and the EMF group ($p<0.05$). There was no dissimilarity among the mean primary follicle numbers of the groups given 2 mg carnosine and the groups given EMF+20 mg carnosine ($p>0.05$). But there was a statistically significant difference between the two groups and the EMF group ($p<0.05$). When the mean preantral follicle numbers were compared statistically, it was determined that there was no meaningful dissimilarity between the control and 20 mg carnosine groups and the other groups ($p<0.05$). But there was an important dissimilarity between the group exposed to 2 mg carnosine and the EMF group ($p<0.05$). When the mean secondary follicle numbers of the control and experimental groups were compared statistically, there was no dissimilarity among the groups ($p>0.05$). When a comparison was made between the groups in terms of tertiary follicle numbers, it was determined that the only dissimilarity was among the control group and the EMF group ($p<0.05$).

The statistical results of primary, preantral, secondary and tertiary follicle diameters of the experimental and control groups were given in Table 5. When primary follicle diameters were compared statistically among groups, there was an important difference among the control group and the EMF and EMF+2mg carnosine groups ($p<0.05$). There was no significant dissimilarity among the group given 2 mg carnosine and the other groups ($p>0.05$). However, when the group given 20 mg carnosine was compared with the other groups, it was determined that the only dissimilarity was with the EMF group ($p<0.05$). In addition, there was a statistically significant dissimilarity among the EMF group and the control, 20 mg carnosine and EMF+20 mg carnosine groups ($p<0.05$). When the preantral follicle diameter was compared statistically between the groups, there was no meaningful dissimilarity among the control and experimental groups ($p>0.05$). It was defined that there was a statistically important dissimilarity

Table 4. Primary, preantral, secondary and tertiary follicle numbers of control and experimental groups.

	Control	2 mg carnosine	20 mg carnosine	EMF	EMF+2 mg carnosine	EMF+20 mg carnosine	p
Number of Primary Follicles	245±33,56^{a}	216±26,42^{ab}	202±19,34^{bc}	172±34,00^{c}	210±30,97^{abc}	214±38,30^{ab}	,000
Number of Preantral Follicles	73±17,55^{abc}	80±13,57^{ac}	69±12,07^{abc}	59±9,96^{b}	66±17,98^{cb}	73±13,74^{ab}	,058
Number of Secondary Follicles	58±5,87^{a}	56±9,52^{a}	53±7,96^{a}	50±6,19^{a}	52±20,56^{a}	58±16,89^{a}	,509
Number of Tertiary Follicles	7±1,44^{a}	9±1,89^{ab}	7±3,77^{ab}	3±3,37^{b}	7±3,52^{ab}	6±1,66^{ab}	,002

The same letters on the same line indicate the similarity between the groups, and different letters indicate the difference.

Table 5. Primary, preantral, secondary and tertiary follicle diameters of the control and experimental groups.

	Control	2 mg carnosine	20 mg carnosine	EMF	EMF+2 mg carnosine	EMF+20 mg carnosine	p
Primary Follicle Diameter (n=70)	0,41±0,17^{a}	0,37±0,12^{abc}	0,38±0,15^{ac}	0,31±0,09^{b}	0,33±0,13^{cb}	0,38±0,10^{ac}	,000
Preantral Follicle Diameter (n=70)	0,61±0,17^{abc}	0,64±0,23^{a}	0,61±0,21^{abc}	0,54±0,12^{b}	0,55±0,11^{cb}	0,60±0,14^{abc}	,004
Secondary Follicle Diameter (n=70)	1,45±0,46^{a}	1,19±0,45^{b}	1,33±0,49^{ab}	1,19±0,49^{b}	1,34±0,47^{ab}	1,30±0,51^{ab}	,012
Tertiary Follicle Diameter (n=30)	3,09±0,60^{a}	2,80±0,45^{a}	3,15±0,71^{a}	2,18±0,36^{b}	2,84±0,58^{a}	3,06±0,67^{a}	,000

The same letters on the same line indicate the similarity between the groups, and different letters indicate the difference.

among the 2 mg carnosine group and the EMF and EMF + 2 mg carnosine groups ($p<0.05$). When the mean secondary follicle diameters were compared between the groups, it was determined that the statistical dissimilarity occurred among the control group and the group given 2 mg carnosine and the EMF group ($p<0.05$). But there was no dissimilarity among the EMF group and the groups given EMF+2 mg carnosine ($p>0.05$). When the mean tertiary follicle diameters were compared statistically, it was found that there was a statistically remarkable dissimilarity among the EMF group and all other groups ($p<0.05$).

Photomicrography of primary, preantral, secondary and tertiary follicles of the experimental and control groups were given in Figure 1. When the light microscopic images of primary, preantral, secondary and tertiary follicles of the control and experimental groups were examined, the histological images of the control, 2 mg carnosine and 20 mg carnosine groups showed similar structures. There were important irregularities in the structure of the oocyte and the granulosa cells surrounding the oocyte, especially in the EMF-treated groups. However, in the EMF+2 mg carnosine group, there were irregularities in the oocyte and granulosa cells around the oocyte, especially in the secondary and tertiary follicles, as in the EMF group. But the structure of the oocyte and the granulosa cells surrounding the oocyte in the EMF+20 mg carnosine group showed a more regular structure compared to the EMF group.

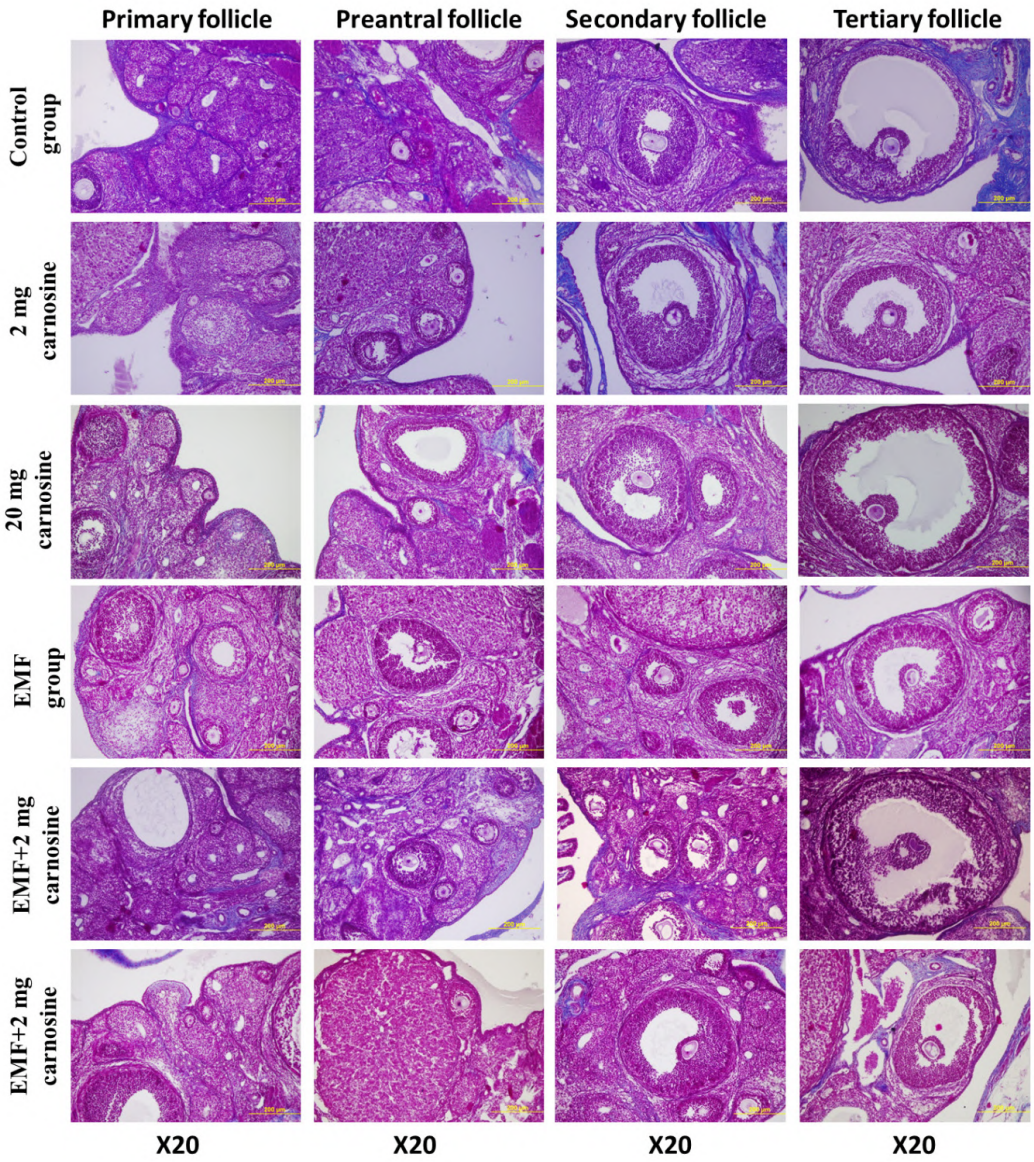

Fig. 1.- Photomicrography of primary, preantral, secondary and tertiary follicles of control and experimental groups (x20).

DISCUSSION

This study demonstrated that exposure to EMF can reduce the mean ovarian follicle count and that this reduction can be ameliorated with higher carnosine dosages. Our findings showed that rats exposed to EMF had a notable lower number of ovarian follicles compared to the control group. Additionally, the structure of the oocyte and the granulosa cells surrounding the oocyte in the groups administered 20 mg carnosine showed a more regular structure compared to the EMF group. The scientists noted that prenatal exposure to 900 MHz EMF could cause pathological changes in the seminiferous tubules in 60-day-old rats and compromise the spermatogenic cycle. They observed that the diameters and epithelial thickness of the seminiferous tubules decreased (Odacı et al., 2016). Studies have shown that environmental electric and magnetic fields can cause deoxyribonucleic acid (DNA) damage and prolong the life of free radicals (Lai and Singh, 2004). Ahmadi et al. (2016) pointed out that EMF radiation has detrimental effects on the implantation of the ovule and the formation of ovarian follicles. Agarwal et al. (2009) examined whether exposure to mobile phones for 1 hour affected human ejaculation of sperm. They concluded that mobile phone may cause male infertility by affecting sperm. Sepehrmanesh et al. (2017) have indicated that exposure to EMF leads to an increase in testicular proteins of adults associated with reproductive damage. Yan et al. (2007) studied the effect on sperm cells of 6 hours a day exposure to EMF from mobile phones for 18 weeks. As a result, they found an increase in sperm cell death in rats. Alchalabi et al. (2015) explained that exposure to 1800 MHz EMF decreasing ovarian follicular development and numbers. Gül et al. (2009) studied the toxic effect of EMF emitted from mobile phones on rat ovarian follicles.

Pregnant rats were exposed to EMF for 11 hours and 45 minutes with mobile phones in standby mode, followed by 15 minutes/12 hours in talk mode with phones during 21 days of gestation. They examined the ovaries of the female offspring. The researchers found a decrease in the number of follicles in the EMF group compared to the control group. Studies in female animals have shown that EMF can cause functional and structural changes in the reproductive system. They reported that EMF exposure can reduce the number of follicles and disrupt the structure of oocytes and follicles in the ovary (Turedi et al., 2016; Khaki et al., 2016; Roshangar et al., 2014). Al-Akhras et al. (2001) significantly reduced fertility was observed in female rats exposed to 50-Hz EMF. Electron microscopy studies have reported that 50 Hz EMF exposure causes degenerative changes in follicle and oocyte cells. Electron microscopy studies have indicated that 50 Hz EMF exposure causes degenerative changes in follicle and oocyte cells (Khaki et al., 2016; Roshangar et al., 2014). Bakacak et al. (2015) reported that ovarian primordial follicle numbers of rats exposed to EMF were notable lower than the control group. Roshangar et al. (2014) investigated the ovarian tissues of 2-day-old mouse pups obtained from mother animals exposed to 50 Hz EMF. They found less developed primordial follicles. In addition, they reported that the integrity of the zona pellucida was disrupted and thinning was observed in the zona pellucida in adult groups.

According to Türedi et al. (2016), rat pups exposed to 900 MHz EMF during prenatal periods were found to show a reduction in the number of primordial and tertiary follicles in the ovarian tissues examined at day 34 postnatally. In addition, they reported an increase in atretic follicle and atretic index, increase in follicle, fibrosis and vasocongestion, albeit at a low level, in the stroma (Turedi et al., 2016). Okatan et al. (2018) stated that there was no dissimilarity among the groups in terms of the number of primordial follicles, primary follicles and Graafian follicles in the EMF group compared to the control group, and only the number of secondary follicles decreased. They reported that exposure to 900 MHz EMF during middle and late adolescence caused changes in ovarian morphology and deterioration in follicle quality. They also concluded that EMF increases oxidative stress and causes a reduce in mitotic activity and secondary follicle numbers. The researchers experimentally analyzed the sepsis-ameliorating therapeutic potential of carnosine against sepsis-induced male albino rats. In their

studies, carnosine was administered in 2 different doses, 25 mg/kg and 50 mg/kg, for 30 sequential days. They reported that after carnosine treatment, the intensity of sepsis was significantly reduced, as evidenced by histopathological analysis (Sun et al., 2017). Oral use of carnosine over a three-month period has been proven to improve the overall appearance of the skin and reduce age-related wrinkles (Babizhayev et al., 2012). In their study, scientists reported that carnosine and vitamin E protect the ovaries from ischemia-reperfusion damage in ovarian torsion, and that carnosine may be especially useful in the treatment of ovarian torsion (Sarac et al., 2018). Studies have shown that carnosine supplementation improves markers of metabolic syndrome in obese diabetic patients (De Courten et al., 2016). One study concluded that carnosine administered intravenously at 100, 500, 1000 and 2000 mg/kg for 14 days was not toxic and reduced cell death (Rajanikant et al., 2007). After reviewing the available literature, we decided to administer 2 mg and 20 mg of carnosine before exposure to EMF for 28 days (Rajanikant et al., 2007). Another study conducted on 75 adult chronic schizophrenia patients aged 18-65 years who were given 2 g of carnosine per day for 3 months showed that carnosine should be considered as an adjunct therapy to improve executive dysfunction in patients with schizophrenia (Chengappa et al., 2012). Khoshbakht et al. (2021) investigated the protective effects of selenium in rat testis tissue exposed to electromagnetic field. They found that serum LH, FSH, GnRH, testosterone level, sperm count, germinal epithelial thickness and seminiferous tubule diameter were significantly decreased in the EM group compared to the control group. In addition, they reported that sperm count, germinal epithelial thickness, seminiferous diameters, serum LH, FSH and GnRH and testosterone levels increased in the EM/SE group compared to the EM group, and sperm abnormality, leptin receptor and apoptotic cells were significantly decreased. Altindag et al. (2017) examined the effects of cell phone exposure on testicular tissue and the protective effects of melatonin use. In the group receiving 2100 MHz radiation, the regular structure of the seminiferous tubules was disrupted, and edema occurred between the seminiferous epithelial cells; they also reported that the seminiferous tubule structure was highly protected in the radiation and melatonin group against the radiation group.

At the end of this study, follicle numbers and diameters were found to be lower in the EMF group and higher in the EMF+20 mg carnosine group. There were marked irregularities in the structure of the oocyte and the granulosa cells surrounding the oocyte in rats exposed to EMF. However, in the EMF+20 mg carnosine group, the oocyte and the granulosa cells surrounding the oocyte exhibited a more regular structure. It was observed that carnosine, which was used as a therapeutic in the experiment, reduced the damage caused by the electromagnetic field. Although we determined that carnosine has a significant effect on preventing electromagnetic field damage, we think that larger studies are needed in terms of long-term effects and possible side effects.

AUTHOR CONTRIBUTIONS

The study was designed by A. Arslan, M. Nisari, E. Balcıoğlu and N. Acer. E. Balcıoğlu, B. Yalçın and M. Ülger collected the data. A. Arslan, E. Balcıoğlu, M. Nisari, and N. Acer analyzed the data. The article was written by A. Arslan M. Nisari, E. Güler and G. B. Uzun. Each author contributed to the evaluation, review of the data, and revision of the manuscript.

ETHICAL STATEMENTS

The study was approved by Erciyes University Animal Ethics Committee with protocol number 13/82 (12.06.2013). Histopathological procedures were performed in Erciyes University Faculty of Medicine, Department of Histology-Embryology.

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