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Özgün Araştırma / Original Article

Determining the effect of an electromagnetic field generated by a high voltage power line on rat spermatogonia cells

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Abstract

Objective: Purpose of the study was to research the effect of an electromagnetic field created by a high voltage line on the testes tissue and the serum biochemistry of Wistar albino male rats. At the same time, in the study also examined the protective effects of melatonin and ganoderma lucidum.

Methods: In the study, 64 rats were divided into eight equal groups (n: 8). In experiments lasting 26 and 52 days, the following groups were used: High voltage (HV), HV + Ganoderma lucidum (GI), HV + Melatonin (MEL), and Sham-control. MEL (10 mg/kg) was administered intraperitoneally and GI (20 mg/kg) as oral lavage. Extremely low frequency electric (80.3 V/m) and magnetic fields (2.48 μT) were applied for eight hours per day.

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Results: The biochemical results of the study show an increase in total oxidant status (TOS) and oxidative stress index (OSI) level and a decrease in total antioxidant status (TAS) level in the 26 and 52 day high voltage groups, compared to the control group. In the high voltage groups, the nitric oxide (NO) value increased with the increase of the exposure period. Testicular mass ($p>0.05$), TAS, TOS, OSI and NO ($p<0.05$) were found at 26 days of comparison. Testicular weight, TAS, TOS, OSI ($p<0.05$), and NO ($p>0.05$) were found in 52 day comparisons. In routine histopathology data, we detected effects on both the 26 and 52 day HV groups, such as degeneration in spermatic cells and full structural deterioration and increasing hyalinisation. We also detected an step up in the ligament cell in the tubular field for the 52-day test groups. In melatonin added group, proliferating cell nuclear antigen (PCNA)-positive cells were more active. TUNEL assay analysis showed that there was no significant difference between the the 26 day experiment groups ($p>0.05$), but that there was a significant dissimilarity between the 52 day experiment groups ($p<0.05$).

Conclusions: In our study, the oxidative effect of created by a high voltage results in changes in serum biochemistry of rats and a number of degenerative deterioration in the histological structure of testes. Aslo, it is shown that the oxidative effect created by a high voltage has negative effects on the creation of spermatogonia. GI partly protects against these effects, while melatonin is more effective in this regard.

Keywords: Melatonin, ganoderma, high voltage, electromagnetic field, spermatogonia, antioxidant, oxidative stress

Yüksek Gerilim Hattı İle Oluşturulan Elektromanyetik Alanın, Rat Spermatogonium Hücreleri Üzerindeki Etkisinin Belirlenmesi

Öz

Giriş: Çalışmanın amacı, yüksek gerilim hattı ile üretilen elektromanyetik alanın testis dokusu ve Wistar albino erkek ratların serum biyokimyası üzerindeki etkisini araştırmaktır. Aynı zamanda çalışmada, melatonin ve ganoderma lucidum' un koruyucu etkilerinin olup olmadığı da incelenmiştir.

Yöntemler: Çalışmada, 64 rat sekiz eşit gruba ayrıldı (n: 8). 26 ve 52 gün süren deneylerde, aşağıdaki gruplar kullanıldı: HV, HV+GI, HV+MEL ve kontrol. MEL (10 mg/kg) günlük intraperitonal ve GI (20 mg/kg) oral lavaj olarak uygulandı. Oldukça düşük frekanslı elektrik (80.3 V/m) ve manyetik alanlar (2.48 μT) günde sekiz saat süreyle uygulandı.

Bulgular: Çalışmanın biyokimyasal sonuçlarında, kontrol grubuna göre 26 ve 52 günlük yüksek gerilim gruplarında toplam oksidan durum ve oksidatif stres indeksi düzeyindeki artış ve toplam antioksidan durum düzeyinde bir azalma görülmektedir. Yüksek gerilim gruplarında, maruz kalma süresinin artmasıyla nitrik oksit seviyesi artmıştır. 26 günlük karşılaştırmalarda testis ağırlığı ($p>0.05$), TAS, TOS, OSI ve NO ($p<0.05$) bulunmuştur. 52 günlük karşılaştırmalarda testis ağırlığı, TAS, TOS, OSI ($P<0.05$), ve NO ($p>0.05$) bulunmuştur. Rutin histopatoloji sonucunda, 26 ve 52 günlük HV grupları üzerinde, spermatik hücrelerde dejenerasyon ve tam yapısal bozulma ve artan hiyalinizasyon gibi etkiler tespit ettik. Aynı zamanda 52 günlük test grupları için tubülar alanda ligament hücresinde bir artış tespit ettik. Melatonin grubunda PCNA-pozitif hücreler daha aktif idi. TUNEL testi değerlendirmesinde, 26 günlük test grupları arasında anlamlı bir fark olmadığı ($p>0.05$), ancak 52 günlük test grupları arasında anlamlı bir farklılık olduğu gösterilmiştir ($p<0.05$).

Sonuç: Çalışmamızda, yüksek gerilimin neden olduğu oksidatif etkinin, ratların serum biyokimyasında ve testislerin histolojik yapısındaki bir takım dejeneratif bozulmalarda değişikliklere neden olduğunu göstermektedir. Ayrıca, yüksek gerilimin oluşturduğu oksidatif etkinin, spermatogonia oluşumu üzerinde olumsuz etkileri olduğu gösterilmiştir. GI kısmen bu etkilere karşı korurken, melatonin bu konuda daha etkilidir.

Anahtar kelimeler: Melatonin, ganoderma, yüksek gerilim, elektromanyetik alan, spermatogonia, antioksidan, oksidatif stres.

INTRODUCTION

Given the widespread use of electrical energy in developed society, society, power lines, household electrical appliances (TV sets, computers) and very low frequency magnetic fields generated by electricity transmission lines are exposed to every day (ELF-MFs below 300 Hz). With the increase of electric and magnetic fields caused by high voltage lines, a relationship has been established in the formation of childhood leukemia, nervous system and lymphoma cancers[1,2]. These negative effects can affect the electrical parameters of the human body. Television, vacuum cleaners, hair dryers, microwave ovens, clock radios, washing machines, refrigerators, coolers, computers, shavers and other electrical appliances are man-made devices that make life easier. However, in the case of close and continuous exposure to the human body, humans may be exposed to magnetic fields at the level of micro tesla[3]. In vitro studies have investigated the effects of very low frequency electromagnetic fields on proliferation, growth, shape and morphology of cells. One study has demonstrated that exposure to ELF-EMFs has negative effects on fertility and development in female mice by decreasing the number of blastocysts and increasing DNA fragmentation in the blastomers of blastocysts. However, it is need to conduct studies related to the human reproductive system of ELF-EMFs.

The response of the organism to oxidative stress can be assessed by its effects on the antioxidant system and apoptosis[5]. PCNA is used as a biomarker in studies of the effects of ELF-EMFs on the reproductive system[6].

Ganoderma lucidum is a medical mushrooms known in Chinesa as 'lingzhi', Korean 'yeongji' and Japanese 'reishi'. These fungi have been used for thousands of years in the treatment and prevention of various diseases in humans[7]. Studies have reported the free radical cleansing and antioxidant effects of Ganoderma lucidum, the use of which has increased in popularity in recent years[8]. Melatonin has also been discovered to be a direct free radical scavenger within the last ten years. In addition to its ability to directly neutralise a few free radicals and reactive oxygen and nitrogen species, it induce several antioxidative enzymes, thus increasing its efficiency as an antioxidant[9]. Due to the scarcity of prior scientific reports, we examine these compounds (ganoderma and melatonin) in the current study. Particularly in recent years, electromagnetic pollution has increased in urban areas, and high voltage lines are causing concern for people living in these areas and working in jobs involving high voltages.

Aims of this study were to investigate the affects of ELF-EMFs on rats, in terms of testicular tissue and serum biochemistry nitric oxide (NO), total antioxidant status (TAS), total oxidant status (TOS), oxidative stress index (OSI), and to investigate the protective effects of melatonin (MEL) and ganoderma lucidum (GI), which are thought to have antioxidant effects that can protect against damage. We therefore analyse whether high voltages show effects on the testicular tissue of rats, using routine histology, immunohistochemistry (PCNA, proliferating cell nuclear antigen) and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay methods.

This study aims to determine the effects of EMFs generated by the high voltage lines on the biological system and to determine what kind of effects can occur in the formation of male spermatogonia in the proliferation event which is the basis of reproduction. At the same time, the effects of the electromagnetic field on the biochemical parameters were examined.

METHODS

This study was carried out at the Sabahattin Payzin (Prof.Dr.) Health Sciences Research and Application Centre at Dicle University,

Diyarbakır, Turkey. The entire study was conducted according to the Helsinki Declaration. This study started after obtaining local ethics authorization for experimental animals, Dicle University (Approval No: 2013/13).

Animals and experimental protocol

In this study, 64 albino Wistar rats were used, weighing 320.45 ± 9.22 g on average. After a one-week adaptation process, we divided the groups into eight equal groups (n: 8). The animals were housed under optimal laboratory conditions (temperature: 23 ± 1 ºC and relative humidity: 45–55%), with 12 h of light and 12 h of darkness. They had access to tap water and food (ad libitum), which was available within the cage. The 26 day experimental groups were as follows: Group 1: HV; Group 2: HV+GI; Group 3: HV+MEL; and Group 4: sham - control group. The 52 day experimental groups were as follows: Group 5: HV; Group 6: HV+GI; Group 7: HV+MEL; and Group 8: sham control group. Melatonin was administered intraperitoneally on a daily basis (10 mg/kg), and GI by oral lavage (20 mg/kg). The 26 and 52day experimental groups (Groups 1–3 and 5–7) were exposed to ELF-EMFs for 8 h daily. To create the ELF-EMFs, in the study, we used two voltage transformer transformers, which produced 10 kV (10,000 V) of high voltage electricity. The input for transformer 1 was 220 Volt, the output was 10 kV, while the input for transformer two was 10 kV, the output was 220 V and 5,000 VA. The experimental design is shown in Figures 1 and 2. The rats were separated into groups and placed into plexiglass cages (size 15 x 42 x 42 cm). The masses of the rats and their testes were measured using a precision scale (Sartorius AZ212, United Kingdom).

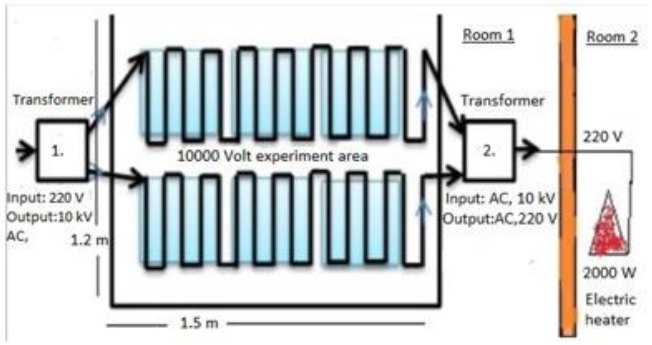

Figure 1a. The experiment setup.

Figure 1b. The experimental apparatus.

Melatonin

For the MEL groups, melatonin (Merck KGaA, Cat. No: 8145370001, Germany) was prepared according to the average mass of each rat. Melatonin was dissolved in pure ethanol (99.8% Riedel - de Haen) after the rats were weighed on a sensitive scale, after diluted with distilled water, proportionally scaled. Melatonin was administered intraperitoneally daily during the experiment.

Ganoderma lucidum

In Ganoderma groups, Ganoderma extracts were prepared according to the masses and standards of rats. Ganoderma lucidum, rats were given orally lavage with distilled water (Gano Excel, Cat. No: Mal1991984T, Industries Sdn. Bhd., Malaysia).

BIOCHEMICAL ANALYZES

Blood collection and storage

When the work is completed, testes tissue and blood were obtained from the rats, which were sacrificed under xylazine-ketamine (Pfizer, Turkey; BioVeta Co., Czech Republic) anaesthesia. Blood sample collection was carried out through an intra-cardiac path in each rat. Blood samples were separated from cells by centrifugation (NF 1200 R Nuve, Turkey) at 5,000 rpm for 5 min. Serum samples after centrifugation were stored at -20°C until analysis time. The serum samples from the rats were analysed for alteration in serum TAS, TOS, OSI and NO.

Total antioxidant status

TAS the serum samples was determined using a method automated (Abbott Architect® c16000) evaluation method developed by Erel. TAS was measured using a commercially available TAS assay kit (Rel Assay Diagnostics, Gaziantep, Turkey). The assay was calibrated with a stable antioxidant standard solution and the assay results expressed in mmol trolox equivalent/l[10].

Total oxidant status

The commercial kit was used to measure the total oxidant status (Rel Assay Diagnostics, Gaziantep, Turkey). The assay was calibrated with hydrogen peroxide and the results expressed in μmol H2O2 equivalent/l[10].

Oxidative stress index

The percentage ratio of TOS to TAS gave the oxidative stress index, an indicator of the degree of oxidative stress. The OSI value was calculated using the formula: OSI (AU) = [TOS (μmol H2O2 equiv./l) / TAS (μmol trolox equiv./l) × 100] 10.

Nitric oxide

Direct measurement is somewhat difficult, because nitric oxide is a highly labile molecule. However, nitric oxide formation has different indicators in vivo and in vitro. Serum nitrite levels were measured based on the Griess reaction. The reduction of nitrate to nitrite was accomplished by a catalytic reaction using cadmium. Absorbance of this complex was measured at 545 nm. A standard curve was established using a set of serial dilutions (10-8 to 10-3 mol/l) of sodium nitrite. Linear regression was carried out using the peak area from the nitrite standard. The resulting equation was then used to calculate the unknown sample concentrations. Results were expressed as micromoles per litre of serum[11].

HISTOLOGICAL EVALUATIONS

Tissue extraction and routine histology

In the final stage of the study, tissue sections taken from rats testes were evaluated and counted under a light microscope using routine histology and immunohistochemistry (PCNA and TUNEL) methods.

Testicular tissue specimens were taken from the rats with Bouin's fixative, which was prepared for 18 hours before fixation was performed. The tissues were cleaned of the Bouin's solution by washing under running water for 12 hours. The dehydration process was then completed by soaking for 2x20 minutes in pure alcohol (99.9) for eight hours using 30%, 50%, 70%, 80%, 90% and 96% alcohol, respectively. To clarify and remove the alcohol, the tissues were soaked for 2x15 minutes in xylolide and then kept in a 1/1 volume of paraffin/xylol for one hour, and then in pure paraffin for four hours. Following the infiltration process (Nüve EN500, in an oven at 60ºC), the tissues were embedded in paraffin blocks. Sections of thickness 4–6 μm were taken from each paraffin block with the aid of a fully automated rotary microtome (Leice RM 2265, Germany) and were transferred to a positively charged slide[12,13]. Haematoxylen-eosin (H-E), TUNEL assay and PCNA methods were applied to the obtained sections. An evaluation of these preparations was carried

out using a Nikon Eclipse 80i microscope (Nikon, Japan) and NIS Elements software v3 09.

Immunohistochemistry: PCNA

Chemicals and solutions including PBS (phosphate buffer solution), EDTA and hydrogen peroxide solution were prepared in order to carry out the immunohistochemistry evaluation. The paraffin blocks were cut using a microtome, and preparations were taken. Samples were left in the machine (oven) overnight. The xylol was purified from paraffin and passed through reduced alcohols, and distilled water was used twice. The microwave was heated for 3x5 min at 800°C (in a citrate buffer solution). The PBS was incubated for 3x5 min. Then, it was placed in hydrogen peroxide and incubated for 20 minutes, then placed in PBS for 3x5 min. An outline of the tissue was drawn in pencil. The blocking solution was allowed to stand for 7-8 min after the drop. The primer antibody was dropped and allowed to stand in the refrigerator at +4ºC overnight; it was then removed from the refrigerator and allowed to rest for 30–60 min outside, and then left in the PBS for 3x5 min (PCNA antibody, lot J1012, F-2: SC-25280, Santa Cruz Bio. Inc., USA). The secondary antibody (biotinylated antibody) was allowed to stand for 15 min and thereafter incubated in PBS for 3x5 min. Streptavidin peroxidase was allowed to stand for 15 min, thereafter washed for 3x5 min in PBS. Diammonium phosphate (DAP) was added dropwise, and the reaction was monitored (for a time adjusted according to the reaction). The sections were incubated in PBS for 3x5 min. Contrast staining was performed for 45 seconds in haematoxylin. Flowing water was then used for 5 min, causing undergo the alcohol series. Transparency was performed with xylol, and was finished with Entellan. The tissue was then submitted for analysis.

TUNEL assay

The in situ deoxyribonucleic acid fragmentation was visualised using the TUNEL technique. Paraffin sections were removed in xylol, passed through decreasing alcohol grades and brought up to distilled water. Two serial five-wash cycles were performed using PBS. Permeabilisation was carried out in a permeabilisation solution (0.1% Triton X-100, lot SLBD3821V Sigma-Aldrich, USA; 0.1% sodium citrate) at +4°C for 10 min. Two serial five-wash cycles were performed in the PBS. An in situ cell death detection kit, AP (Roche, Cat. No: 11684 809 910, Germany) was used as the TUNEL kit. An enzyme solution (vial 1) and labelling solution (vial 2) were prepared in a ratio of 1:9 and diluted 1:1 with TUNEL dilution buffer (Roche, Cat. No: 11966006001, Roche, Germany). The prepared solution was applied for one hour at 37ºC in a dark place. Two serial five-wash cycles were performed using the PBS. The AP converter (vial 3) supplied with the kit was applied to the sections for 30 minutes. Two serial five-wash cycles were performed using PBS. Fast Red Tablet (Roche, Cat. No: 11496549001, Germany) that substrate was prepared and sectioned. The positive signal was checked under a light microscope and the reaction was stopped with PBS as seen. Two serial five-wash cycles were then performed using PBS. Anti-staining with haematoxylin was performed. It was streamed and two serial five-wash cycles were performed using PBS. The sections were closed with Keizer's glycerol gelatin (Cat. No: 1.092.42.0100, Merck KGaA, Germany). Apoptotic germ cells were quantified by counting the number of TUNEL stained nuclei per seminiferous tubular cross section. Cross sections of 100 tubules per specimen were evaluated, and the mean number of TUNEL positive germ cells per tubule cross section was computed[14-16].

STATISTICAL EVALUATION

Histological, immunohistochemistry, TUNEL assay and biochemical data were evaluated stastistically using Statistical Package for Social Sciences 15.0 software (SPSS Inc., Chicago, IL, USA). Measured variables were presented with

mean and standard deviation, and categorical variables with number and percentage. The Kolmogorov Smirnov Test was performed to see if the data fit the normal distribution. After this test, it was determined that the data did not fit the normal distribution. The mann-whitney-u test was used to compare the two groups without normal distribution, the kruskal-wallis test and the Bonferroni-corrected mann-whitney-u test were used to compare multiple groups. Hypotheses were bidirectional and $p<0.05$ was considered statistically significant. However, statistically significant results were accepted when the Bonferroni corrected mann-whitney-u test was $p\leq0.008$.

RESULTS

Electric and magnetic field measurement data

With reference to the measurement methods, the arithmetic mean of daily measurements was taken. In plexiglass cages, the electric and magnetic field intensity measurements in experimental setup were determined as 80.3 V/m and 2.48 μT, respectively. The electromagnetic field was measured with a Spectran device NF5035 (AARONIA AG, Strickscheid, Germany), using the method of six-minute measurement proposed by the International Commission on Non-Ionizing Radiation Protection. The necessary warning/caution signs were hung on the door of the room where the experiment was performed. Data from these measurements are given in Table 1.

Table 1: Data on electric and magnetic fields

	*Electric Field (V/m)	*Magnetic field (μT)
Mean ± std deviation	80,35 ± 1,44 V/m	2,48 ± 0,55 μT

*Values were calculated by taking 10 measurements per day and taking the mean ± standard deviation

The Testis Masses of Rats in the Study

At the end of the study, the left testis of each rat in the control and experimental groups was taken, and measurements were made using sensitive scales. Although there was no significant chance between the 26-day groups ($P>0.05$), there was a significant difference between the 52-day groups ($P<0.05$). There was a significant difference between group 6 (HV + GI) and group 7 (HV + MEL). The findings of the study in terms of left testis mass are given in Table 2.

Table 2: Left testis masses (g) data (26 and 52 day groups)

Group/time	Mean ± SD	Median	Min	Max	P
26 day	1.377 ± 0.115	1.350	1.212	1.555	0,394
52 day	1.494 ± 0.109	1.473	1.347	1.680	0,023

Mean ± standard deviation, median, minimum, maximum and P value was given.
The kruskal wallis test was used in the comparison of 26 ($p>0.05$) and 52 day groups ($p<0.05$).

Serum Biochemistry Analysis Data

Blood serum from rats, Rel Assay kit for TAS and TOS analysis were studied using full automatic analysers. OSI values were also formally calculated. The level of nitric oxide was determined manually, and the values were read on a spectrophotometer; statistical analyses were carried out based on these results.

In our study 26 and 52 day of experiment, a important change was found within the TAS, TOS and OSI groups ($p<0.05$). Data on the serum TAS, TOS and OSI in rats in the 26 day and 52 day groups are shown in Tables 3 and 4. In terms of the nitric oxide findings, there was a significant difference between the 26 day groups and an insignificant difference between the 52 day groups. These findings are given in Table 5.

Table 3: Statistical analysis of serum TAS, TOS and OSI in rats in the 26 day group

*Groups	TAS (µmol trolox eq/l)	TOS (µmol H_2O_2 eq/l)	OSI (AU)
Group 1 (HV)	1.127 ± 0.082	27.792 ± 1.855	24.70 ± 1.487
Group 2 (HV + GI)	1.340 ± 0.038	24.552 ± 1.504	18.87 ± 0.773
Group 3 (HV + MEL)	1.360 ± 0.046	22.305 ± 3.007	16.42 ± 2.276
Group 4 (Control)	1.187 ± 0.048	19.321 ± 1.950	16.28 ± 1.560
P	0.000	0.000	0.000

* Kruskal-Wallis variance analysis was used in the comparison of the four groups ($p<0.05$). The results are expressed as P significance values, mean and standard deviation

*Group	(1 - 2)	(1 - 3)	(1 - 4)	(2 - 3)	(2 - 4)	(3 - 4)
TAS	*0.001	*0.001	ns0.114	ns0.369	*0.001	*0.001
TOS	*0.005	*0.002	*0.001	ns0.141	*0.001	ns0.059
OSI	*0.001	*0.001	*0.001	ns0.027	*0.006	ns0.674

A Bonferroni-corrected Mann-Whitney U test was used to compare the two groups within the four groups. * indicates a significant difference between the groups ($p<0.008$); ns indicates no significant difference between the groups ($p>0.008$)

Table 4. Statistical analysis of serum TAS, TOS and OSI in rats in the 52 day group

Groups	TAS (µmol trolox eq/l)	TOS (µmol H_2O_2 eq/l)	OSI (AU)
Group 5 (HV)	1.070 ± 0.063	35.898 ± 2.728	38.87 ± 2.971
Group 6 (HV + GI)	1.227 ± 0.060	33.732 ± 3.124	27.44 ± 1.565
Group 7 (HV + MEL)	1.320 ± 0.068	31.747 ± 2.945	24.29 ± 3.266
Group 8 (Control)	1.110 ± 0.029	29.293 ± 1.318	26.40 ± 1.270
P	0.000	0.001	0.000

* Kruskal-Wallis variance analysis was used in the comparison of the four groups ($p<0.05$). The results are expressed as P significance values, mean and standard deviation

Group	(5 - 6)	(5 - 7)	(5 - 8)	(6 - 7)	(6 - 8)	(7 - 8)
TAS	*0.001	*0.001	ns0.268	ns0.013	*0.001	*0.001
TOS	ns0.172	ns0.021	*0.001	ns0.207	*0.003	ns0.115
OSI	*0.001	*0.001	*0.001	ns0.172	ns0.172	ns0.345

A Bonferroni-corrected Mann-Whitney U test was used to compare the two groups within the four groups. * indicates a significant difference between the groups ($p<0.008$); ns indicates no significant difference between the groups ($p>0.008$)

Table 5: Serum nitric oxide parameters in the 26 day and 52 day groups of rats

Groups	*NO (mol/l)	P	Groups	*NO (mol/l)	P
Group 1	6.762 ± 1.128	0.001	Group 5	12.793 ± 3.952	0.109
Group 2	9.312 ± 3.174		Group 6	8.850 ± 2.837	
Group 3	11.775 ± 2.514		Group 7	9.162 ± 2.427	
Group 4	13.912 ± 2.355		Group 8	9.237 ± 0.692	

* Kruskal-Wallis variance analysis was used in the comparison of the four groups. The results are expressed as P significance values, mean and standard deviation. P significance values were found to be $p<0.05$ at 26 days and $p>0.05$ at 52 days.

Group	(1 - 2)	(1 - 3)	(1 - 4)	(2 - 3)	(2 - 4)	(3 - 4)
NO	^{ns}0.093	*0.002	*0.001	^{ns}0.074	^{ns}0.012	^{ns}0.206

A Bonferroni-corrected Mann-Whitney U test was used to compare the two groups within the four groups. * indicates a significant difference between the groups ($p<0.008$); ns indicates no significant difference between the groups ($p>0.008$)

In addition, for TAS, TOS, OSI and NO, the 26 and 52 day exposures for the same groups were compared using the Mann-Whitney U test. There was a significant difference in TOS, OSI and NO levels between the 26 and 52 day HV groups ($p<0.05$) and an insignificant difference in TAS level ($nsp>0.05$). There was a significant difference in TAS, TOS and OSI levels between the 26 and 52 day high voltage groups (HV) + Ganoderma L. group ($p<0.05$) and a meaningless difference in NO level ($nsp>0.05$). There was a significant difference in TAS and OSI levels between the 26 and 52 day HV groups + melatonin groups ($p<0.05$) and an insignificant difference in TAS and NO level ($nsp>0.05$). A decrease in the TAS values and an increase in the TOS and OSI values were observed as the dose value increased.

Histopathological Data

The seminiferous tubules were normal in the 26 day control group for the HV rats; however, in the HV group, basal membrane thickening in the total cross sections, impaired integrity in the spermatic cells and hyalinisation towards the lumen, spermium and tail extensions in the form of a spiral were observed. These findings are presented in Figure 2 (A). On comparing the HV group and the HV+GI group, it was observed that spermatic cells near basal membranes showed increased chromatin density and degenerative changes in lumen-like cells, although cellular integrity was preserved in seminiferous tubules. These results are illustrated in Figure 2 (B). In the HV + melatonin group, the seminiferous tubule cells were locally vacuolated, and cells were arranged regularly, although intertubular area connective tissue growth and hyalinisation were detected. These results are illustrated in Figure 2 (C). Histopathological data for the 26 day sham control groups are shown in Figure 2 (D).

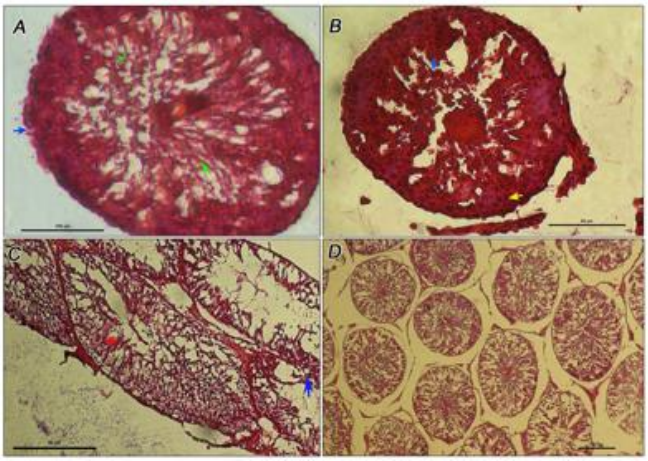

Figure 2. (26 day groups). **(A)** HV group, showing consistency deterioration (blue arrow) with thickening in the basilar membrane of the seminiferous tubule, hyalinisation on the lumen near side, threaded stretching lumen, and structural features such as gigantic spermium tail extensions (green arrow). Bar: 100 µm. **(B)** HV+GI group, showing degenerative changes (blue arrow) in spermatic cells close to the lumen, dense chromatin in the nucleus of spermatic cells near the basal membrane, and preservation of cellular integrity (yellow arrow). Bar: 50 µm. **(C)** HV+MEL group, showing vacuolisation (red arrow) in spermatic cells in seminiferous tubules, and sperm cells in the lumen of some tubules (blue arrow). In the intertubular area, connective tissue and hyalinisation were observed. Bar: 50 µm. **(D)** sham control group, showing normal appearance of seminiferous tubules. Bar: 100 µm. (H-E staining).

In a histopathological evaluation of the 52 day HV group, pyknosis in spermatic cell nucleus, an abnormal appearance of spermatids towards the lumen and hyalinisation in the upper part of some seminiferous tubules were observed, with significant deterioration and degeneration in the basal membrane structure, as illustrated in Figure 3 (A). In the 52 day HV+GI group, a certain order was observed in the basal layer of spermatic cells, whereas in the vicinity of the lumen, pyknotic nuclei were found. The interlobular area showed increased

fibrous tissue and occasional vacuolisation, as illustrated in Figure 3 (B). In the 52 day melatonin group (HV+MEL group, 52 day), spermatic cells in the seminiferous tubules showed regular placement, although some cells showed slight degeneration. These results are illustrated in Figure 3 (C). Histopathological data for the 52 day sham control group are shown in Figure 3 (D).

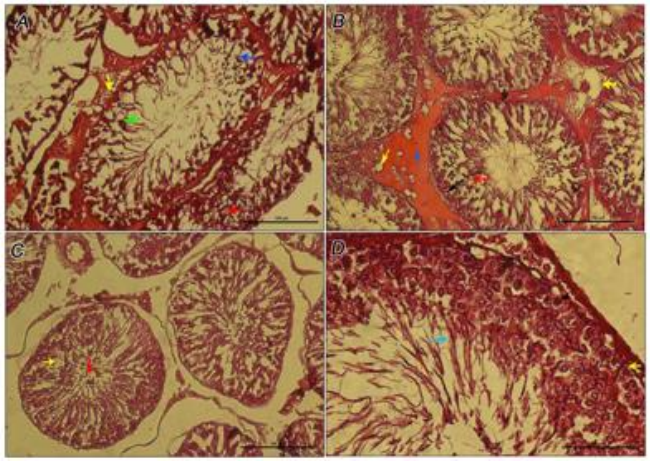

Figure 3. (52 day groups). **(A)** HV group, showing degeneration of basal membrane structure (yellow arrow), pyknosis in the nucleus (blue arrow) in spermatic cells, deterioration in spermatid structures towards the lumen and abnormal sperm (green arrow), and hyalinisation in the lumen of some seminiferous tubules (red arrow). **(B)** HV+GI group. In the intertubular area, pineal nuclides (red arrow) were observed in the cells towards the lumen, while a marked increase in fibrous tissue (blue arrow), occasional vacuolar structures, dilation in the blood vessels (yellow arrow), and spermatic cells in the basal part of the seminiferous tubule were clearly observed (black arrow). **(C)** HV+MEL group, showing spermatic cells within the seminiferous tubules, slight degeneration towards the lumen (yellow arrow) and luminal spermium (red arrow). **(D)** sham control group, showing spermium cell to create into the lumen of spermatic cells (blue arrow) and normal appearance of sertoli cells (yellow arrow). (Bar: 100 µm. H-E staining).

Immunohistochemical (Pcna) Analysis Data

Proliferative cell nuclear antigens (PCNA) showed a positive reaction in spermatic cells that were damaged throughout the seminiferous tubule in the 26-day HV group, as shown in Figure 4 (A). In the HV+GI group in particular, impaired spermatic cells were seen on the side close to the lumen. PCNA-positive cells and regular spermatic cell lineage were observed in the basal part. These results are illustrated in Figure 4 (B). PCNA-positive basal cells were observed in the group given 26 days of melatonin, as illustrated in Figure 4 (C). Immunohistochemical data for the 26 day sham control group are shown in Figure 4 (D).

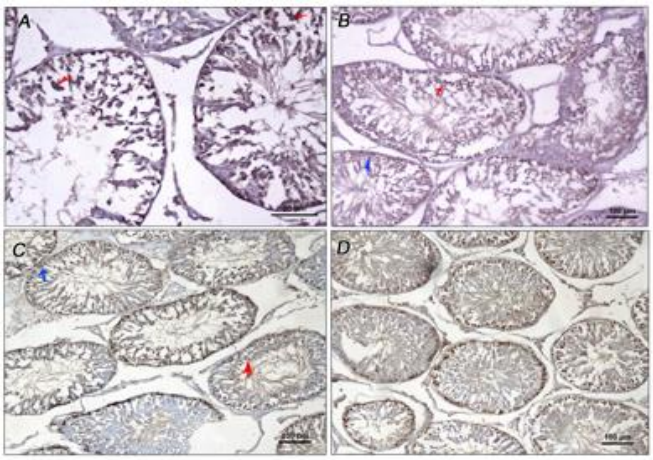

Figure 4. (26 day groups PCNA). **(A)** HV group, showing distortion in spermatic cells toward the lumen and PCNA-positive cells (red arrow). Bar: 50 µm. **(B)** HV+GI group. Intensive PCNA-positive cells were observed in the cells in the second row and the lumen (red arrow) while basal cells showed improvement (blue arrow). Bar:100 µm. **(C)** HV+MEL group. Cells in the lumen of some seminiferous tubules showed a negative PCNA reaction (red arrow), while basal cells showed a PCNA-positive reaction in all of the seminiferous tubules (blue arrow). Bar: 100 µm. **(D)** sham control group. Basal cells reacted positively to PCNA throughout the seminiferous tubule, while PCNA-positive cells were observed in some of the second-line cells. Bar: 100 µm. (PCNA immun staining).

In the 52 day HV group, PCNA was observed to be positive in basal cells, which were mostly located near the lumen, as illustrated in Figure 5 (A). In this group (the 52 day HV+GI group), more basal cell PCNA-positive cells were observed on the pyknotic lumen side, as illustrated in Figure 5 (B). In the 52 day HV+MEL group, while the cell line was regularly observed in the basal part, PCNA showed a positive reaction in these cells. However, in some of the shrinking nuclei, the lumen also showed a PCNA-positive nucleus. These results are illustrated in Figure 5 (C). Immunohistochemical data for the 52 day sham control group are shown in Figure 5 (D).

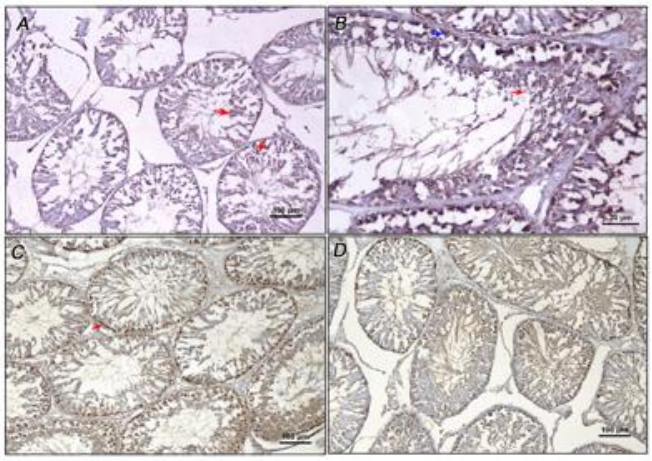

Figure 5. (52 day groups PCNA). **(A)** HV group. The disrupted spermatic cells were seen to have a positive PCNA mark near the lumen. Bar: 100 µm. **(B)** HV+GI group. The spermatic cells showed a partial positive PCNA reaction in the direction of the basal membrane (blue arrow), but it was observed that the pyknotic structures in the direction of the lumen were intense (red arrow). Bar: 20 µm. **(C)** HV+MEL group, settled by the regular cells in the basal portion in the positive reaction to PCNA (red arrow). whereas in cells has been shown to be significantly close to the lumen of ripening. Bar: 100 µm. **(D)** sham control group. Basal cells throughout the seminiferous tubule showed a normal PCNA-positive reaction, while PCNA-positive cells were observed in some of the second-row cells. Bar: 100 µm. (PCNA immun staining).

TUNEL Assay Analysis Data

Spermatogonium cells giving positive signals in the tubules were counted from eight samples from each group. The counting process was performed by adding positive signal numbers to 100 randomly selected tubs in each preparation. Positive signal counts per tubule were obtained and percentage values were generated. For the TUNEL assay, no important change was observed between the 26 day groups ($p>0.05$), while there was a significant difference between the 52 day groups ($p<0.05$). The findings from the TUNEL data (percentage of positive signals) are given in Table 6.

When comparing the same groups over time (for 26 and 52 days), there was a significant difference ($p<0.05$) between the positive signaling cell counts of the HV groups (Groups 1–5) and the HV + GI groups (Groups 2–6). There was no difference between the HV + MEL groups (Group 3–7) and the control groups (Group 4–8) ($p>0.05$). Analyses were performed using the Mann-Whitney U test. Positive and negative signal calculations from TUNEL assay staining for 26 and 52 days of application are shown in Figures 6 (A), (B), (C), (D) to Figures 7. (A), (B), (C), (D).

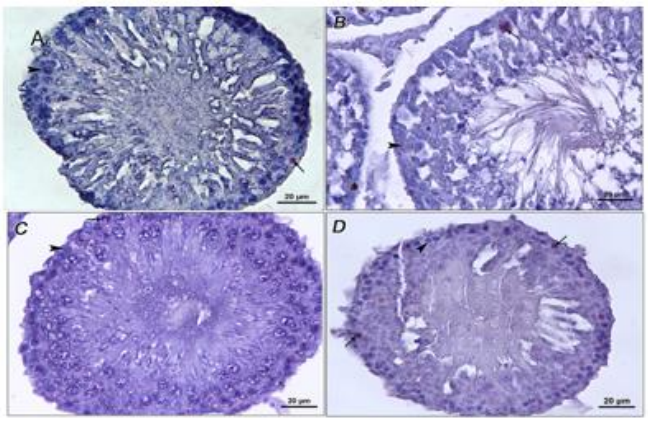

Figure 6. (26 day groups TUNEL). (A) HV group. In Group 1, both positive (arrow) and negative signalling cells (arrow head) are seen. (B) HV+GI group. In Group 2, both positive (arrow) and negative signalling cells (arrow head) are seen.. (C) HV + MEL group. In Group 3, positive (arrow) and negative signalling cells (arrow head) are seen. (D) sham control group. In Group 4, positive (arrow) and negative signalling cells (arrow head) are seen. (Bar: 20 µm, TUNEL staining).

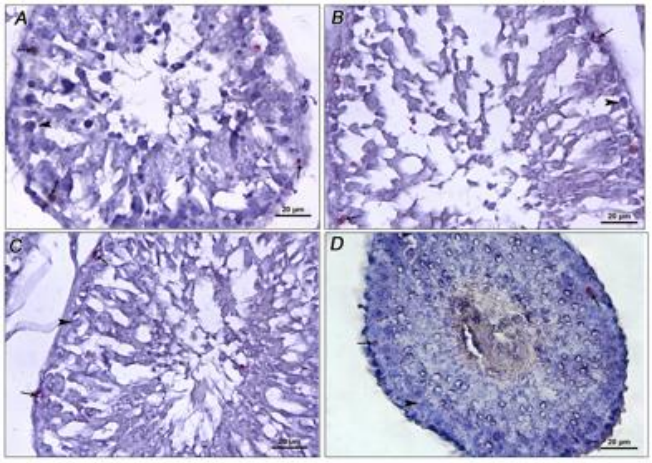

Figure 7. (52 day groups TUNEL). (A) HV group. In Group 5, positive (arrow) and negative signalling cells (arrow head) are seen. (B) HV+GI group. In Group 6, positive (arrow) and negative signalling cells (arrow head) are seen. (C) HV+MEL group. In Group 7, positive (arrow) and negative signalling cells (arrow head) are seen. (D) sham control group. In Group 8, positive (arrow) and negative signalling cells (arrow head) are seen. (Bar: 20 µm, TUNEL staining).

DISCUSSION

Exposure to a static magnetic field failed to influence the relative mass of testes[17]. A study by Rajaei et al. showed effects from exposure to very low frequency electromagnetic fields. The testis masses was found to be significantly decreased when the rats in the experimental group were compared to those in the control group[18]. In our study, no significant change in testicular mass ($p>0.05$) was observed between the 26 day experimental groups. However, testicular mass was found to be significantly changed ($p<0.05$) among the 52 day experimental groups. This significant difference was only between group 6 (HV+GI) and group 7 (HV+MEL). Testicular masses for the 52 day experimental groups (HV, HV+GI and HV+MEL) were found to be lower than for the control groups.

Akdağ et al. observed a decrease in the level of nitric oxide in a study of extremely low-frequency magnetic fields over a long period of time (100 μT and 500 μT for 10 months, two hours per day)[19]. In our study, the decreased levels of nitric oxide in the 26 day experimental group may be a result of the adverse effects of increased oxidative stress on the rats' physiology. There was no significant difference in nitric oxide levels in the 52 day experimental group of rats ($p>0.05$). Based on data in the literature, we could not establish a correlation with nitric oxide due to a lack of HV studies of Ganoderma and melatonin. While the nitric oxide levels in our study did not show a difference from the control group in either the 26 or 52 day HV+MEL groups, a decrease was observed in the 26 day HV+GI group.

In this study, we show that oxidative stress (OS) in the reproductive system is caused by potential damage from high levels of reactive oxygen species (ROS)[20]. Akpınar et al. exposed Wistar rats to extremely low frequency electric fields (ELF-EF) (12 kV/m and 18 kV/m) for one hour per day over 14 days. As a result of the study, it was determined that there was a significant increase in the mean values of TOS and OSI and a significant decrease in the mean values of TAS in the experimental groups compared to the control groups[21]. In our study, the values of TAS decreased ($p<0.05$), and the TOS and OSI values increased compared to the control group for the HV group over 26 and 52 days of application ($p<0.05$). This result shows that HV application can increase the damage caused by oxidative stress. The results of our study seem to be compatible with those in the literature.

Researchers have found that Ganoderma has the ability to cleanse ROS, showing strong antioxidant activity and increased serum antioxidant activity[22]. In our study, an increase in the mean value of TAS ($p<0.05$) and a decrease in TOS and OSI values ($p<0.05$) were found statistically for both the 26 and 52 day HV and HV+GI groups. Particularly in the application of electric and magnetic fields, melatonin has been found to effectively remove oxidative damage in the human haematopoietic system[23]. In our study, we found that melatonin could protect against oxidative damage.

Table 6. TUNEL assay data for all experimental groups

*Groups	(%) percentage positive signal	P	*Groups	(%) percentage positive signal	P
Group 1	0.965 ± 0.201	0.325	Group 5	1.275 ± 0.116	0.000
Group 2	0.850 ± 0.207		Group 6	1.110 ± 0.185	
Group 3	0.800 ± 0.075		Group 7	0.897 ± 0.135	
Group 4	0.847 ± 0.167		Group 8	0.787 ± 0.237	

* Kruskal-Wallis variance analysis was used in the comparison of the four groups ($p>0.05$). The results are expressed as P significance values, mean and standard deviation

Groups	(5 - 6)	(5 - 7)	(5 - 8)	(6 - 7)	(6 - 8)	(7 - 8)
Percent positive signal	ns0.061	*0.001	*0.001	ns0.028	*0.004	*0.001

A Bonferroni-corrected Mann-Whitney U test was used to compare the two groups within the four groups. * indicates a significant difference between the groups (p<0.008); ns indicates no significant difference between the groups (p>0.008)

A long-term electromagnetic field study found that testicular tissue showed no histological or morphological effects[24]. ELF-EMFs can induce histological changes in the dental pulp of rats, and the use of MLT and GI may have a protective effect against these changes[25]. In a study of mouse testes, observed degeneration and spermatogenesis in germ cells, a decrease in sertoli cells, necrosis in the epithelial germinal cells of seminiferous tubules, and atropia in seminiferous tubules[26]. In our study, using routine histopathology, an increase in the degeneration of spermatic cells in seminiferous tubules was observed in the testis structure between the 26 day and 52 day HV groups. In the 52 day group, it was observed that the structural integrity totally deteriorated, hyalinisation increased, and haemorrhage and dilatation in vessels increased, with an increase in connective tissue between the tubules. In the Ganoderma group, cell degeneration and structural integrity were partially improved, whereas in the melatonin group, spermatic cells were correctly luminally differentiated along the basement membrane and showed maturation in the spermium direction.

The results of other studies show that the application of a 4.5 miliTesla, 120 Hz ELF-EMFs effects the early stages of carcinogenesis chemically induced in rat livers, through the reduction of PCNA, Ki-67 and cyclin D1 expression and without inducing apoptosis[27]. It is understood that antioxidants are significant in the prevention and cure of various types of tissue damage due to oxidative stress[28]. An immunohistochemical (PCNA) evaluation undertaken as part of our study showed that the results are compatible with the histopathological findings. In the 26 day group, PCNA-positive cells were observed to have proliferated, especially in the Ganoderma group. In the HV+MEL group, it was observed that PCNA-positive cells were located throughout the seminiferous tubule in spermatic cells, and this proliferation was more widespread than for the Ganoderma lucidum group. Compared with HV and HV+GI at 52 days, it was observed that the proliferation of PCNA-positive cells was partially arranged, with two rows arranged alongside the basal membrane. However, PCNA-positive proliferation was found to be more effective when the HV and HV+MEL groups of the same group were compared. The TUNEL method is a technique that allows apoptotic cells to be labelled in situ[29]. In a study conducted by Kim and colleagues, apoptosis was assessed in testis germ cells under 60 Hz MFs of 2.5, 5, 10, and 20 μT for 24 h over 20 weeks. The TUNEL method was used in their study, and it was found that apoptosis may be affected in testicular germ cells when applied above 20 μT [30]. We used the TUNEL assay method in our study, since this method is widely used in electromagnetic applications. We found that the HV group showed an increased percentage of positive signals for apoptosis relative to the control group. Our results were consistent with those of previous studies, suggesting that the effect is dose-time dependent. Studies of Ganoderma and melatonin have shown that the apoptotic increase is reduced for damage caused by the application of an electromagnetic field, and these results are compatible with those from our work. Dose residuals have been shown to increase the number of apoptotic cells. The amount of increased apoptosis outside the normal cycle of the cell is likely to indicate oxidative damage due to the HV field.

Using biochemical, histopathological and immunohistochemical analyses of the data, it was shown that the damage to the HV group is higher than that in the control group, and that an increase in the exposure period triggers the formation of oxidative damage. When the findings of our study are examined, the results of antioxidant capacity and oxidative stress show us that ganoderma maintains oxidative damage and melatonin is more effective in protection. There is a need for more detailed, long-term investigations at the molecular level, as these would reveal the effects on the reproductive system of living organisms exposed to extremely low frequency HV electromagnetic fields. We believe that this study offers a contribution to research in this area.

Bu çalışma, 24-27 May 2016 tarihinde Isparta, Türkiye' de, "6th World Congress of Oxidative Stress, Calcium signaling and TRP Channels" kongresinde ingilizce olarak sözlü sunum olarak sunulmuştur.

Declaration of Conflicting Interests: The authors declare that they have no conflict of interest.

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