Extremely Low Frequency-Electromagnetic Fields (ELF-EMF) Can Decrease Spermatocyte Count and Motility and Change Testicular Tissue

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ABSTRACT

Background: Substantial evidence indicates that exposure to extremely low frequency-electromagnetic fields (ELF-EMFs) affects male reproductive system.

Objective: The goal of this study was to evaluate the effects of long-term irradiation with ELF-EMF on sperm quality and quantity and testicular structure.

Material and Methods: In this case-control study, sixty male Sprague-Dawley rats were randomly divided into six groups. Experimental groups were exposed to ELF-EMF (50 Hz EMF, 100 µT) for either 1 h/day for 52 days (Group 1), 4 h/day for 52 days (Group 3), 1 h/day for 5 days (Group 5), 4 h/day for 52 days (Group 7). Groups 2, 4, 6 and 8 were only sham exposed at durations equal to Groups 1, 3, 5 and 7, respectively.

Results: Both count and motility of sperms were significantly decreased in animals exposed to ELF-EMF (1 h/day for 52 days, 4 h/day for 52 day, and 4 h/day for 5 days) compared to the sham-exposed groups (P<0.05). Serum testosterone levels showed a significant decrease in the animals exposed to ELF-EMF (4 h/day for 5 days) compared to the control groups (P<0.05). A significant decrease was observed in the volume of the seminiferous tubules, seminiferous tubules epithelium and interstitial tissue in the animals exposed to ELF-EMF for 4 h/day for 5 days. Tubules length was also reduced by 18% in animals exposed to ELF-EMF (4 h/day for 5 days).

Conclusion: Our results show that ELF-EMF can reduce spermatocyte count and motility and is able to induce structural changes in testicular tissue.

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Keywords

Low Frequency; Electromagnetic Fields; Spermatocytes; Stereology; Testis

Introduction

Today, exposure to Electromagnetic Fields (EMF) emitted from different sources is a part of modern life that cannot be avoided. Today, exposure to ELF-EMF with frequencies below 300 Hz is widely common in our daily life [1]. Using electrical appliances in residential and work places has increasingly exposed humans to ELF-EMFs. Electrical power lines, house hold appliances, and computer monitors are among the main sources of ELF-EMFs. ELF-EMFs may have potential risk for human health by being associated to some can-

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cers, and congenital defects [2-4]. Some studies reported different effects of ELF-EMFs on male reproductive system [5, 6]. In fact, testes along with the brain and blood are among the most sensitive organs to molecular changes induced by ELF-EMF exposure. Testes are prone organs that can be damaged by potential deleterious effects of radiofrequency radiation such as inflammation, oxidative stress, heat, and apoptosis [7]. Male infertility is a concerning health issue in the modern world. This problem has been estimated to affect 5% of males around the globe [8]. Although the reason for this is not understood in about half of the individuals, increased rate of infertility in men in recent years has been debated as a consequence of predisposing environmental conditions. Several studies have been conducted to assess the influence of various intensities of radiofrequency radiation on the fecundity of male animal models.

The process of spermatogenesis is a highly sophisticated and well-coordinated phenomenon comprising of developing germ cells with different nucleic acid copy numbers. Derangement in production each of these cells at different stages of spermatogenesis can result in abruption of the process and subsequently defected spermatocyte production [8]. Multiple parameters related to sperm specification such as count, motility and morphology are influenced by EMFs. Also, reproductive system may be affected by EMFs through changes in sexual hormones [9, 10]. ELF-EMF of 60 Hz is involved in inhibition of regenerative spermatogenesis after heat shock to testes [11]. There are disputes regarding the role of radiofrequency on reproductive function. Some authors have stated that ELF-EMF have no detrimental effects on reproductive organ of male animal models [12].

The uncertainty about the biological effects of ELF-EMF on reproductive system, indicate the need for more studies. Since, there is a limited body of knowledge on long and intermediate time-courses of ELF-EMF exposure on reproductive capacity. Therefore, we aimed to investigate the biological effects of long-term exposure to 50 Hz, 1 μT ELF-EMFs on sperm qualities and testicular structural features. The main goal of this study was to investigate the biological effects of different ELF-EMF on the quantitative structural aspects of the testis, and to find the answers to the following questions:

1- Does sperm quality change after being exposed to ELF-EMF?

2- Does the testicle volume (connective tissues and seminiferous tubules) alter after being exposed to ELF-EMF?

3- Does the count of spermatogenic cells (spermatogonia, spermatocytes and spermatids cells) alter after exposure to ELF-EMF?

4- Do the numbers of Sertoli and Leydig cells change after exposure to ELF-EMF?

5- Does the tubules' length change after exposure to ELF-EMF?

Material and Methods

Animals

In this case-control study, we used 60 male mature Sprague-Dawley rats (200-250 grams). Animals were purchased from the "SUMS Animal Care Centre". Our experiments were performed according to the standard ethical protocols approved by Shiraz University of Medical Sciences.

Experimental design

We designed six experimental groups and randomly allocated 10 rats in each group. Helmholtz coils were provided by Medical Physics Department of SUMS [13]. The coils were capable of creating an electromagnetic field with an intensity of 100 μT. The key specifications of the Helmholtz coils are discussed in detail in our previous publications [13]. Our experiment continued eight weeks (exposing the animals to various ELF-EMF levels). The rationale behind choosing this time interval was the point that one period of rat spermatogenesis takes about 48-56 days

[14]. Sixty male Sprague-Dawley rats were randomly divided into six groups. Experimental groups were exposed to ELF-EMF (50 Hz EMF, 100 μT) for either 1 h/day for 52 days (Group 1), 4 h/day for 52 days (Group 3), 1 h/day for 5 days (Group 5), 4 h/day for 52 days (Group 7). Groups 2, 4, 6 and 8 were only sham exposed at durations equal to Groups 1, 3, 5 and 7, respectively.

Spermatozoa analysis

At the end of experiment, a tissue section of 10 mm was spliced from the vas deferens (distal parts) from rats in both control and intervention groups. The specimens were immediately submerged into 3 ml normal saline solution in a Petri dish. The solution was gently shaken (5-10 minutes) to obtain a homogenous suspension. The procedure was performed at 37 °C [15].

Spermatozoa count

Spermatozoa enumeration was carried out using a Hemocytometer. For each subject 200-300 cells were counted under ×40 objective magnification of optical microscope [15].

Spermatozoa motility

To assess the motility, the suspension containing spermatozoa was applied on pre-warmed (37 °C) slides. The motility was checked in at least 200-300 spermatozoa per rat. The criteria for motility were either rapid, slow progressive (moving either fast or slow in a linear direction), non-progressive for spermatozoa moving in a circular pattern, and non-motile for those with no movements. The spermatozoa were assessed in 10 randomly chosen microscopic fields. The ratio of motile spermatozoa was calculated as below [15]:

Motile spermatozoa = Counted motile spermatozoa×100/ total number of spermatozoa.

Percentage of normal and abnormal spermatozoa

Morphological abnormalities were evaluated in at least 200-300 spermatozoa. We used Eosin Y (1%, 5-10 minutes) for staining the spermatozoa suspension. After rinsing the stain, the specimens were allowed to be air-dried, and then the assessment was carried out under light microscopy. Spermatozoa with head and tail abnormalities were counted. Head abnormalities were considered as amorphous head, while other abnormalities included bicephal spermatozoa, or those with fused body. Spermatozoa with disconnected tail were enumerated as abnormal cells as well. Finally, the normal-shape ratio of spermatozoa was calculated [15]:

Normal spermatozoa = Counted normal spermatozoa×100/ total number of spermatozoa.

Hormone assay

Chemiluminescence immunoassay kit (Catalog Number: L2KTW2 was purchased, and the procedure was carried out according to the instructions provided by the manufacturer. Serum samples were obtained from the blood drawn from the heart by centrifugation at 2500 rpm for half-hour. The serum samples were stored at -20 °C until use. The measurement was performed both before and after the exposure.

Stereological study

On the last day of our experiment, the testicles were removed and weighed. Testicle volume (V) was determined according to the Scherle's method, and immersion method at the end of experiment (Figure 1A). For eliminating the need to incessant sectioning of the tissues (that is inevitable, based on Cavalieri's Principle), we used the shrinkage degree (d shr) and the length of tubule instead of preparing uniform isotropic tissue sections based on the orientator method (Figures 1B, C and D). An average of 8-12 slabs were prepared from each testicle, afterward a circular area was removed from a random slab using a trocar (Figure 1E and F). Then, we calculated the surface area of the circular section. Further

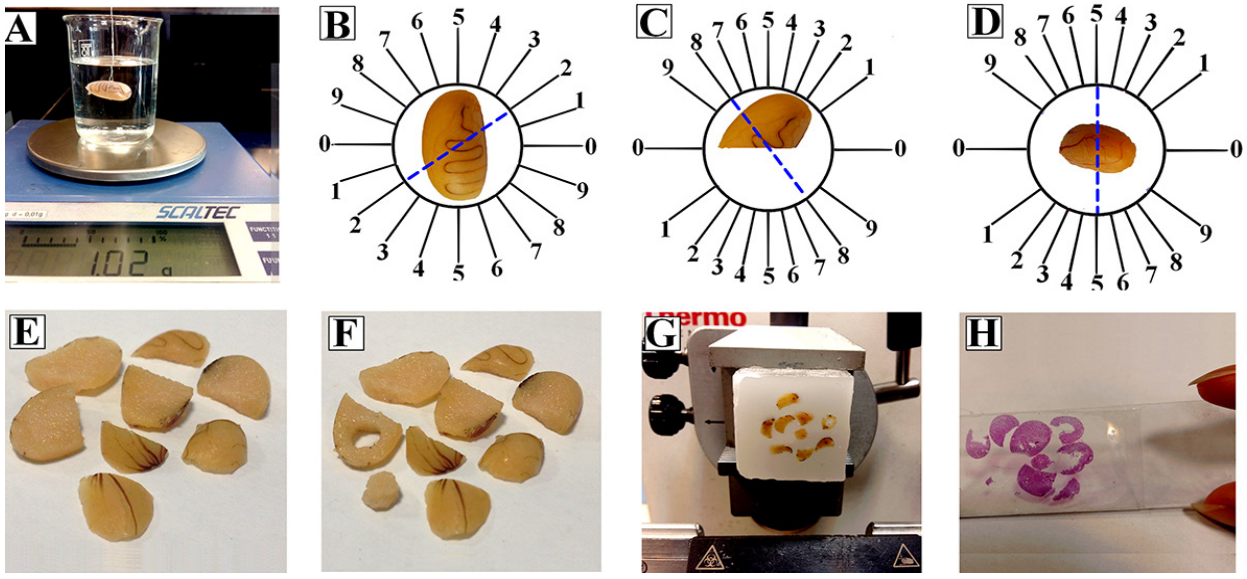

Figure 1: Application of stereological techniques. Determining testis volume according to the Scherle's method and immersion method (A). Obtaining uniform isotropic tissue sections according to the orientator method (B, C and D). Obtaining a collection of isotropic uniform random sections (E). Punching out a circle through a random slice (F). Embedding and sectioning (G). Tissue slide preparation (H).

analysis on the slabs and circular-shape sections (4-25 µm thickness) was performed by staining through Hematoxylin-Eosin [15-18]. The following formula 1 was used to determine the surface area of circular tissue sections and d (shr).

$$d\,(shr)=1-((AA/AB)\times 1.5) \qquad (1)$$

Where AA and AB are the areas of the circular piece before and after processing. The tissue samples were evaluated using video microscopy. For detailed stereological analysis, special guiding frames (point grid and unbiased counting frame) were applied.

Estimating the volume of the testicle components

The "point-counting method" was the method of choice for estimating the volume density of testicles [15-18]. This method includes testicle structures including tubular structures, as well as germinal and interstitial sections) (Figure 2A). The following formula was used to determine the total volume index for individual structures:

$$V(structure)=Vv\,(structure/testicle)\times V(testicle)$$

Estimation of tubules length

Two parameters, the tubular length density and the total tubular length were determined (Figure 2B). The first parameter was measured using the following formula 2:

$$Lv=2\Sigma Q/[\Sigma P\times(a/f)] \qquad (2)$$

Which "ΣQ" denotes the total counts of tubules per testicle, and "ΣP" is the total counts of frames, and "a/f" is the area of the counted frames. The second index was determined using the formula 3 previously described [15-18]:

$$L(tubules)=(Lv(tubules/testicle)\times(1-d(shr))^{2/3}\times V(testicle)) \qquad (3)$$

Estimating the cells number

We used tissue sections with thickness of 25 µm to determine the numbers of different cells in testicles. The enumerated cells were the total counts of spermatogonia, spermatocytes, round and long spermatids, as well as Sertoli

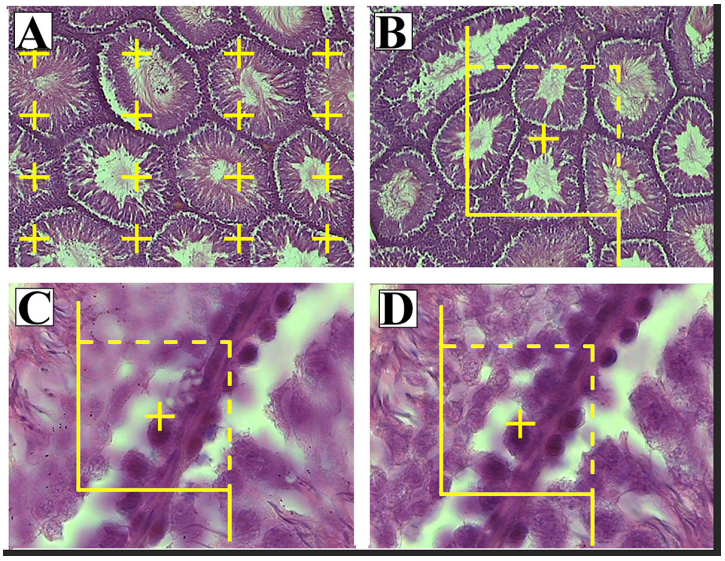

Figure 2: Application of stereological techniques. Point-counting technique to estimate the volume density of the structures (A). Estimation of the length density of the seminiferous tubules by unbiased counting frame (B). Optical disector technique used to estimate the numerical density of the different cells (C and D).

and Leydig cells (Figure 2C and D). The numbers of these cells per volume of germinal epithelial were calculated as follows formula 4:

$$Nv(cells/testicle)=(\Sigma Q/(\Sigma A\times h)\times(t/BA)) \quad (4)$$

This formula is applicable by applying "optical disector" method using stereology software as previously described.

In this method, a microcenter (Heidenhain MT-12, Leipzig, Germany) and a lens with high numerical aperture for oil immersion were used. To determine the guard zones and dissector's height of the tissue section, after recording the distribution of all sampled cells at different focal planes, a plot was prepared for the z-axis distribution.

In the above mentioned formula, ΣQ denotes the number of focused nuclei, ΣA is the surface area of the unbiased counting frame, "h" shows the "dissector's height", "t" is the average section thickness, and BA indicates the microtome setting [15-18]. The total number of cells was estimated using the formula 5 below:

$$N(cells)=Nv(cells/testicle)\times V(epithelium)\times[1-d(shr)] \quad (5)$$

Statistical Analysis

Statistical procedures were performed by using SPSS (Ver16, SPSS, Chicago, IL, USA). Shapiro–Wilk test was used to verify the normal distribution of the variables. Kruskal-Wallis (comparison of stereological features between different groups) and Mann-Whitney U tests (for comparison of sperm characteristics and hormone levels between different groups) were used as inferential statistical methods with $P<0.05$ as the statistically significant cut-off point.

Results

Spermatozoa count, motility and morphology

A significant decrease was observed in the count number as well as motility of the sperm in the animals exposed to ELF-EMF (4 h/52 day), ELF-EMF (1 h/52 day) and ELF-EMF (4 h/5 day) compared to the related control groups ($P<0.05$). However no change was observed in the parameters of normal sperm morphology in all of the experimental groups (Table 1).

Serum testosterone levels

Table 2 shows significant reduction in the serum testosterone levels in the animals exposed to ELF-EMF (4 h/day) compared to the control groups (P<0.05). However, we failed to find significant differences in the serum testosterone levels of the other experimental groups.

Weight and volume of the testis

Our findings show that weight and volume of testis were not significantly different among the experimental groups and the control groups (Figure 3A and B).

Volume of the seminiferous tubules epithelium and interstitial tissue

In Figure 3C and D, revealed a significant decrease in the Volume of the seminiferous tubules, seminiferous tubules epithelium and interstitial tissue in the animals exposed to ELF-EMF (4 h/5 day) compared with the control groups (P<0.05). However no significant differences were found in the seminiferous tubules of other experimental groups.

Table 1: Mean±standard deviation of the spermatozoa count (×10^6), motile (%) and normal morphology (%) of the different groups including Control (4 h/52 day, 1 h/52 day 4 h/5 day) and Extremely Low Frequency-Electromagnetic Fields (ELF-EMF) (4 h/52 day, 1 h/52 day, 4 h/5 day) groups.

Groups	Count	Motility	Normal morphology
Control (4 h/52 day)	*7.4±1.3	**57.5±12.8	91.1±9.2
ELF-EMF (4 h/52 day)	2.7±0.9	40.9.2±13.4	89.3±12.1
Control (1 h/52 day)	*4.1±1.3	**49.5±8.7	91.3.0±5.1
ELF-EMF (1 h/52 day)	2.9±1.1	37.7±9.3	88±7.3
Control (4 h/5 day)	*4.4±2	**54.1±15.5	71.5±15.4
ELF-EMF (4 h/5 day)	2.1±.1.3	38.8±13.6	59.5±24.2

*P<0.05, (Control 4 h/52 day vs. Extremely Low Frequency-Electromagnetic Fields (ELF-EMF) 4 h/52 day), (Control 1 h/52 day vs. ELF-EMF 1 h/52 day), (Control 4 h/5 day vs. ELF-EMF 4 h/5 day)

**P<0.05, (Control 4 h/52 day vs. ELF-EMF 4 h/52 day), (Control 1 h/52 day vs. ELF-EMF 1 h/52 day), (Control 4 h/5 day vs. ELF-EMF 4 h/5 day)

Table 2: Mean±standard deviation of the reproductive hormone testosterone of the different groups including Control (4 h/52 day, 1 h/52 day, 4 h/5 day), Extremely Low Frequency-Electromagnetic Fields (ELF-EMF) (4 h/52 day, 1 h/52 day, 4 h/5 day).

Groups	Testosterone (IU/L)
Control (4 h/52 day)	3.7±3.2
ELF-EMF (4 h/52 day)	2.7±1.4
Control (1 h/52 day)	4.8±3.4
ELF-EMF (1 h/52 day)	3.1±3.3
Control (4 h/5 day)	*6.7±5
ELF-EMF (4 h/5 day)	2.8±1.4

*P<0.05, (Control 4 h/5 day vs. Extremely Low Frequency-Electromagnetic Fields (ELF-EMF) 4 h/5 day)

Length of the seminiferous tubules

Our findings also revealed 18% reduction in the length of tubules of the animals that were exposed to ELF-EMF (4 h/5 day) compared to the control groups (P<0.05). However, no significant differences were observed in the length of seminiferous tubules of other experimental groups (Figure 4C).

Number of cells

The results indicated that spermatogonia, spermatocytes, round and long spermatids, Sertoli and Leydig cells had no significant differences among the experimental groups and the control groups (Figure 4A and B), (Figure 5A, B and C).

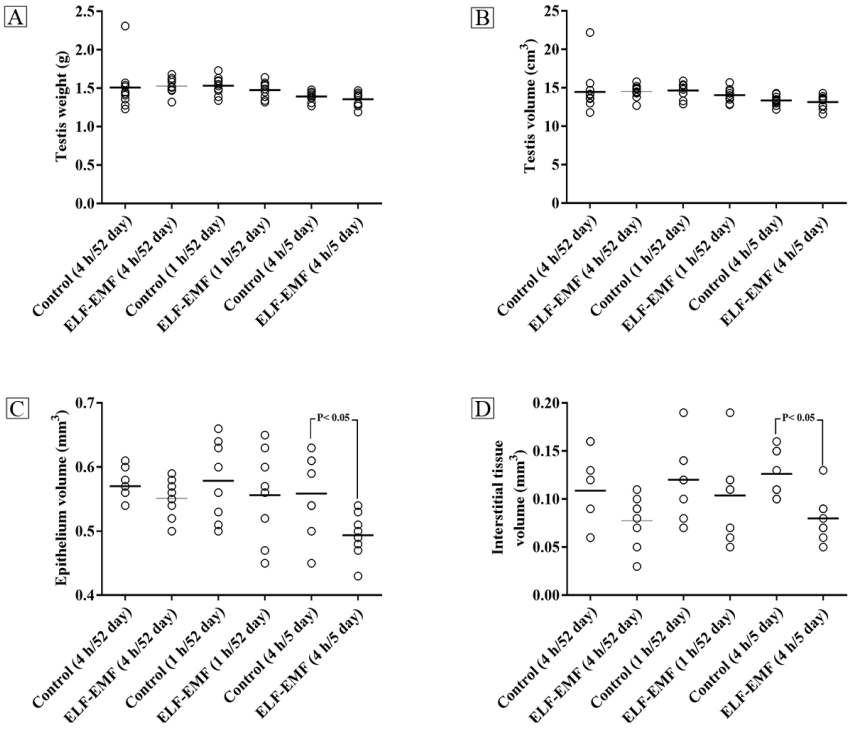

Figure 3: The aligned dot plot of the testis weight (A). Volumes of the testis (B), epithelium (C) and interstitial tissue (D). (ELF-EMF: Extremely low frequency-electromagnetic field)

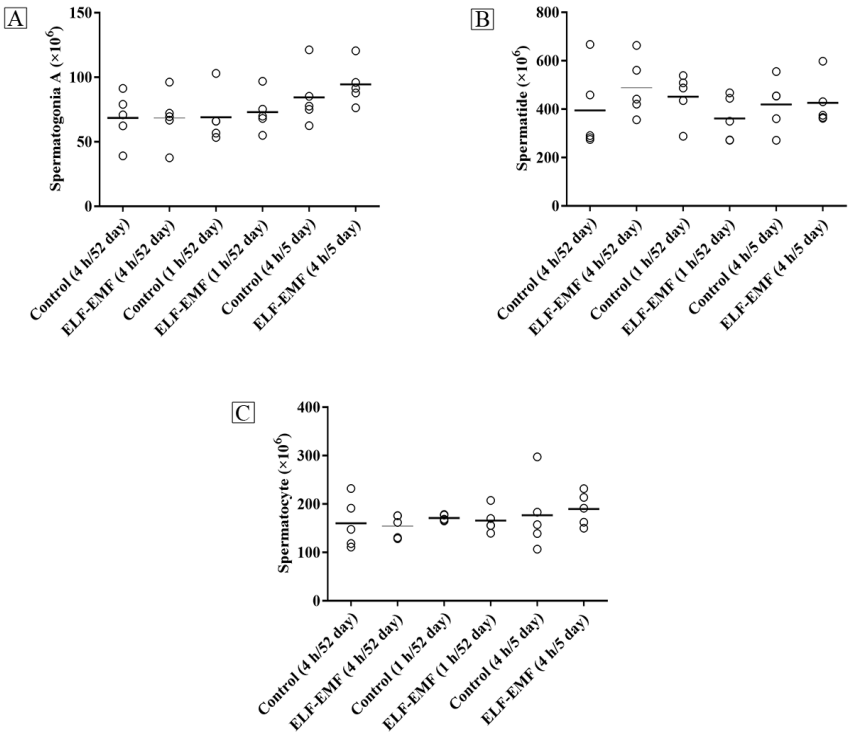

Figure 4: The aligned dot plot of the number of the spermatogonia cells (A), spermatide cells (B), and spermatocyte cells (C). (ELF-EMF: Extremely low frequency-electromagnetic field)

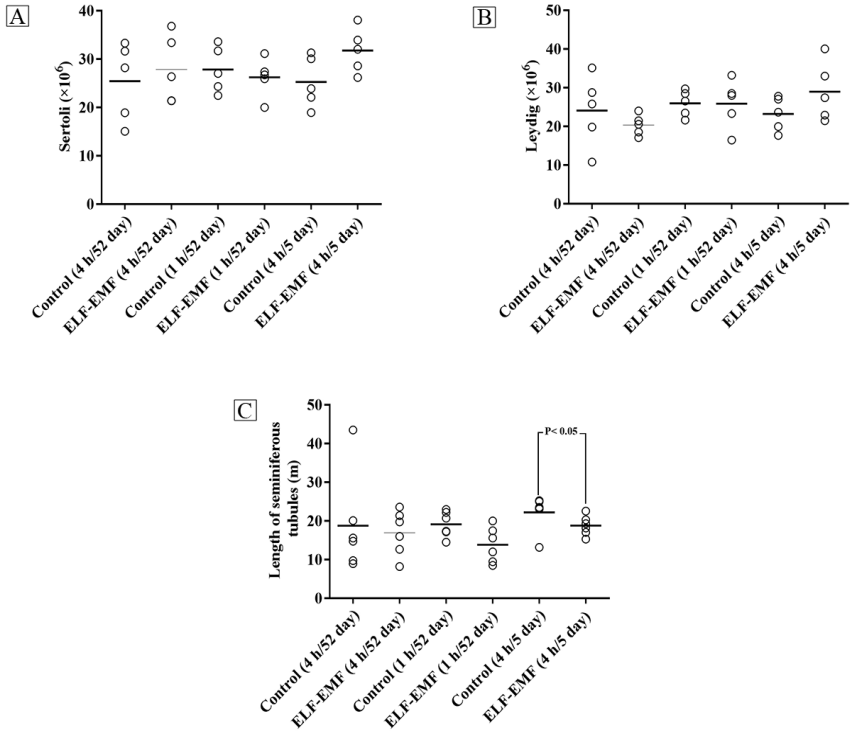

Figure 5: The aligned dot plot of the number of the sertoli (A), and Leydig cells (B) and length of seminiferous tubules (C). (ELF-EMF: Extremely low frequency-electromagnetic field)

Discussion

Electromagnetic fields are a global health concern [19-23]. In the present research, we investigated the potential effects of ELF-EMF on sperm characteristics and testicular tissue integrity in rat model. We observed that sperm quantity and motility were significantly attenuated in the subjects exposed to 100 µT, 50 Hz ELF-EMF for either 4 hours or 1 hour per day for 52 days. However, there were no significant changes in sperm morphology in these conditions. In line with our findings, exposing male rats to ELF-EMF significantly reduced sperm motility, and quantity in previous studies [7, 24, 25]. Moving to radiofrequency electromagnetic fields (RF-EMFs), mobile phone radiofrequency (900 MHz) has been reported to be associated with low sperm count, sperm morphologic defects, as well as spermatocyte genomic alternation, and cell cycle arrest [8]. Despite these reports, neither of one, two, or ten months of exposure of male rats to 50 or 60 Hz ELF-EMF did not show any significant effects on sperm count, morphology and motility [12, 26-30]. In the study by Tas et al. they did not find any significant adverse effects on sperm specification in rats exposed to 900 MHz radiation after 12 months [31]. Variabilities observed in the results of these studies may somehow be a factor of different animal features, different radiofrequency doses, duration, and type of radiation applied [32]. The mechanisms responsible for low sperm count following radiation are unclear. Depletion of seminiferous epithelium structures and shedding cells into lumen structures have been proposed as a possible reason [8]. Furthermore, higher germ cell apoptosis rate were observed in testes exposed to ELF-EMFs [29, 32-34]. However, nor apoptosis rate, or cell cycle arrest were noted in GC–2 cells exposed to ELF-EMF of 50 Hz [34, 35]. On the other hand, radiofrequency irradiated sertoli cells may participate in deranged spermatogenesis by producing and secretion of inflammatory cytokines, and in turn negatively

affecting the survival of testicular germ cells [36]. Another participating factor may be the altered expression of specific adhesion molecules on surface of either developing germ cells or epithelial and Sertoli cells [37]. Molecular studies are required to further characterize mediators involved in regulating sperm development after being exposed to EMFs. Sperm morphological changes are assumed to be the result of chromosomal changes in spermatids during spermatogenesis [8], alternation in epigenetic mechanisms resulting in different gene expression patterns, and peroxidation of membrane structures of sperm precursors [27, 34, 35]. The role of altered expression of specific micro RNAs (miRNA) in dysfunctional spermatogenesis in the context of being exposed to ELF-EMF has been described [38]. In line with this, a total of 55 miRNA were identified with significantly different expression pattern in GC–2 mouse spermatocyte cell line exposed to 1 mT, 2 mT and 3 mT of 50 Hz ELF-EMF [34, 35]. Although molecular determinants and signalling routes involved in the regulation of gene expression are not well known, potential role of epigenetic mechanisms and altered DNA methyl-transferases have been proposed [34, 35]. Morphological alternation of spermatocytes is important as these may predispose to low functionality of the cells and subsequently leading infertility. Nevertheless, molecular mediators associated with morphological defects are yet to be assessed.

In present study, testosterone level diminished in all ELF-EMF exposed groups. However, a significant decline of testosterone level was seen in animals exposed to 4 hours per day for 5 days. In agreement with our finding, exposing male rats to 1mT intensity of 50 Hz ELF-EMF for 85 or 126 days significantly reduced testosterone level [7, 25]. In a similar way, mobile-derived EMF was associated with the reduced testosterone levels [34, 35]. In contrast, either four or eight weeks of exposure of male rats to 50 Hz ELF-MF with magnetic flux intensity of 500 μT failed to show a significant effect on serum testosterone level [12]. Also, three months of exposure to 60 Hz ELF-EMF with intensity of 14-200 μT exerted no prominent changes in testosterone level of BALB/c mice [32]. Hormonal changes following radiofrequency exposure and their impact on reproductive function could be a determining factor in fertility. Different results regarding testosterone level in the above mentioned conditions may be due to different developmental stages of the experimental animals, or due to different radiofrequency intensities and durations.

We noticed a significant decrease in seminiferous tubules volume, length, and seminiferous epithelial and interstitial tissues in animals with 4 hours for 5 days exposer to ELF-EMF. While, we observed no such differences in animals exposed to ELF-EMF either 1 hour or four hours for 52 days. Type A and B spermatogonia, spermatocytes, round spermatids, sertoli cells, and leydig cells showed no significant differences among the exposed and non-exposed animals in our study. In comparison, mobile-derived EMF was associated with reduced testes weight in rats exposed to radiation during fetal period [9]. Exposing male rats to 50 Hz ELF-EMF significantly reduced the seminiferous tubules diameter, while it increased the number of seminiferous tubules [7]. Likewise, mice exposed to 60 Hz ELF-EMF revealed diminished seminiferous tubule diameter and disarrangement of seminiferous tubules structures [32, 33]. Seminiferous epithelium atrophy was reported in mice exposed to 900 MHz radiofrequency for 35 consecutive days [8]. In contrast, either four or eight weeks of exposure of male rats to 50 Hz ELF-MF with magnetic flux intensity of 500 μT did not have any significant effects on seminiferous tubes diameter [12, 28]. There were also no significant differences in seminiferous tubular diameter, spermatids, and Sertoli's cells in animals exposed to 915 MHz radiofrequency compared to non-exposed subjects [30].

The reduction in tubular diameter following ELF-EMF exposure may be due to higher apoptosis rate in seminiferous epithelium [30]. Altered testicular architecture, and deranged morphology of seminiferous tubules have also been noted as possible mechanisms [26, 31]. Radiofrequency induced oxidative stress was proposed as a potential mechanism that can be accounted for adverse cellular and histological defects in testes following exposure to EMF [1, 8]. However, oxidative markers, TAC, myeloperoxidase, catalase, and malondialdehyde did not differ significantly in exposed vs. non-exposed groups in animals exposed to 100 and 500 μT of 50 Hz ELF-MF for 10 months (7 days a week and 2 hours per day) [29]. In accordance, no significant difference was described in oxidative status of testes in male rats treated with 50 Hz ELF-EMF with intensity of 1 mT for 2 months [27]. Regarding these disputes, there is a need to reveal more evidences on the role of oxidative stress in pathogenesis of spermatogenesis and testes derangements in these conditions.

Conclusion

Today, exposure to ELF-EMF with frequencies below 300 Hz is widely common in our daily life. Exposure to ELF-EMF (4 h/52 day, 1 h/52 day, and 4 h/5 day) significantly decreased both count and motility of sperms in irradiated animals compared to the control groups. Moreover, serum testosterone levels were significantly reduced in the irradiated animals (4 h/5 day) compared to those of the control groups. In addition, a significant decrease was observed in the volume of the seminiferous tubules, seminiferous tubules epithelium and interstitial tissue in the animals exposed to ELF-EMF for 4 h/5 day. Tubules length showed a 18% decrease in animals exposed to ELF-EMF (4 h/5 day). Altogether, our results show that exposure to ELF-EMF can reduce spermatocyte count and motility and is able to induce structural changes in testicular tissue. Further studies are needed to reveal the different aspects of such exposures.

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Authors' Contribution

SMJ. Mortazavi and S. Karbalay-Doust conceived the idea. All experiments were mainly conducted by M. Darabyan with the assistance of M. Sisakht, Gh. Haddadi, N. Sotoudeh, and M. Haghani, under the supervision of SMJ. Mortazavi and S. Karbalay-Doust. All the authors read, modified, and approved the final version of the manuscriptript.

Ethical Approval

This study was approved by the Medical Ethics Committee of Shiraz University of Medical Sciences (Ethics Committee approval Code: IR.SUMS.REC.1395.169).

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Conflict of Interest

None

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