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Arch. Esp. Urol. 2015; 68 (6): 562-568

BIOCHEMICAL AND HISTOPATHOLOGICAL EFFECTS ON THE RAT TESTIS AFTER EXPOSURE TO ELECTROMAGNETIC FIELD DURING FETAL PERIOD

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Summary.- *OBJECTIVES: Electromagnetic radiation (ER) emitted from cell phones may exert a detrimental influence on human health and may affect the man reproductive system. We aimed to study the biological and morphological effects on the testes of 60-day-old male rats after ER exposure (900 MHz), which was applied continuously throughout embryogenesis.*

METHODS: A total of six pregnant Sprague Dawley rats were included in the study. Three pregnant rats (experimental group) were exposed to radiation from a cell phone set to talking mode for 24 hours a day for 20 days, and the other 3 pregnant rats (control group) were not to exposed to radiation. Newborn male rats were included from the experimental group (n=7) and the control group (n=7). At the end of 60 days, the rats' testes were excised, and testis length, width, depth, and weight were measured. Histopathological examinations were compared and serum testosterone (T) levels were assayed biochemically.

RESULTS: While serum T level (3.51±0.21 ng/ml) of ER Exposed group was significantly lower than the control group (4.04±0.47 ng/ml, p=0.018), Caspase-3 enzyme activity (2.00±0.88) was significantly higher than the control group control (1.00±0.63, p=0.026). Johnsen score (8.4±0.5) of ER group was fairly lower than the control group (9.4±0.5, p= 0.010).

CONCLUSION: Our study demonstrated that ER exposure throughout embryogenesis may cause reductions in serum total T levels and in the size and weight of the testes of male rats, while causing modest increase in apoptosis.

Keywords: *Electromagnetic radiation. Testis. Testosterone. Apoptosis. Caspase-3.*

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Accepted for publication: December 1st, 2014

Resumen.- *OBJETIVOS: La radiación electromagnética (RE) emitida por los teléfonos móviles puede tener una influencia deletérea sobre la salud en humanos y puede afectar al sistema reproductor masculino. Buscamos estudiar los efectos biológicos y morfológicos en los testículo de ratas macho de 60 días de edad después de la exposición continua a RE (900 MHz) aplicada continuamente durante el periodo embrionario.*

MÉTODOS: En el estudio se incluyeron un total de seis ratas Sprague Dawley embarazadas. Tres ratas embarazadas (Grupo experimental) fueron expuestas a la radiación de un teléfono móvil en modo conversación las 24 horas diarias durante 20 días, y las otras tres (grupo control) no fueron expuestas a radiación. Fueron incluidas las ratas macho nacidas del grupo experimental (n=7) y del grupo control (n=7). Al final de los 60 días se extirparon los testículos de las ratas y se midieron la longitud, anchura, profundidad y peso testiculares. Se compararon las valoraciones histopatológicas y se detectaron bioquímicamente los niveles de testosterona sérica.

RESULTADOS: Mientras que los niveles de testosterona sérica del grupo expuesto a RE (3,51±0,21ng/ml) eran significativamente menores que los del grupo control (4,04±0,47 ng/ml, p=0,018), la actividad de la enzima Caspasa 3 era significativamente superior a la del grupo control (1,00±0,63, p=0,026). El score de Johnsen del grupo de RE (8,4±0,5) fue bastante más bajo que el del grupo control (9,4±0,5, p= 0,010).

CONCLUSIONES: Nuestro estudio demostró que la exposición a RE durante la embriogénesis puede provocar reducción de los niveles séricos de Testosterona total y del tamaño y peso de los testículos de las ratas macho, causando a la vez un aumento modesto de la apoptosis.

Palabras clave: *Radiación electromagnética. Testículo. Testosterona. Apoptosis. Caspasa 3.*

INTRODUCTION

Electromagnetic radiation (ER) emitted from cell phones may affect the reproductive system via its specific efficacies or its thermal molecular actions, or both. The effects of cell phone exposure are different, depending on the exposure time, exposure conditions, gender, and the type of tissues exposed (1). In the literature, there are publications that describe detrimental effects on reproductive health and fetal development (especially regarding the likelihood of genetic defects associated with high-dose exposure), and others that report a lack of any significant action (2,3). Since the younger segment of the population uses cell phones the most intensively, it is important to reveal the likely negative effects of long-term cell phone exposure on reproductive health (particularly in males, who carry their cell phones in pockets close to the reproductive organs) (4).

Histopathological changes in the testis, in particular, were studied to investigate the effects of cell phone-emitted radiation exposure on fetal development, reproductive health, and embryonic and fetal growth (5). As ER could decrease the weight of testis, epididymis and seminal vesicles, decrease seminiferous tubule diameter and tunica albuginea thickness, lead to irregularity of basal membrane of seminiferous tubule and its epithelium, it could cause hypospermatogenesis and stop maturation of spermatozoa (1,4,5,6). ER may decrease testicular function and obvious decrease in serum testosterone (T) levels by deteriorating the differentiation of Leydig cells (7,8). Furthermore, it has been reported that ER induces apoptosis by damaging DNA directly, increasing lipid peroxidation and reactive oxygen radicals (5,9).

We also aimed to study the biological and morphological effects on the testes of male rats occurring subsequent to ER exposure (900 MHz), which was applied continuously throughout embryogenesis.

MATERIALS AND METHODS

Ethic issue

The study protocol was reviewed and approved by the Local Ethical Committee for Animal Experiments, School of Medicine, Recep Tayyip Erdogan University, Turkey (approval number: 2014/2).

Animals

A total of six pregnant Sprague Dawley rats, weighing 280-300 grams each, were included in the study. The rats were maintained on a 12-hour light-dark cycle regimen at a constant temperature of 20-22ºC with 50-55% relative humidity. All of the animals were fed ad libitum with free access to tap water. All procedures involving the animals were designed and performed according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

Gestational period

Female rats were housed in plastic cages sized 36 cm x 23 cm x 21 cm (three rats per cage). One male rat was placed in each cage for mating with the three female rats. One day later, the male rats were removed from the cages. After inserting a pipette into the vagina of each female rat, a 0.5 mL physiological saline solution was administered and vaginal secretions of the rats were collected. The samples were examined with the vaginal smear method by light microscopy. If the microscopic

examination showed sperm, the rat was considered to have conceived and to be at postnatal day 0 of pregnancy. The rats were considered pregnant without the need for microscopic examination of the vaginal secretion if the vaginal membrane was observed on vaginal inspection. After the mating day, three pregnant rats (experimental group) were exposed to radiation emitted from the digital signal generator and the other three pregnant rats (control group) were not exposed to radiation.

Exposure ER

Three pregnant female rats were exposed to cell phone set on talking mode radiation for 20 days continuously (24 hours). The other three pregnant female rats were not exposed to any radiation. The device used for ER; a generator with an external antenna was placed under the cage centrally. A digital signal generator (Anritsu MG3670 B type, Japan), which produces 900 MHz radiofrequency radiation, was used in the present study to represent exposure by global systems for mobile communications. Peak power of the digital signal generator was fixed at 2W during exposure. For the generator used in this study, the carrier frequency was 900 MHz, modulation frequency was 217 Hz, pulse width was 577 μsec, and maximal peak power was 2W.

Performing the experiment and the creation of the experimental groups

After the delivery of the female rats the newborn male rats were determined. Seven male rats that were exposed to ER in fetal period were included in the ER group and 7 male rats that were not exposed to ER in fetal period were included in the control group randomly. The male rats were waited to enter puberty for 60 days after birth. The pubertal stage is accepted as the certain stage whether there is rat testis maturation, at the end of 60 days, the male rats (130-140g) were intraperitoneally administered a combination of 10mg/kg of 2% xylazine hydrochloride (Rompun, Bayer, Germany) and 50mg/kg ketamine hydrochloride (Ketalar, Eczacibasi, Turkey). The animals were killed by intracardiac perfusion with 4% formaldehyde. Blood samples (3.0 ml) were removed from the left ventricle of each animal on decapitation.

HISTOPATHOLOGICAL EVALUATION

Histological Criteria for the Modified Johnsen Scoring

This scoring system from 1 to 10 as follows: complete spermatogenesis Score is 10. Slightly impaired spermatogenesis including many late spermatids or disorganized epithelium has a score of 9. If there are less than five spermatozoa per tubule or few late spermatids has a score of 8. Score 7 stands for Score no spermatozoa, no late spermatids or many early spermatids. Score 6 stands for no spermatozoa, no late spermatids or few early spermatids. If there is no spermatozoa or spermatids or many spermatocytes it has a score of 5. If there is no spermatozoa/spermatids or few spermatocytes it has a score of 4. Score 3 stands for spermatogonia only. Score 2 stands for no germinal cells or Sertoli cells only. Finally score 1 stands for no seminiferous epithelium (10).

Evaluation of Caspase-3 and Hematoxylin-eosin (H&E)

The testes were excised and the specimens were prepared for histopathological analysis. In addition, testis length, width, depth, and weight were measured. The testes were stored in buffer formaldehyde (10%) at 4ºC for 24 hours. For immunohistochemical staining 3 μm thick sections of the testis tissues were cut, collected on glass slides

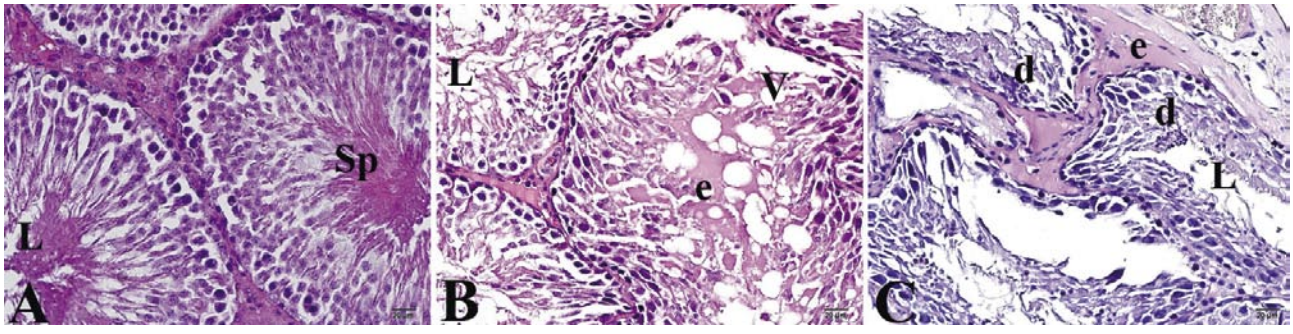


Figure 1. Histopathological examination of testis tissue by light microscopy with H&E stain. A: control group; B and C: experimental group; Sp: spermium, e: edema, L: lumen, d: degeneration, v: vacuolization.

and allowed to stand in xylene for 20 minutes before the application of an alcohol series (50–100%) then allowed to stand for 10 minutes in an H_2O_2 solution. After being washed with PBS, these sections were heated in a citrate buffer solution at 800Wfor 4-5 minutes and allowed to stand in secondary blocker substance for 20 minutes. The primary antibody Caspase-3 (Rabbit Polyclonal antibody to Anti-active Caspase-3, ab13847, 1/50 dilution ratio) and a biotinylated secondary antibody (Universal LSAB Kit-K0690, DAKO), sections were incubated with primary antibodies used for 60 min. at room temperature (22-24 C0) and then incubated with Streptavidin-Horseradish Peroxidase (Universal LSAB Kit-K0690, DAKO) for 30 min. A diaminobenzidine solution was used as an achromogen, Mayer's hematoxylin as a counterstain for 3–5 minutes, and PBS as a negative control. The treated sections were then mounted with Entellan (Code107960, Merck, Darmstadt, Germany). Employing a blind evaluation protocol performed by two histologists and two pathologists, the percentage positivity of the Caspase-3 immunoreactivity were separated into four categories: weak (+), mild (++), moderate (+++), and severe (++++). The blocked tissues were cut into 4- μm thick sections before being stained with H&E, and then examined under a light microscope (BX51; Olympus, Tokyo, Japan) with a digital camera (DP72; Olympus, Tokyo, Japan) and photographed at relevant magnifications.

Hormone analysis

The concentration of T was measured using the enzyme-linked immunosorbent assay (ELISA) method. We used commercially available human T ELISA kits (MyBiosource, USA). The procedure for the ELISA method was followed according to the instructions provided by the manufacturer. Absorbance was measured at a wavelength of 450 ηm using the ELISA reader. The levels of T are presented as ng/ml. The intra-assay and inter-assay coefficients of variation were 8.06% and 9.3%, respectively. The limit of detection (LOD) for the T assay was 0.066ng/ml.

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS; version 18 for Windows, IBM, IL, USA). The data are presented as mean ± standard deviation. Mann Whitney-U test was used for the comparison of Elisa and histopathologic data in both group.

RESULTS

While serum T level (3.51±0.21ng/ml) of ER exposed group was significantly lower than the control group (4.04±0.47ng/ml, p=0.018), Caspase-3 enzyme activity (2.00±0.88) was significantly higher than the control group control (1.00±0.63, p=0.026) (Table I). Johnsen score (8.4±0.5) of ER group was fairly lower than the control group (9.4±0.5, p=0.010) (Table I). In control group, the testis-wrapping tunica albuginea had not lost their fibrous connective tissue properties and were composed of cells with normal histological morphology. The tubulus seminiferus germinative epithelia were preserved from the lumen to the periphery, there was no spilling of cells, and the lumina were full of spermiums. The interstitial spaces between the tubules were filled with Leydig cells that stained acidophilic with eosin, and there was no degeneration of cell nuclei (Figure 1A).

In ER group, in areas exhibiting normal tunica albuginea thickness, cellular losses were lower among the spermatogenic series in the tubuli seminiferi contorti near the exterior surface, and cellular degeneration and spilling was lower among these regions. In regions showing variation in the tunica albuginea thickness, tubuli lost their proper morphology and lumina were mostly empty and occasionally filled with fluid, while intracellular vacuolization and cellular loss in the spermatogenic series was also observed (Figure 1B and 1C).

In ER group, the testis-wrapping tunica albuginea areas neighboring the epididymis had not

Table I. Serum testosterone levels, Caspase-3 activity and Johnsen scores in both group.

Group	Testosterone	Caspase-3	Johnsen scores
	median± SD	median± SD	median± SD
Control	4.04 ± 0.47	1.00 ± 0.63	9.4 ± 0.5
ER	3.51 ± 0.21¥	2.00 ± 0.88≠	8.4 ± 0.5¶

¶ p=0.010, ¥ p=0.018, ≠ p=0.026 vs. control group.

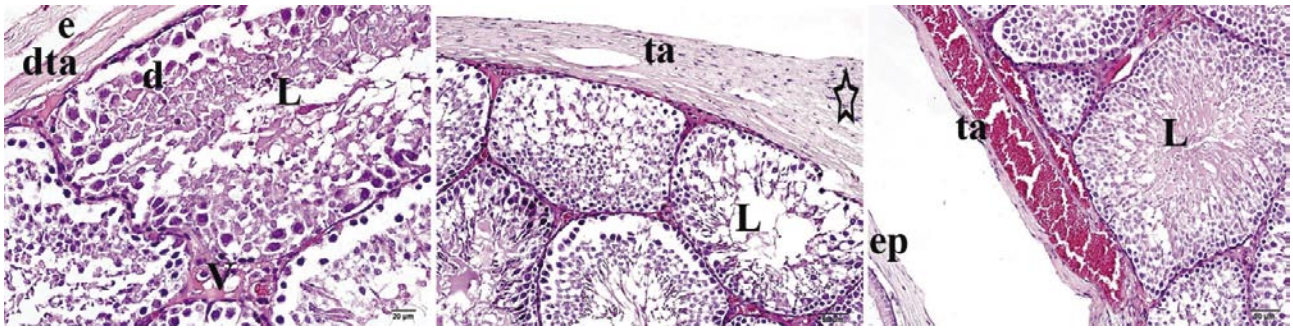


Figure 2. Photomicrograph of rats' testis tissue by H&E stain. All figure were experimental group; e: edema, L: lumen, ta: tunica albuginea, d: degeneration, ep: epididymis, dta: degenerative tunica albuginea, star: thickening of tunica albuginea, v: vacuolization.

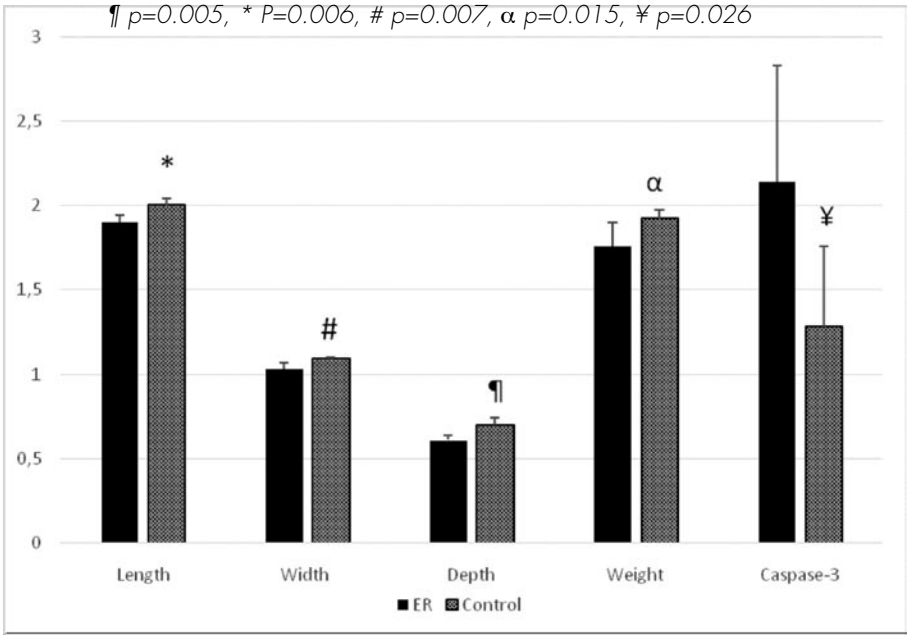
lost their fibrous connective tissue properties and were composed of cells with normal histological morphology. There were thickness differences in discrete regions (local thickening or thinning) with concomitant dilatations in the tunica vasculosa (Figure 2).

In addition to the existence of local tubular injuries and interstitial Leydig cell vacuolization, mild increases in Leydig cell loss were also observed in ER group. Despite the lower cell loss (5%), there was no statistical difference ($p>0.05$). Vacuolization of Leydig cells induced cell loss. Moreover, our study revealed that there were significant reductions ($p<0.05$) in testicular size and weight in the experimental group compared to the control group (Graphic 1).

In ER group, apoptotic increases in Leydig cells were defined by Caspase-3 immunopositivity (Figure 3). Staining of the testis tissue using the immunoperoxidase method revealed the Caspase-3 immunopositivity to be 72% (+), 20% (++), and 8% (+++) in the control group and 28% (+), 50% (++), 15% (+++), and 7% (++++) in the experimental group (Figure 3). A statistical difference was found in the ER-exposed group compared to the control group for Caspase-3 immunopositivity.

DISCUSSION

According to the results of the present study serum T level of the rats which were exposed to ER during fetal life was significantly lower than those which were not exposed to ER. When testis dimensions were evaluated during puberty -the best stage in which pubertal maturation can be observed- testis dimensions of ER group were significantly lower than the control group. In other words the exposure to ER led to immaturity of testis. Additionally; the significant lower Johnsen scores of ER group than the control group suggests the interruption of testicular maturation. The histopathological and immunohistochemical examination revealed that beside the damage of testicular tissue Caspase-3 enzyme activity also were increased in relation to ER exposure. The number of cell phone users has progressively increased globally due to the mobile phone revolution of the last twenty years (11) This has caused increased concern regarding the health effects of radiation emitted from cell phones, base stations, and transmitters. Accordingly, many animal studies



Graphic I. Effects of ER on testicular morphometric size, weight and Caspase-3 activity.

have been conducted to reveal the likely effects of cell phone-emitted radiation on reproductive health (2,5,12). In many studies, the effect of exposure to a 900 MHz cell phone on apoptosis, sperm motility, and male hormones was investigated, and it was reported that there is no definite proof of a negative effect (13,14). However, some authors have reported that long-term 900 MHz cell phone exposure causes decrements in the weight of testes, the diameter of seminiferous tubules, the height of germinal epithelia, and the thickness of tunica albuginea (1,5,15). They reported that exposure to mobile phones may primarily affect Leydig cells, seminiferous tubules, and spermatozoa. It is assumed that cell phone exposure causes damage via scrotal hyperthermia and oxidative stress (1,16). As it is known that the major function of the testis is the synthesis and secretion of T, which is essential for spermatogenesis and the production of spermatozoa; therefore, cell phone exposure specifically may causes deterioration of spermatogenesis and T level reductions, while it is assumed that the decrements of serum T levels may occur due to the differentiation block of Leydig cells originating from mesenchymal cells and decreases in testicular function (7,8). In the current study, serum T levels in the experimental group was prominently reduced than control group, this phenomenon is explainable by minor increases in apoptotic rates despite the observed Leydig cell vacuolization; thus, a few percentage points (5%) of cellular loss was likely compensated for by ongoing cell renewal, explaining significantly reductions in T levels.

Ozguner et al. observed reductions in testicular weight in an ER-exposed group in comparison to controls, albeit with no significance (1). In present study, reductions both in testicular size and weight were revealed in the experimental group in comparison to the control group, and these changes occurred with statistical significance. It has been shown in various studies that long-term exposure to cell phone radiation diminishes serum T level (12,17,18) Hitherto, none of these studies assessed the effect of cell phone-generated ER on Leydig cells in utero. In the present study, it was found that in utero long-term exposure to ER generated by cell phones was significantly decrease serum T levels in exposure-ER rats compared to the matched control rats. Our study is the first study that shows ER exposure to lower serum T level and testis dimensions.

In a study conducted by Dasdag et al., the weight of the epididymis and vesicula seminalis, the diameter of the seminiferous tubuli, and the thickness of the tunica albuginea were decreased following exposure to long-term radiation (2.4 GHz) in comparison to the control group (6). Also, Hanci et al. detected reductions in the diameter of seminiferous tubules and in thickness of the seminiferous epithelia (5). In addition, Tas et al. reported that seminiferous tubule thickness was similar in ER-exposed and control groups, while tunica albuginea thickness was significantly reduced in the former (10). In our study, it is demonstrated that the tunica albuginea demonstrated thickness variations (decrements or increments) in regions exposed to more-intense radiation, while no thickness differences of the tunica albuginea were seen in regions near the epididymis. It is assumed that this phenomenon occurred due to lower radiation exposure and subsequent lower radiation influence due to the shedding of the overlying epididymal layer in the tunica albuginea regions neighboring the epididymis.

Caspases play an important role in male infertility via interference with sperm differentiation and testis maturation (19). In our study, it is observed that the apoptosis-indicating Caspase-3 immunopositivity in the rat testis is significantly higher in the experimental group compared to the control group, similar to the study results of Hanci et al. (5). Moreover, apoptotic cell death is very important in

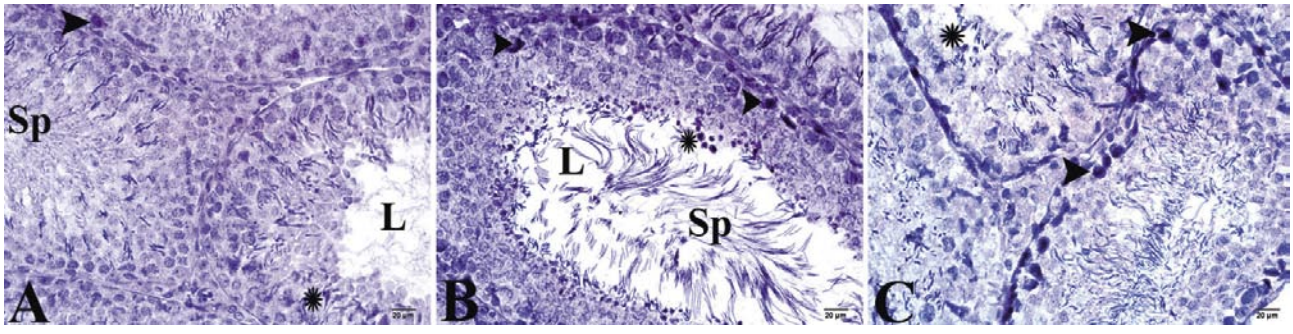


Figure 3. Immunostaining of anti-Caspase-3 in testis tissue. A: control group, B and C: experimental group; black arrow head: strong positivity, asterix: positive stained spermatid cells, L: lumen, Sp: spermium.

the maturation and differentiation of germ cells, and reductions in T levels may occur due to increases in germ cell apoptosis (2). In our data was shown that serum T levels decreased in accompaniment with increases of Caspase-3 immunopositivity in the experimental group compared to the control group. Also, remarkably difference was detected regarding the T level between the experimental and control groups.

Our finding of high Caspase-3 enzyme level in ER group suggests that long term exposure to ER increases apoptosis by activating Caspase-3 enzyme which leads to cellular injury of testis.

CONCLUSION

Our study demonstrated that chronic ER exposure throughout embryogenesis can causes reductions in serum T levels and in the size and weight of the testes of male rats, while causing only a modest increase in apoptosis. According to these results, our opinion is that exposure to ER during fetal period may cause male infertility via deterioration in testicular maturation.

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