

EFFECT OF ROSMARINIC ACID ON SERTOLI CELLS APOPTOSIS AND SERUM ANTIOXIDANT LEVELS IN RATS AFTER EXPOSURE TO ELECTROMAGNETIC FIELDS

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Rosmarinic acid belongs to the group of polyphenols; it has antioxidant, anti-inflammatory and antimicrobial activities and help to prevent cell damage caused by free radicals. The objective was to study the effect of Rosmarinic acid on sertoli cells apoptosis and serum antioxidant levels in rats after they were exposed to electromagnetic fields. Male Wistar rats (n=40) were allocated into three groups: control group (n=10) that received 5cc normal saline (0.9% NaCl) daily by gavage method, Rosmarinic acid group that received 5mg/rat (gavage) (n=10), electromagnetic fields (EMF) group that had exposure with 50hz (n=20) which was subdivided to two groups of 10; EMF group and treatment group. Treatment group received 5mg/rat (gavage) Rosmarinic acid daily for 6weeks, respectively. However, the control group just received an equal volume of distilled water daily (gavage). On the 42nd day of research, 5cc blood was collected to measure testosterone hormones, total antioxidant capacity (TAC), levels from whole group's analysis. Level of malondialdehyde (MDA) levels and sertoli cells apoptosis significantly decreased in the group that received 5mg/rat of Rosmarinic acid ($P<0.05$) in comparison with experimental groups. Level of testosterone, total antioxidant capacity (TAC), significantly increased in groups that received Rosmarinic acid ($P<0.05$). Since in our study 5mg/rat of Rosmarinic acid showed significantly preventive effect on cell damages especial sertoli cells apoptosis that caused with EMF, it seems that using Rosmarinic acid as food additive can be effective for supporting people living under EMF environmental pollution.

Keywords: Apoptosis, EMF, Rosmarinic acid, Sertoli cells, Testosterone**Introduction**

Rosmarinic acid used in European folk medicine to treat numerous ailments is a major anti-oxidant compound. Rosmarinic acid (RA) is used to treat bronchial asthma, peptic ulcers, cataract, arthritis, cancer and rheumatoid arthritis. It is found in large quantities in oregano, lemon balm, sage, marjoram, and rosemary (Kuo et al., 2011). Many studies have shown that rosemary extracts play important roles in anti-inflammation, anti-tumor, and anti-proliferation in various in vitro and in vivo settings (Cheng et al., 2011). Rosemary extracts have been used as an antioxidant to improve sperm quality and fertility (Malo et al, 2010).

A number of research studies on the potential health effects of electromagnetic fields (EMFs) have been performed in Europe, North America, Asia and Iran. The mechanism of the EMF interactions is not well known, although a few studies have suggested the involvement of lipid peroxidation and free radical formation (Khaki et al., 2008), as well as biochemically induced oxidative stress. Many studies by Khaki et al. showed that EMF had harmful side effects on reproductive organs in mammals and could be baneful on spermatogenesis specially on Sertoli cell (Khaki et al., 2010; Khaki et al., 2011). Sertoli cells' early disruption of maturation and differentiation in young males could cause future spermatogenesis and infertility problems (Tarulli et al, 2013). Antioxidants secreted by the reproductive tract protect spermatozoa against the toxic effects of reactive oxygen species (ROS) after ejaculation (Koziorowska-Gilun et al., 2011). Vitamin E can regulate apoptosis-related protein Bcl-2, Bax expression and confront free radical damage which in turn contributes to a protective effect for ovarian granulosa cells (Lu et al., 2009). Previous studies have shown that the extract of rosemary leaves known as antioxidants belongs to the group of polyphenols (Cheng et al., 2011). The aim of this study was to see antioxidant effect of Rosmarinic acid on dysfunctional in sertoli cells change after exposure to electromagnetic fields (EMF) in rats.

Material and Methods**Animals**

A total of 40 male adult Wistar rats with weight 250 ± 10 g were maintained for one week prior to use in this study. Rats were housed together (10 per cage) and fed on a compact diet in the form of granules and water. The diet contained all the essential ingredients, including, vitamins and minerals. The environmental conditions (temperature and humidity) in all the animal holding areas were continuously monitored. Temperature was maintained in the range of 23°C and humidity was maintained at 35 to 60%. Light was provided on a 12 h light/dark cycle from 0700 to 1900 h. All animals were treated in accordance to the Principles of Laboratory Animal Care (NIH) throughout the study. The experimental protocol was approved by the Animal Ethics Committee in accordance with the guide for the care and use of laboratory animals prepared by Tabriz University of Medical Sciences. Rats were allocated randomly to four groups, a control group (n = 10) that received 5cc normal saline (0.9%) daily by gavage methods and three treatment groups (total = 30) and n= 10 in each. The first treatment group received rosmarinic acid (5 mg/kg body weight), the second extract group received rosmarinic acid (5 mg/kg body weight) and EMF exposure at 50 Hz for 42 consecutive days, while the fourth group received only EMF exposure for 42 consecutive days. Animals were maintained under standard conditions with respect to humidity, illumination and temperature for 42 consecutive days.

Pharmacological Procedure

Rosmarinic acid was purchased from Sigma Chemicals (St. Louis, Mo., USA). Rosmarinic acid was dissolved in normal saline (0.9%) (5mg/rat) and administered by gavage models.

EMF-producing system

The equipment was based on the Helmholtz coil, which operated following Fleming's right hand rule. The equipment produced an alternating current of 50 Hz, which created an EMF of 80G. The intensity of the EMF was controlled using a transformer. The equipment had two main parts. In the first part, there were two copper coils that were placed, one above the other and separated by a distance of 50 cm. A cylindrical wooden vessel was placed between the coils (the exposure area), the interior of which contained a chamber for holding the caged experimental animals. The second part was the transformer, which controlled the input and output voltage using a voltmeter and the current with an ampere meter. A fan was used as required to prevent increase in temperature inside the chamber. Four cages at a time were placed within the chamber, with ten rats per cage.

Surgical Procedure

On day 42, a sodium pentobarbital solution (40 mg/kg) was administered intra-peritoneal as an anesthetic, and the peritoneal cavity was opened with a lower transverse abdominal incision. Both testes were then immediately removed from the control and experimental groups. The weight of the testes for each group member was recorded. Animals were then decapitated between 10:00 and 12:00 h. At the end of 4 weeks of treatment, testis was dissected from each rat, 24 h after the last administration.

TUNEL analysis of apoptosis

The *in-situ* DNA fragmentation was visualized by TUNEL method (Khaki et al., 2008). Briefly, de-waxed testis tissue sections were predigested with 20 mg/ml proteinase K for 20 min and incubated in phosphate buffered saline (PBS) solution containing 3% H₂O₂ for 10 min to block the endogenous peroxidase activity. The sections were incubated with the TUNEL reaction mixture, fluorescein-dUTP (*in situ* Cell Death Detection, POD kit, Roche, Germany) for 60 min at 37°C. The slides were then rinsed three times with PBS and were incubated with secondary anti-fluorescein-POD-conjugate for 30 min. After washing three times in PBS, diaminobenzidine- H₂O₂ (DAB, Roche, Germany) chromogenic reaction was added on sections and counterstained with hematoxylin. As a control for method specificity, the step using the TUNEL reaction mixture was omitted in negative control serial sections, and nucleotide mixture in reaction buffer was used instead. Apoptotic sertoli cells were quantified by counting the number of TUNEL stained nuclei per seminiferous tubular cross section. Cross sections of 100 tubules per specimen were assessed and the mean number of TUNEL positive sertoli cells per tubule cross-section was calculated.

Measurement of Serum Total Antioxidant capacity (TAC)

TAC was measured in serum by means of a commercial kit (Randox Co-England). The assay is based on the incubation of 2, 2'-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS) with a peroxidase (methmyoglobin) and hydrogen peroxide to produce the radical cation ABTS⁺, which has a relatively stable blue-green color, measured at 600 nm. The suppression of the color is compared with that of the Trolox, which is widely used as a traditional standard for TAS measurement assays, and the assay results are expressed as Trolox equivalent (mmol/L).

Measurement of Serum Malondialdehyde levels (MDA)

Tissue MDA levels were determined by the thiobarbituric acid (TBA) method and expressed as nmol MDA formed/mL. Plasma MDA concentrations were determined with spectrophotometer. A calibration curve was prepared by using 1,1', s3, 3'-tetramethoxypropane as the standard.

Total serum testosterone hormone measurement

Total serum concentration of testosterone was measured using a double-antibody RIA kit (Immunotech Beckman Coulter Co., USA). The testosterone detection sensitivity per assay tube was 0.025 ng/ml.

Statistical analysis

Statistical analysis was done using the ANOVA and test for comparison of data in the control group with the experimental groups. The results were expressed as mean $\pm$ S.E.M (standard error of means). P-value less than 0.05 was considered significant and is written in the parentheses.

Results

Number of Apoptotic sertoli cells colored brown, in EMF group was (11.12 $\pm$ 0.05) and in R.A, received group was (2.05 $\pm$ 0.05) and in R.A+EMF was (8.05 $\pm$ 0.05), and in control group was (2.01 $\pm$ 0.03) respectively. These changes were significant as p value less than 0.05 (P<0.05), (Table 1).

Results of MDA (malondialdehyde) level in blood

MDA level in EMF group was (7 $\pm$ 0.05) and in R.A, received group was (3 $\pm$ 0.05) and in R.A+EMF was (6 $\pm$ 0.05) and in control group was (5 $\pm$ 0.05) mmol/ml respectively. These changes were significant as p value less than 0.05 (P<0.05). Statistical analysis Dunnett (one side) shows significant differences between experimental groups in comparison to control group (P<0.05), (Table 1).

Results of total blood anti-oxidant capacity (TAC)

TAC level in EMF group was (0.66 $\pm$ 0.05) and in R.A, received group was (2.95 $\pm$ 0.05) and in R.A+EMF was (1.1 $\pm$ 0.05) and in control group was (1.8 $\pm$ 0.05) mmol/ml respectively. These changes were significant as p value less than 0.05 (P<0.05). Statistical analysis Dunnett (one side) shows significant differences between experimental groups in comparison to control group (P<0.05), (Table 1).

Result of Testosterone levels

Testosterone level in EMF group was (0.75±0.05) and in R.A, received group was (4.1±0.05). in R.A+EMF was (3±0.05) and in control group was (2.2±0.05) ng/ml respectively. These changes were significant as p value less than 0.05 (P<0.05). Statistical analysis Dunnett (one side) shows significant differences between experimental groups in comparison to control group (P<0.05), (Table 1).

Result of sertoli cells Apoptosis levels

Apoptosis level in EMF group was (11.05±0.05) and in R.A, received group was (3.1±0.05) and in R.A+EMF was (6±0.05) and in control group was (1.1±0.05) respectively. These changes were significant as p value less than 0.05 (P<0.05). Statistical analysis Dunnett (one side) shows significant differences between experimental groups in comparison to control group (P<0.05), (Table 1).

Table 1: Sertoli cells Apoptosis, TAC, MDA, Testosterone levels of rats exposed to 50 Hz EMF and 5mg/rat Rosmarinic acid

Groups	Control Normal saline (0.9% NaCl)	Rosmarinic acid (5mg/rat)	EMF (50Hz)	EMF with Rosmarinic acid (5mg/rat)
Testosterone (ng/ml)	2.2 ±0.05	4.1 ±0.05*	0.75 ±0.05*	3 ±0.05
MDA(mmol/ml)	5 ±0.05	3 ±0.05*	7 ±0.05*	6 ±0.05
TAC(mmol/ml)	1.8 ±0.05	2.95 ±0.05*	0.66 ±0.05	1.1 ±0.05
Apoptosis Sertoli cells(100 numbers/cross section)	1.1±0.05	3.1±0.05	11.05±0.05*	6±0.05*

Data are presented as mean ± SE.

* Significantly different at p< 0.05 level (compared with the control group)

Discussion

Sertoli cells are 'nurse' cells of the testes that are part of a tubule. They are activated by follicle-stimulating hormone and have FSH-receptor on their membranes. They are specifically located in the convoluted seminiferous tubules. Their main function is to nourish the developing sperm cells through the stages of spermatogenesis, the seminiferous tubules and Leydig cells, respectively. This structural partitioning has often led to a functional separation, especially in view of the fact that LH controls Leydig cell secretion of testosterone, which, together with FSH, controls spermatogenesis (Cheng et al., 2011). Leydig cells have two different populations (LCs), namely a) foetal and b) adult types. During developmental process, they can be identified in the testis. Decreases in number of LCs after birth will occur in the rat (Khaki et al., 2011). The adult-type LCs emerges during pubertal sexual development. Endocrine and paracrine signals control the LCs population (Koziorowska-Gilun et al., 2011). In the adult rat, once a critical mass of mature LCs is achieved, the proliferative activity of the LC population is negligible (Lu et al., 2009). LH plays a pivotal role in the control of LC development, during normal puberty (Martínez-Sámano et al., 2010).

Testosterone biosynthesis is dependent on the stimulation of testicular Leydig cells through activation of the LH receptor (LHR) by LH and the placental LH homolog human chorionic gonadotropin (hCG). The increased use of power lines and modern electrical devices is of concern as a public health hazard, and chronic exposure to EMF has attracted considerable attention. Exposure to EMF adversely affects spermatogenesis by the Sertoli and Leydig cells (Martínez-Sámano et al., 2010). Magnetic fields of 50 Hz also induce cytotoxic so cytostatic changes in the differentiating spermatogonia of mice (Khaki et al., 2008). EMF is able to generate destructive reactive oxygen species including superoxide, hydrogen peroxide and hydroxyl radical and frequently used to produce oxidative and necrotic damages (Khaki et al., 2010). Our results revealed that 50 Hz EMF could significantly increase level of MDA in serum and respectively lead to sertoli cells apoptosis. These biochemical and pathological changes are due to decreasing serum total antioxidants levels, and this can lead to the exposition of these cells to cell injury by acting ROS. These results agree with previous study that oxidative stress occurs from an imbalance between ROS species and antioxidant actions (Limón-Pacheco and Gonsébat, 2009). In addition, using 50 Hz EMF in this study could influx on serum LH and testosterone and sperms parameters levels and can lead to decrease of these items. These results are also consistent with other results by Khaki et al. (2011).

In the last few years, a great deal of interest has been particularly addressed to phenolic compounds, between the major class of phytochemical antioxidants exist in fruits and vegetables. Dietary polyphenols have received tremendous attention among nutritionists, food scientists and consumers due to their roles in human health. Research in recent years strongly supports a role for polyphenols in the prevention of degenerative diseases, particularly cancers, cardiovascular diseases and neurodegenerative diseases (Khaki et al., 2012a) label this ref since there are more than one Khaki et al 2012). Rosmarinic acid belongs to the group of polyphenols which are strong antioxidants that complement and add to the functions of antioxidant vitamins and enzymes as a defense against oxidative stress caused by excess reactive oxygen species (ROS). In addition to the above possible mode of antioxidant actions, other mechanisms such as inhibition of xanthine oxidase and elevation of endogenous antioxidants are also considered important (Cuerda et al., 2011). The *in vitro* capacity of polyphenols to act as both primary and secondary antioxidants has been probably the best described property of almost every group of flavonoid and non-flavonoid compounds. This concept, however, appears now to be an oversimplified view of their mode of action (Limón-Pacheco and Gonsébat, 2009). In fact, suggesting a variety of other potential mechanisms of action of polyphenols in cells and protection against oxidative stress. In our results this herb could show beneficial role by its increase of serum antioxidants, serum testosterone and sperms parameters. So, we can conclude that these benefits belong to Ros-A, antioxidants' effects and its flavonoid which could have useful effects on sex hormones and increase sperm population (Limón-Pacheco and Gonsébat, 2009; Martínez-Sámano et al., 2010; Khaki et al., 2012b). There are also prior confirmed studies about effect of polyphenols in Ros-A on the proliferation and apoptosis of activated hepatic stellate cells (HSC-T6). It has been shown to be beneficial on apoptosis and to protect hepatic cell damage (Zhang et al., 2011). A better understanding of underlying mechanisms in fertility and better study results clarifying the effectiveness of nutritional and biochemical factors are important to improve diagnosis and treatment. The tight junctions of Sertoli cells form the blood-testis barrier, a structure that partitions the interstitial blood compartment of the testis from the adluminal compartment of the

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seminiferous tubules. Because of the apical progression of the spermatogonia, the tight junctions must be dynamically reformed and broken to allow the immunoidentical spermatogonia to cross through the blood-testis barrier so they can become immunologically unique. Sertoli cells control the entry and exit of nutrients, hormones and other chemicals into the tubules of the testis, as well as make the adluminal compartment an immune-privileged site. In our study, significant increase was observed in sertoli cells apoptosis and this is caused by the decrease in the cell population in testis and decrease in serum testosterone (Khaki et al., 2012a). The present study points to the possibility that electromagnetic fields (EMF) also lower serum testosterone levels (Khaki et al., 2013) and through induction of apoptosis in sertoli cells cause damage to blood testes barrier. While direct and indirect antioxidant activities of polyphenols may play important roles in reducing oxidative stress via the above mentioned mechanisms, the actual roles at the cellular level of these compounds may be more complicated and Natural antioxidants, whether consumed before or after radiation exposure, is not so clear.

In conclusion, Rosmarinic acid via increasing serum-testosterone level to antioxidant protective effects in radiation. These effects may result in the increase of cell proliferation in the EMF group; so, we suggest that using Rosmarinic acid has beneficial effect in protection of cell injuries in life area population exposed to radiation such as electric power.

Conflict of interest statement: We declare that we have no conflict of interest.

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