

## Antioxidant Effect of Rosmarinic acid on Sertoli cells Programed cell death after exposed with electromagnetic fields (EMF) in Rats

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**Abstract:** 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. Wistar male rat (n=40) were allocated into three groups, control group (n=10) that received 5cc Normal saline (0.9% NaCl) daily by gavage method and Rosmarinic acid group that received 5mg/rat (gavage)(n=10), and EMF group that 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). In 42th day of research, 5cc blood was collected for measure Testosterone levels from whole group's analysis. Sertoli cells apoptosis significantly decreased in group that has received 5mg/rat of Rosmarinic acid (P<0.05) in comparison to experimental groups. Level of testosterone significantly increased in groups that received Rosmarinic acid (P<0.05). Since in our study 5mg/rat of Rosmarinic acid, have significantly Preventive effect on cell damages especial sertoli cells apoptosis that caused with EMF, so it seems that using Ro as food additive can be effective for supporting peoples that living in EMF environmental pollution.

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

### 1. Introduction

Rosmarinic acid is used to treat bronchial asthma, peptic ulcers, cataract, arthritis, cancer and rheumatoid arthritis. It is found in big quantities in oregano, lemon balm, sage, marjoram; 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). A number of research studies on the potential health effects of electromagnetic fields (EMFs) have been performed in Europe, North America, and Asia such as 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 showed EMF had harmful side effects on reproductive organs in mammal's and could be baneful on spermatogenesis (Khaki et al., 2010; Khaki et al., 2011). 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 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 exposed with electromagnetic fields (EMF) in Rats.

### 2. Material and Methods

#### 2.1. Animals

A total of 40 male wistar rats were maintained for 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). 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 to four groups, a control group (n = 10), that received 5cc Normal saline (0.9% NaCl) daily by gavage methods and three treatment groups (n = 30). 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.

## 2.2. Pharmacological Procedure

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

## 2.3. 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 80 G. 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.

## 2.4. 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. The 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.

## 2.5. TUNEL analysis of apoptosis

The *in-situ* DNA fragmentation was visualized by TUNEL method (Khaki et al., 2008). Briefly,

dewaxed tissue sections were predigested with 20 mg/ml proteinase K for 20 min and incubated in phosphate buffered saline (PBS) solution containing 3% H<sub>2</sub>O<sub>2</sub> 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- H<sub>2</sub>O<sub>2</sub> (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 leydig 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 germ cells per tubule cross-section was calculated.

## 2.8. 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.

## 2.9. 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 were considered significant and are written in the parentheses.

## 3. Results

### 3.1. TUNEL staining

Number of Apoptotic 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 was significant as p value less than 0.05 (P<0.05), (Table 1).

### 3.2. Testosterone

Testosterone level in in EMF group was (0.75 $\pm$ 0.05) and in R.A, received group was (4.1 $\pm$ 0.05) and in R.A+EMF was (3 $\pm$ 0.05) and in control group was (2.2 $\pm$ 0.05) ng/ml respectively. These changes was 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 and Testosterone levels of rats with 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
Sertoli cells apoptosis(ng/l)	2.01 ±0.03	2.05 ±0.05	11.12 ±0.05*	8.05 ±0.05*

Data are presented as mean ± SE.

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

#### 4. Discussion

Sertoli cells are 'nurse' cell of the testes that is part of a tubule. It is activated by follicle-stimulating hormone and has FSH-receptor on its membranes. It is specifically located in the convoluted seminiferous tubules. Because its main function is to nourish the developing sperm cells through the stages of spermatogenesis, the seminiferous tubules and Leydig cells, respectively. It is activated by follicle-stimulating hormone and has FSH-receptor on its membranes. It is specifically located in the convoluted seminiferous tubules. 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 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 (Lu et al., 2009; Martínez-Sámano et al., 2010). 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 and 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 cells injuries and respectively cause to sertoli cells apoptosis, this biochemical and

pathological changes due to decreasing serum total antioxidants levels and this cause to predispose this cells to cell injury by acting ROS, this results are agree with previous study that oxidative stress occurs from an imbalance between ROS and antioxidant actions (Cheng et al., 2011; Limón-Pacheco and Gonsebatt, 2009). in additional using 50 Hz EMF in this study could influx on serum LH and testosterone and sperms parameters levels and cause to decreasing these items, this results are agree with others results (Koziorowska-Gilun et al., 2011; Khaki et al., 2011). In the last few years, a great deal of interest has been particularly addressed to phenolic compounds, among the major class of phytochemical antioxidants 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., 2012). Rosmarinic acid belongs to the group of polyphenols 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 Gonsebatt, 2009). in fact, suggest a variety of other potential mechanisms of action of polyphenols in cells protection against oxidative stress. in our results this herbal could show beneficial role by increasing in

serum antioxidants, serum testosterone and sperms parameters, so we can conclude that these are belonged to Ros-A, antioxidants effects which flavonoid can useful effects on sex hormones and increase sperms populations (Limón-Pacheco and Gonsebatt, 2009; Martínez-Sámano et al., 2010; Khaki et al., 2012b) and confirmed prior studies about effect of it on the proliferation and apoptosis in activated hepatic stellate cells (HSC-T6), which is useful to decrease this cell population (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 luminal compartment of the 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 in our study significantly increased was observed in sertoli cells apoptosis and this cause to decreasing in this cells 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 T 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, so it will be suggestion that using Rosmarinic acid has beneficial effect in protection of cell injuries in life area population that involve with radiation such as electric power.

#### Conflict of interest statement

We declare that we have no conflict of interest.

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