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Full Length Research Paper

Effects of rosmarinic acid on male sex hormones (testosterone-FSH-LH) and testis tissue apoptosis after exposure to electromagnetic field (EMF) in rats

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Rosmarinic acid (RA) is a polyphenol derived from many common herbal plants of the Lamiaceae group: Rosemary, sage, Spanish sage, oregano, basil, marjoram, thyme, the mint group, lavender, perilla and lemon balm. It is currently being studied for its effects on Alzheimer's disease and some other diseases. Rosmarinic acid in its natural state as part of a herb has been used to strengthen the memory and to improve mood by dispersing melancholy. Male Wistar rats (n = 40) were allocated to four groups, a control group (n = 10) 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 electromagnetic field (EMF) exposure at 50 Hz for 40 consecutive days, whilst the forth group received only EMF exposure for 40 consecutive days. Animals were maintained under standard conditions. At the conclusion of the test period, rat testes tissues were removed from all group members and animals were maintained under standard conditions. Tissue preparation was performed and analyzed for apoptosis. There was a significant increase in apoptosis in EMF group and significant increase in testosterone serum level in RA group when compared with other groups (P < 0.05). EMF has negative effect on testis histology in rats. However, these side effects are less seen in the EMF group that received RA. Therefore, it is recommended that usage of rosmarinic acid in modern country has fewer industrial side effects on male fertility.

Key words: Apoptosis, electromagnetic field (EMF), male fertility, rosmarinic acid, testis.

INTRODUCTION

The antioxidant capacity of phenolic compounds, flavonoids and foods rich in these compounds, has been repeatedly demonstrated in various *in vitro* and *in vivo* systems (Alexandopoulou et al., 2006). Rosmarinic acid (RA), a water-soluble polyphenolic component isolated from Dansam-Eum (DSE), has been reported to have anti-oxidative (Huang and Zhang, 1992; Firdose et al., 2011), anti-inflammatory (Osakabe et al., 2004) and anti-depressive activities (Takeda et al., 2002). Li et al. (2007) reported that RA was found in the brain following intravenous administration of *Salvia miltiorrhiza*. Hence, RA can be considered as an active compound that improves the health of auditory regulation organs. An electromagnetic field (EMF or EM field) is a physical field produced by electrically charged objects. EMFs affect the behavior of charged objects in the vicinity of the field. The EMF extends indefinitely through space and determines electromagnetic interaction (Schüz et al., 2009; Emre et al., 2011). EMF is one of the four fundamental forces of nature; the others are gravitation, the weak interaction and the strong interaction. The EMF can be viewed as a combination of an electric field and a magnetic field. 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

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differentiating spermatogonia of mice (Khaki et al., 2008). Little is known about the effect of EMF on the cytoarchitecture of the boundary tissue of the seminiferous tubules, which perform a number of crucial functions, including, mechanical support and transport of nutrients to the spermatozoa (Roychoudhury et al., 2009) and sperm discharge by maintaining pressure on the tubules (Iorio et al., 2007). Other studies have investigated transitory effects of EMF on the testes, but no previous study has reported the possibility of recovery from the potentially harmful effects of EMF exposure after an exposure-free period. Furthermore, studies by Khaki et al. (2011) showed that many antioxidants can moderate harmful effects of EMF waves in testis tissues. The present study was designed to investigate the protective effects of RA on EMF effects on testis apoptosis.

MATERIALS AND METHODS

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) 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 40 consecutive days, while the forth group received only EMF exposure for 40 consecutive days. Animals were maintained under standard conditions.

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.

Surgical procedure

On day 40, a sodium pentobarbitall solution (40 mg/kg) was administered intra-peritoneally 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. Then tissue preparation was performed to investigate, vein congestion and apoptosis by TUNEL.

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_2O_2 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_2O_2 (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 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 assessed and the mean number of TUNEL positive germ cells per tubule cross-section was calculated.

Statistical analysis

Data were expressed as means with standard error of mean (SEM). Analyses of detected parameters were done by one-way ANOVA test followed by Tukey's (honestly significant difference (HSD) test. P-value < 0.05 was considered to be significant.

RESULTS

Compared to the control group, apoptotic cells percentage decreased following administration of *Ocimum basilicum* extract (1.5 g/kg body weight). Exposure to 50 Hz of EMF caused a significant increase in the apoptotic cells percentage. When 50 Hz of EMF was administrated together with *O. basilicum* extract (1.5 g/kg body weight), apoptotic cells percentage decreased significantly ($P < 0.05$) from 18.12 ± 1.05 to 10.05 ± 0.01 in spermatogonia, and decreased significantly ($P < 0.05$) from 20.11 ± 0.05 to 9.05 ± 0.05 in primary spermatocytes, respectively, vein congestion decreased significantly ($P < 0.05$) from 8 ± 0.03 to 0.5 ± 0.01. These results indicate the protective effect of *O. basilicum* against EMF-induced apoptosis (Table 1).

DISCUSSION

Plants and natural products are extensively used in

Table 1. Means of germinal cells apoptosis and male sex hormones (Testosterone-FSH-LH) of rats testis exposed to EMF and rosmarinic acid.

Groups	Control	EMF (50 Hz)	Rosmarinic acid (5 mg/kg body weight)	Rosmarinic acid + EMF
Spermatogonia apoptotic cell (%)	8 ± 0.05	22.11 ± 0.05*	4.25 ± 0.03	12.05 ± 0.02*
Primary spermatocytes apoptotic cell (%)	5 ± 0.05	19.02 ± 0.05*	3.75 ± 0.01	10.05 ± 0.05*
Testis weight's(Gram)	1.40 ± 0.821	1.10 ± 0.001	1.49 ± 0.001	1.39 ± 0.371
Testis vein congestion (%)	1 ± 0.01	10 ± 0.02*	6 ± 0.03	0.5 ± 0.01*
Serum testosterone levels (ng/ml)	1.70 ± 0.01	1 ± 0.01	2.99 ± 0.210**	1.70 ± 0.01
LH levels (ng/ml)	1.51 ± 0.138	0.91 ± 0.128	1.73 ± 0.164	1.31 ± 0.128
FSH levels (ng/ml)	20.17 ± 1.005	18.17 ± 1.005	20.17 ± 1.005	21.17 ± 1.005

Data are presented as mean ± SE. *Significantly different at $P < 0.05$ level (compared with the control group). **Significantly different at $P < 0.001$ level (compared with the control group).

several traditional systems of medicine, so, screening these products for radio-protective compounds has several advantages, because they are usually considered non-toxic and are widely accepted by humans. Many natural antioxidants, whether consumed before or after radiation exposure, can confer some level of radioprotection. In addition to beneficial effects accrued from established antioxidants, such as, vitamin C and E, and their derivatives, vitamin A, beta carotene, curcumin, *Allium cepa*, quercetin, caffeine, chlorogenic acid, ellagic acid and bixin, protection is also conferred by several novel molecules, including, flavonoids, epigallocatechin, and other polyphenols (Kirtikar and Basu, 1991; Khaki et al., 2009a, b, c). Fertility is one of the major problems in a man's life; about 25 and 35% of infertility is regarded to be man and woman receptivity (Mosher and Pratt 1991). The importance of many of these factors is not yet clearly understood. 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. Smart choices for better foods might prevent body from many diseases (Reddy et al., 2006; Suryavathi et al., 2005). As all spermatogenesis stages occur in seminiferous tubule of testis, it is possible to evaluate the extent of spermatogenesis by the determination of the number of spermatozoa produced per one gram of testicular parenchyma (Acharya et al., 2008; Hew et al., 1993). The sperm count is considered as important parameter to assess the effects of chemical on spermatogenesis (Yousef, 2005). It has also been reported that there is a direct correlation between the epididymal sperm count and motility with fertility in animals (Dawson et al., 1992; Timmermans, 1989; Yu et al., 2005). The oxidative damage, elevated lipid peroxidation and the alteration of membrane properties can lead to germ cell death at different stages of development and the sperm count decrease (Bestas et al., 2006). Accordingly, it is expected that antioxidant therapy acts as a protective defense against oxidative stress and improve fertility parameters. The ability of antioxidants, such as ascorbic acid in semen to protect spermatozoa from oxidative damage has been shown by some authors (Timmermans, 1989). The main pharmacological actions of ginger and compounds isolated include immuno-modulatory, anti-tumorigenic, anti-inflammatory, anti-apoptotic, anti-hyperglycemic, anti-lipidemic and anti-emetic action. Ginger is a strong antioxidant substance and may either mitigate or prevent generation of free radicals. It is considered a safe herbal medicine with only few and insignificant adverse/side effects (Dawson et al., 1992). Oxidants and antioxidants have attracted widespread interest in nutrition research, biology and medicine. It has become clear that constant generation of pro-oxidants, including oxygen free radical, is an essential attribute of aerobic life (Acharya et al., 2008). A disturbance in the pro-oxidant/antioxidant system has been defined as oxidative stress. Reactive oxygen species (ROS) are very reactive molecules ranked as free radicals owing to the presence of one unpaired electron, such as a superoxide ion, nitrogen oxide and hydroxyl radical, administration of this extract with gentamicin was also able to counterbalance the negative effect of gentamicin on sperm count. However, oxidative stress occurs from an imbalance between ROS and antioxidant actions. During chronic oxidative stress caused by environmental factors (that is, UV light, ionizing radiation and toxic substances), infections, diabetes or lack of dietary antioxidants, an inequity of cellular reducing equivalents capable of detoxifying the increased burden of ROS has marked effects on normal cellular processes. However, in times of oxidative stress, normal cellular respiration is also, still functioning, resulting in dysregulated mitochondrial free radical production and disparity between ROS generation and antioxidant defenses (Schriner et al., 2005; Limón et al., 2009). A previous study showed that 2 h of 60 Hz EMF exposure immediately altered the metabolism of free radicals, decreased superoxide dismutase (SOD) activity in plasma, decreased glutathione (GSH) content in the heart and kidney, but did not induce immediate lipid

peroxidation (Khaki et al., 2008), EMF is able to generate destructive reactive oxygen species including superoxide, hydrogen peroxide and hydroxyl radical, and is frequently used to produce oxidative and necrotic damages (Khaki et al., 2008). The role of EMF in the induction of apoptosis and oxidative damage has also been reported. This could be indicative of free radical scavenging properties of *O. basilicum* (Polat et al., 2006). The results of other study showed the ability of *O. basilicum* in the enhancement of protective effects of rats resulting from decrease of apoptosis in testis, testis weights increasing, vein congestions decreasing and also increased testosterone levels (Khaki et al., 2011). Our previous study, demonstrated that the administration of *O. basilicum* can overcome reproductive toxicity of EMF (Khaki et al., 2011). Rosmarinic acid can reduces blood glucose and balance sex hormones and re-uptake sexual behaviors in diabetic rats (Farzadi et al., 2011; Ghasemzadeh et al., 2011).

Conclusion

Rosmarinic acid was also able to reduce apoptosis in testis and balance sex hormone levels in groups that are exposed to 50 Hz EMF waves, and the results in this research confirmed that rosmarinic acid could moderate harmful effects of environmental exposed ring, such as EMF, and using this antioxidant in mammals, such as human and animal nutrition can reduce reactive oxygen species effects and treatment of male reproductive disorders.

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