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RESEARCH ARTICLE

PROPHYLACTIC ROLE OF MELATONIN AGAINST X BAND MICROWAVE RADIATION INDUCED TOXICITY IN TESTIS OF SWISS ALBINO MICE

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ABSTRACT

Purpose: The aim of present study was to investigate the protective role of melatonin (Mel) against 10 GHz microwave radiation induced adverse effects on testes of mice.
Materials and methods: 6-8 weeks old 24 male Swiss albino mice were procured from inbred colony and were divided into 3 groups. Group I Sham exposed (control), Group II microwaves (MW) exposed, Group III (MW + Mel) treated with melatonin (2mg/kg body weight). The power density was measured 0.25 mW/cm^2 and the SAR was calculated 0.12 W/kg.
Results: After completion of exposure period the animals were sacrificed and testes were excised to study various stress related parameters. Analysis of data revealed that long-term 10 GHz exposure resulted in significant decrease ($P < 0.001$) in Superoxide dismutase (SOD),Glutathione peroxidase (GPx) and testosterone whereas Catalase (CAT), Malondialdehyde (MDA) and ROS increased significantly($P < 0.001$). Meanwhile, melatonin reversed adverse effects of MW radiation and it increased SOD, GPx, testosterone and decreased CAT, MDA and ROS significantly in MW + Mel group ($P< 0.001$).
Conclusion: It may be concluded that free radicals causing oxidative stress could be responsible for detrimental effects in testes whereas, melatonin proved to be a strong antioxidant and it reversed the adverse effects of MW radiation.

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INTRODUCTION

Microwave (MW) radiation has been gradually increasing owing to growing demand of electronic devices: Radar, Bluetooth, mobile phone, microwave oven and cell phones which may adversely affect the reproductive pattern. These devices work on microwaves and are constant source of microwave radiations. MW radiation has been reported to have posed severe health concerns. X-band of microwaves (8-10 GHz) has been using for years in telecommunication systems for civil and military purposes: aircraft, weather forecast system and various types of radars. Indiscriminate practice of microwaves in working environment poses a severe threat to human health and sustainable environment. Stuchley *at el.,* (1988), proposed that electron magnetic fields (EMF) radiation leads to increase in temperature and causes thermal effects to cells. Paulraj and Behari (2004), suggested that EMF causes non-thermal effects in tissues to a great extent.

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A plethora of studies have reported that male reproductive organs are highly sensitive to the extremely low frequency magnetic fields (Agarwal *at el.,* 2003; Aitken *at el.,* 1991, 1993; Forgacs *et al.,* 2006; Macleod, 1943; Pasqualotto *at el.,* 2000; Shen *et al.,* 2000; Sukcharoen *et al.,* 1995). The male reproductive system has been found highly vulnerable to various environmental assaults and exposure to such assaults may lead to reproductive disorders like testicular abnormalities, sperm abnormalities, chromosomal aberrations and congenital defects in offspring (Havas, 2000; Kim *et al.,* 2007; Ramadan *et al.,* 2002). Many authors reported that infertility and adverse effects on male reproductive system are caused due to EMF exposure (Kesari *et al.,* 2010, 2011; Nisbet *et al.,* 2012). Electromagnetic field (EMF) exposure harmfully affects the quality of semen and permeability of blood testis barrier in the form of decreasing sperm count, motility, viability and morphology of sperms which may be one of the reason of male infertility (Agarwal *et al.,* 2007; Kesari and Behari, 2010a; Wang *et al.,* 2008). A few studies have suggested that increased oxidative stress leads to biochemically increased lipid peroxidation and free radical formation (Kumar *et al.,*

2011; Meral *et al.,* 2007; Rao *et al.,* 2008). The cellular activities of male germ cells (spermatozoa) are adversely affected by over production of free radicals due to MW radiation (Balci *et al.,* 2007; Kumar *et al.,* 2010; Meral *et al.,* 2007; Rao *et al.,* 2008). Several other studies have observed that, toxicity of MWs is mediated by oxidative stress (De-Iullis *et al.,* 2009; Oktem *et al.,* 2005). Some studies showed that chronic exposure to MWs stimulates oxidative stress which in turn decreases the total anti-oxidative capacity in testicular cells by decreasing activity of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and increasing levels of Malondialdehyde (Kesari and Behari, 2010a,b; Kesari *et al.,* 2012). Malondialdehyde (MDA) is one of the main parameters widely assessed to determine the severity of depletion of lipid concentration of cell membrane due to exposure to MWs (Draper and Hadley, 1990). Spermatozoa cell membrane is rich in polyunsaturated fatty acids and is highly susceptible to damage by lipid peroxidation (Alvarez *et al.,* 1987).

The exposure to MW radiation affects the balance between production and neutralization of reactive oxygen species (ROS) levels dramatically. Over production of ROS may cause damage to the cell structures leading to histopathological and biochemical alterations in testes. However cells have their own set of antioxidant defense mechanism to fight against the detrimental effects of highly reactive oxygen species such as Superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), vitamin E and C. EMF mainly damages leydig cells, seminiferous tubules, sperm DNA, and may reduce testosterone biosynthesis (La Vignera *et al.,* 2012).Testosterone is needed for completion of meiosis, formation of spermatids and maintenance of structural morphology and physiology of seminiferous tubules (Sharpe, 1994;Steinberger,1971).EMF affects spermatogenesis and damages the testicular function through defects in male germ line, decreased sperm count, narrower seminiferous tubules and reduction of intrascrotal testosterone levels (Dasdag *et al.,* 1999). Melatonin, an effective antioxidant is a neurohormone which is synthesized and released from the pineal gland during the dark period (Karasek, 2006). Hence, a strong and effective anti-oxidant supplementation could be beneficial in preventing or improving some complications of MW radiations in male reproductive system (Al- Damegh, 2012; Ilhan *et al.,* 2004; Oral *et al.,* 2006; Sokolovic *et al.,* 2008). Melatonin acts as a strong free radical scavenger and fight against most ROS (Zang *et al.,* 1998). Melatonin regulates seasonal reproductive cycles and reproductive behavior in animals (Awad *et al.,* 2006). Melatonin effectively protects sperm's mitochondria from ROS because it has been found to be a potent antioxidant and its protective actions against oxidative damage are believed to be from its direct free radical scavenging and indirect antioxidant activities, possibly from its ability to limit free radical generation at the mitochondrial level(Shang *et al.,* 2004).The purpose of the present study is to evaluate the intensity of oxidative stress mediated damage in 10 GHz microwave radiation exposed testicular tissue and potential protective role of melatonin against oxidative stress.

MATERIALS AND METHODS

Materials

All chemicals and melatonin were of analytical grade (Merck and Sigma) and procured from Rahsiff scientific, Delhi. All reagents were prepared on the day of experiment and stock solution of melatonin was made for oral administration to mice. The concentration of melatonin was made according to the body weight (2 mg/kg body weight) following the work of (Sokolovic *et al*., 2008).

Experimental animals

24 adult male Swiss albino mice, 6-8 weeks old, weighing 37 ± 2g, were used for the present study. Mice were maintained in the animal house as per the norms established by Institutional Animal Ethical Committee (IAEC). The mice were housed in clean polypropylene cages and maintained under controlled conditions of temperature and light (12L: 12D) and provided with standard mice feed and water ad libitum. The research was approved by the Departmental Ethical Committee (DEC) of Department of Zoology, University of Delhi for animal use.

Microwave radiation source

A klystron set-up consists of klystron power supply, isolator, variable attenuator, frequency meter, horn antenna and a specially designed animal cage was used for the experimental exposure (Fig. 1). A graphite sheet was used to minimize the reflection of scattered beam. The cage was properly partitioned and well ventilated with holes of 1 centimeter diameter. The dimensions of the box (4.5×9×9cm) were such that animals were comfortably placed, though they could not move much. The horn antenna was kept in H-plane configuration so that electric field of the waves was perpendicular to the ground surface. The electric field in the box was almost uniform because the dimension of the box was of the order of one wavelength. Every day four mice were housed at a time in the box which was placed at the same location facing the horn antenna. No animal blocked the radiations falling on other animals. All the experiments were performed and repeated in a blind manner. The temperature in the chamber was maintained around 25-27ºC throughout the experiment. The power density was 0.25 mW/cm^2. The emitted power of microwaves was measured by a power meter which is a peak sensitive device ('RF power sensors 6900 series' and 'IFR 6960B RF power meter'; made of Aeroflex Inc., Wichita, KS, USA).Mice were exposed to 10 GHz source for 2hrs/day for 30 days.SAR was calculated as 0.12 W/Kg following the work of Durney *et al.* (1984).

Experimental design: 24 mice were divided into three groups (n=8).

Group I: Sham exposed (Control)

Mice were kept in a plexiglas cage and placed in-front of the horn antenna aperture without energizing the system for 2 hrs/day for 30 days. Mice were administered distilled water as control.

Group II: Microwaves exposed

Mice were kept in a plexiglas cage and placed in-front of the horn antenna aperture and exposed to 10 GHz microwaves for 2 hrs/day for 30 days, Mice were administered distilled water as control.

Group III: (Mel + MW exposed)

Mice were kept in a plexiglas cage and placed in-front of the horn antenna aperture and were supplemented with melatonin

(2mg/kg)once daily 1 hr before exposure to 10 GHz for 2hr/day for 30 consecutive days. Melatonin was administered orally at 08.00 AM to avoid its effects as neurotransmitter or neuromodulator (Drago *et al.,* 2001). Other researchers used the same dose of melatonin in their studies (Meena *et al.,* 2013; Koc *et al.,* 2003; Sokolovic *et al.,* 2008). At the end of exposure period the animals were sacrificed. For biochemical studies testes were quickly excised and homogenate was made to process various assays.

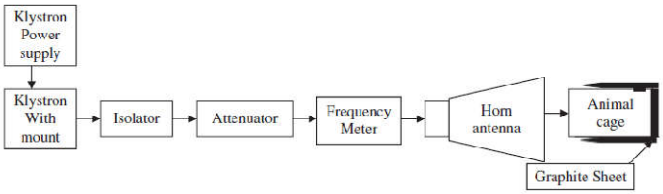


Fig. 1. Schematic diagram of 10 GHz radiation source

Lipid peroxidation (LPO) assay

For LPO assay Buege and Aust (1978) protocol was followed. A 10% testicular tissue homogenate was prepared in KCl. Tissue homogenate was mixed with solution of TCA, TBA & HCl. This final mixture was heated in a water bath for 30 min at 90°C and cooled then freshly made NaOH solution was added to it. After centrifugation the absorbance was recorded at 532 nm using a UV spectrophotometer. The LPO level was expressed in n mole MDA/gm tissue.

Glutathione (GSH) assay

The GSH level was measured by the method of Moron *et al*., (1979). A testicular tissue sample was homogenized in the sodium phosphate–EDTA buffer then DTNB solution was added to it. The optical density of the complex developed by the reaction of GSH and DTNB was measured at 412 nm using a UV spectrophotometer. The results were expressed as n mol GSH/100 mg of tissue.

Superoxide Dismutase (SOD)

SOD was calculated following the work of Marklund & Marklund (1974). Testicular tissue homogenate was prepared in NaCl. Then it was centrifuged for 10 minutes then 0.1 ml of supernatant was taken and mixed with Tris buffer and then pyragallol was mixed to this solution. Absorbance was taken at 420 nm. The results were expressed as µmole/mg tissue.

Catalase (CAT)

Catalase enzyme was estimated by Aebi *et al*., (1984). Testicular tissue homogenate was prepared in phosphate buffer and cold centrifugation was done for 10 minutes then 0.1ml of supernatant was mixed with 1 ml. PBS and 0.4 ml. H_2O_2. Absorbance was measured at 420 nm. The results were expressed as nmole/ml.

ROS (Reactive oxygen species)

ROS was measured by method described earlier (Lee *et al.,* 2006). Briefly 50 mg of testis tissue from each mouse was taken out and it was homogenized in 1 ml of PBS (0.1M Na_2HPO_4, 0.1M KH_2PO_4, 1.37M NaCl, 2.7mM KCl, pH 7.4) using homogenizer. It was passed through 100 µ pore sized cell strainer to get single cell suspension. Then 1 ml of RBC lysis buffer added with single cell suspension and kept for 10 minutes at RT. The suspension was centrifuged at 1200 rpm, for 10 minutes at 4ºC. The pellet was resuspended in 1 ml of PBS and the cells were treated with 2' 7'- dichlofluorescein diacetate (3.3µM) (DCFH-DA; Molecular probes, Eugene, OR, USA). DCFH-DA is permeable to cell membrane it enters the cell and hydrolyzed to DCFH by cell esterases. DCFH is non-fluorescent, impermeable to the cell membrane and readily reacts with intracellular hydrogen peroxide in the presence of cell peroxides and change into fluorescent- DCF, whose florescence can be measured using flow cytometry (Rastogi *et al.,* 2010; Szejda *et al.,* 1984). The cells were incubated at RT for 10 minutes in dark and washed using PBS. The cells were examined using flow cytometry (Guava technologies, CA and USA) total 5000 cells were analyzed in each sample to get the mean fluorescence intensity and were corrected for auto fluorescence of the unlabeled cells.

Testosterone

A serum testosterone assay was performed by ELISA kit. For this, 50 ml of testosterone standard, 50 ml of the testosterone AChE tracer and 50 ml of the testosterone antiserum were added to the wells of an ELISA plate containing 100 ml of EIA buffer. The sensitivity of the assay was 6 pg/ml. The optical density was read at 405–420 nm using a spectrophotometer.

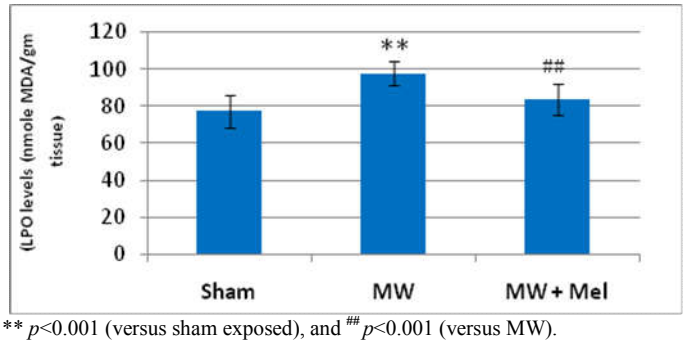
Statistical analysis

The values were expressed as mean ± SD. Data were analyzed using One-way ANOVA with Bonferroni post-hoc test. *P* value at 0.05 was considered as the level of significance.

RESULTS

Effects of MW on lipid peroxidation (LPO)

Amount of MDA due to lipid peroxidation (LPO) was measured by thiobarbituric acid reaction showed a highly significant ($P< 0.001$) increase in LPO in MW exposed group (97.86 ± 6.59) compared to sham exposed group (77.35 ± 8.65; Fig. 2). Melatonin treatment to MW + Mel group (83.67± 8.47) significantly ($P < 0.01$) inhibited the enhanced LPO levels.



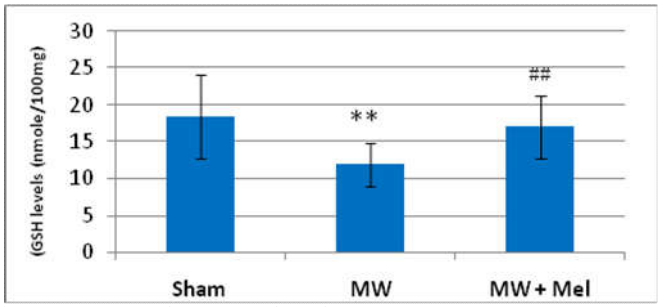
** $p<0.001$ (versus sham exposed), and ## $p<0.001$ (versus MW).

Fig. 2. Effects of10 GHz microwave radiation on MDA in testis of Swiss albino mice

Effects of MW on GSH

MW exposure resulted in highly significant ($P< 0.001$) decrease in GSH in MW exposed group (11.88± 2.97)

compared to sham exposed group (18.43± 5.71; fig. 3). Melatonin treatment to MW + Mel group (17.03± 4.19) resulted in significant increase ($P< 0.01$) in GSH.

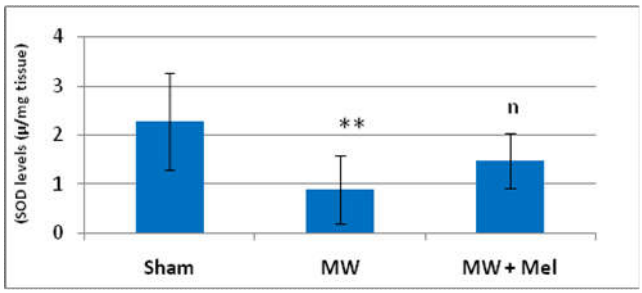


** $p<0.001$ (versus sham exposed), and ## $p<0.001$ (versus MW).

Fig. 3. Response of 10 GHz microwave radiation to GSH levels in testis of Swiss albino mice

Effects of MW on superoxide Dismutase (SOD)

MW exposure resulted in highly significant ($P< 0.001$) decrease in SOD in MW exposed mice group (0.89± 0.68) compared to sham exposed group (2.27± 0.98; fig. 4). Melatonin supplementation to MW + Mel group (1.47± 0.56^{n}) resulted in Non-significant levels of SOD.

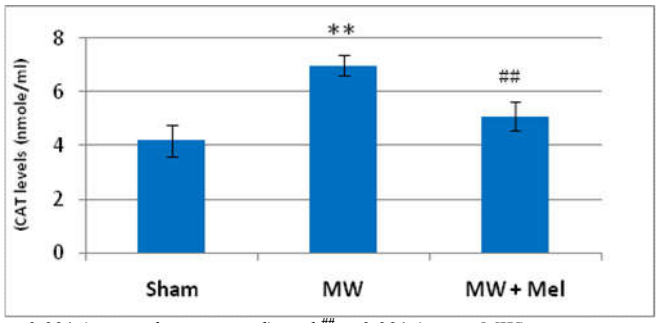


** $p<0.001$ (versus sham exposed), and n-non-significant (versus MW)

Fig. 4. Response of 10 GHz microwave radiation to SOD levels in testis of Swiss albino mice

Effects of MW on catalase (CAT)

MW exposure showed a highly significant ($P< 0.001$) increase in CAT in MW exposed group (7 ± 0.4) compared to sham exposed group (4.2± 0.58; Fig. 5). Supplementation of melatonin to MW + Mel group (5.1± 0.54) significantly ($P<0.01$) inhibited the enhanced Catalase levels.

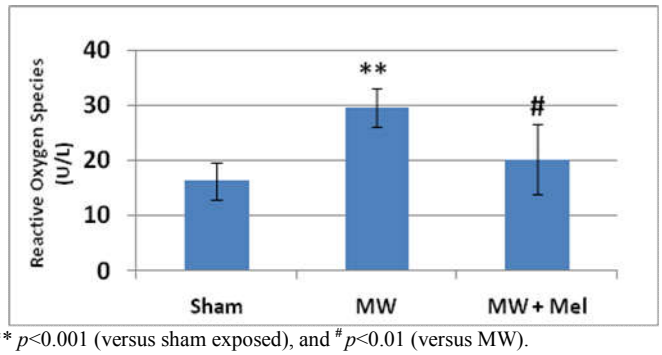


** $p<0.001$ (versus sham exposed), and ## $p<0.001$ (versus MW).

Fig. 5. Response of 10 GHz microwave radiation to CAT levels in testis of Swiss albino mice

Effects of MW on ROS

MW exposure resulted in highly significant ($P< 0.001$) increase in ROS in the MW exposed group (29.6 ± 3.5) compared to sham exposed group (16.3 ± 3.4; Fig. 6). Whereas Melatonin treatment prior to irradiation resulted in significant decrease ($P< 0.001$) in ROS in MW + Mel group (20.2 ± 6.3) compared to the MW exposure group.

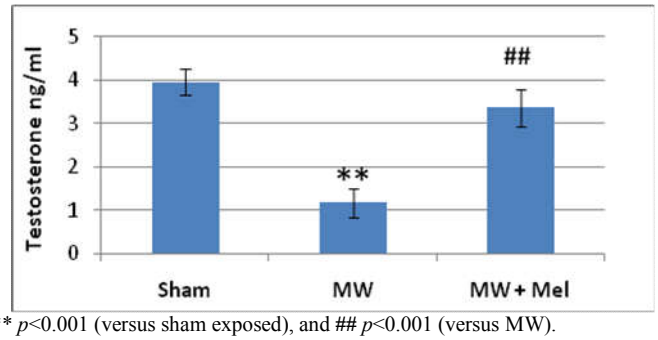


** $p<0.001$ (versus sham exposed), and # $p<0.01$ (versus MW).

Fig. 6. Preventive effects of melatonin on ROS in testis of Swiss albino mice exposed to 10 GHz microwave radiation

Effects of MW on testosterone

MW exposure resulted in highly significant ($P< 0.001$) decrease in testosterone in MW exposed group (1.18 ± 0.33) compared to sham exposed group (3.95 ± 0.3; Fig. 7). Whereas Melatonin treatment prior to irradiation resulted in highly significant increase ($P< 0.001$) in MW + Mel group (3.37 ± 0.43) compared to the MW exposure group.



** $p<0.001$ (versus sham exposed), and ## $p<0.001$ (versus MW).

Fig. 7. Preventive effects of melatonin on testosterone level in Swiss albino mice exposed to 10 GHz microwave radiation

DISCUSSION

Our results showed that melatonin proved to be a strong antioxidant and it reversed the toxic effects of MW radiation. The male germ cells (spermatozoa) in the seminiferous epithelium of mammals are among the major cell systems at risk when animals are exposed to irradiation (Cordelli *et al.,* 2003). The generation of ROS in the cells consequent upon microwave exposure may be a possible interaction between EMF radiation and biological system. ROS and free radicals are generated in the cells by energy transfer or by electron transfer reactions induced by EMF exposure. A formation of highly reactive singlet of oxygen atom results in the sequential reduction to various molecules of the cell such as superoxide, hydrogen peroxide, and hydroxyl radical (Georgiou, C. D. 2010). The EMF radiation emitted from various sources leads to generation of ROS and causes male infertility (Kumar *at el.,* 2010). ROS are able to damage many biomolecules, including DNA, enzyme, lipids and may lead to oxidative stress in human semen (Agarwal *et al.,* 2009). Each healthy cell is fortified with self-defense mechanism containing antioxidant

enzymes: SOD, GPx and catalase so as to maintain a healthy balance of intracellular ROS these antioxidant convert harmful substances into less harmful molecules. EMF exposure to male reproductive system was found to have developed testicular abnormalities: atypical sperm, chromosomal aberrations and congenital defects in offspring (Havas, 2000). Jung and Schill (2000), proposed that excessive exposure to EMF could impair spermatogenesis in testes. Dasdag *et al.* (1999), reported that EMF affects spermatogenesis and damages the testicular function through defects in male germline, decreased sperm count, narrower seminiferous tubules and reduction of intrascrotal testosterone levels. Many studies proposed that over production of ROS has harmful effects on sperms and may lead to male infertility (Macleod, 1943; Aitken *et al.,* 1991,1993; Sukcharoen *et al.,*1995; Pasqualotto *et al.,* 2000; Shen *et al.,* 2000; Agarwal, *et al.,*2003). In support of above studies we have assessed the activities of antioxidant enzymes: SOD, CAT, GPx, in mice testes and our results are in line of earlier findings. An increased oxidative stress coupled with disturbed levels of antioxidant enzymes confirmed the relationship between MW radiation and male infertility. An increase in generation of reactive superoxide ions resulted in decreased level of SOD activity in cells due to MW exposure reported by Alvarez *et al.* (1987). GPx is a relatively stable enzyme it can be inactivated under conditions of severe oxidation stress. Over production of free radicals might have resulted in decreased GPx activity. CAT activity in oxidative stress induced cells has been found enhanced when H_2O_2 levels are particularly high (Condell *et al.,* 1993). Mammalian sperm membranes contain highly unsaturated fatty acids and are also sensitive to oxygen induced damage mediated by lipid peroxidation and free water-induced oxygen (Russo *et al.,* 2006).

The increase in MDA levels in our study seems to support the effects of electromagnetic field on spermatozoa. Lipid peroxidation induces cellular injury to the spermatozoa due to leakage of sperm membrane fluidity (Aitken *et al.,* 1994). The extent of microwave damage to the membrane was monitored by measuring the amount of thiobarbituric acid reactive material (MDA) produced when polyunsaturated fatty acids in the membrane undergo peroxidation and the amount of solute leakage from cells. In our experiment the MDA levels increased significantly compared to the control ones. This occurs due to generation of charge imbalance in unsaturated fatty acids that affects membrane structure and properties under microwave radiation and enhances production of free radical due to its trigger action. Our findings showed that 10 GHz exposure increases concentration of MDA in testes which is associated with unsteady antioxidant enzyme activity under oxidative stress induced by ROS (Amara *at el.,* 2006). Long-term exposure to EMF has adverse effects on proliferation and differentiation of spermatogonia and this may be important in understanding the pathogenesis of EMF induced male infertility proposed by Kim *et al*. (2007). On the basis of these findings it is suggested that oxidative stress results in male germ cells damage because spermatozoa lack antioxidants and any capacity for DNA repair on its own, and would be removed from the germinal epithelium carrying the damages (Aitken and Koppers, 2011). Melatonin is a very potent scavenger for oxygen derived free radicals. It can also protect molecules from oxidative damage by stimulating GSH-Px activity which metabolizes hydrogen peroxide to water (Reiter *et al.,* 1999; Baydas *et al.,* 2001).

Conclusion

It is concluded that 10 GHz microwave radiation decreases the oxidative defense potential of male germ cells and it adversely affects the testicular tissues of Swiss albino mice. Reduction in GPx, SOD and testosterone along with increased CAT, ROS and MDA levels, observed in our study are clear indication of infertility pattern owing to overproduction of ROS under microwave field exposure. An improvement was seen in the enzymatic activities of antioxidants after treatment with melatonin which suggests that melatonin acts as potent free radical scavenger against free radicals and ROS.

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