

# LPO and ROS Production in Rat Brain Exposed to Microwaves: Computational Elucidation of Melatonin in Repair System

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**Abstract** It is widely accepted that non-ionizing electromagnetic fields are present in the environment and are alarming as a major pollutant or electro-pollutant for health risk. The present study aimed to investigate the protective measures of melatonin against exposure of microwave radiations. Study also explored the mechanistic correlation among microwave radiation, melatonin and biological effects by computational method. For this, 60-day-old male Wistar rats were divided into four groups ( $n = 4/\text{group}$ ): sham exposed (control), Melatonin (Mel) treated (2 mg/kg), 2.45 GHz microwave (MWs) exposed and MWs + Mel treated. Exposure took place in Plexiglas cages for 2 h a day for 35 days where, power density ( $0.2 \text{ mW/cm}^2$ ) and specific absorption rate (SAR- $0.14 \text{ W/kg}$ ) were estimated. Results show that melatonin prevent oxidative damage biochemically by significant decrease ( $p < 001$ ) the levels of lipid peroxide (LPO) and

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reactive oxygen species (ROS) in the brain. However, exposure of microwave individually shows significant changes in LPO and ROS level. The effective dose of melatonin was validated by *in silico* method and which reveals the interaction of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) Enzymes of Central Nervous System (CNS) with melatonin. Where, AChE showed better interaction with the binding energy of  $-9.01$  kcal/mol and inhibition constant  $3.11$   $\mu$ M by comparing with BuChE. These results concluded that the melatonin has strong antioxidative potential against microwave radiation, which could be achieved by an implementation of computational approach.

**Keywords** LDH-X · Apoptosis · ROS · Microwave radiation · Cell cycle

## 1 Introduction

Fast growth in the field of telecommunications has led to markedly increase by a parallel increase in electromagnetic field (EMF) density. Human exposure to radiofrequency (RF) EMF occurs wherever electric and/or magnetic fields are generated, transmitted or used by cell phones or its towers or emitted from various electronic gadgets. There has been an increasing concern about the potential hazards of microwave radiations and possible health outcomes of electric and magnetic fields are being discussed. Although, the primary mechanism of interaction between such fields and living matter (brain or neuronal cells, sperm cells etc.) is unknown. In 2011, the International Agency for Research on Cancer (IARC) classified RF-EMF as “possibly carcinogenic to humans” (Group 2B) (Baan et al. 2011). Carcinogenicity has a significant link between long term RF-EMF exposure and cancer risk (Coureau et al. 2014; Hardell and Carlberg 2013; Hardell et al. 2013a, b, c; Mead 2008).

The effects of EMFs emitted by mobile phones on the central nervous system (CNS) have become a particular focus of concern owing to the fact that mostly mobile phones are kept near head during talking mode and or in close proximity to the brain (Mausset et al. 2001, 2004; Odaci et al. 2008). Therefore, the absorption of SAR is always more in brain and also found more sensitive to microwave exposure. In 1998, the International Commission on Non-Ionizing Radiation Protection (ICNIRP) released guidelines and reported that the specific absorption rate (SAR) of mobile phones could be legally limited to  $2.0$  W/kg (ICNIRP 1998). Schönborn et al. (1998) investigated that RF-EMFs emitted by cell phones are absorbed in the brain within a range that could influence neuronal activity. Microwave radiations are potentially strong to penetrate the cranium, and nearly 40% of these can reach deeper into the brain (Barnett et al. 2007; Kang et al. 2001), where penetration depth is assumed to be 4–5 cm deep into the brain (Dimbylow and Mann 1994; Rothman et al. 1996). Several studies from our group based on *in vitro* and *in vivo* model show the fact that MF (magnetic field) or RF-EMF exposure causes neurological damage (Kesari et al. 2010, 2011, 2013, 2014, 2015, 2016). Consequently, an increased blood-brain barrier permeability and oxidative

damage, which are associated with brain cancer and neurodegenerative diseases, have also been reported by many researchers (Xu et al. 2010; Zhao et al. 2007; Nittby et al. 2009; Awad and Hassan 2008; Leszczynski and Joenvaara 2002).

Therefore, the aim of the present study was to further explore the computational elucidation of melatonin in repair system induced by microwave radiation exposure. In this study, we selected melatonin which is reported as acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitor used in the microwave induced cancer treatment. The knowledge of AChE and BuChE structure is essential for understanding its high catalytic efficacy and the molecular basis for the recognition of ACh by other ACh-binding protein (ACh receptors). Therefore, we have selected these enzymes to see the interaction pattern analysis of melatonin. For the validation of present study, the structural interaction by introducing computational approaches explores the binding/inhibition pattern of melatonin with AChE and BuChE enzymes. Considering the current evidences, we have examined two most relevant brain biomarkers like, lipid peroxidation (LPO) and production of reactive oxygen species (ROS). Computational elucidation of melatonin in repair system has also been considered by using docking method.

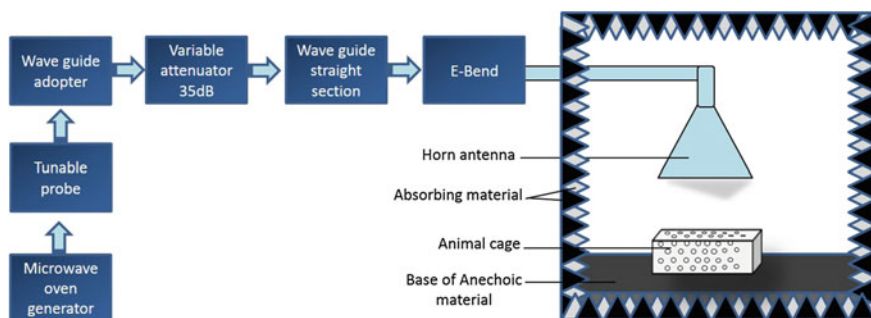
## 2 Methodology

### 2.1 *Animal Exposure*

Sixty-day-old male Wistar rats ( $180 \pm 10$  g) were obtained from an animal facility of Jaipur National University, Jaipur, Rajasthan, India. Animals were divided into four groups: sham exposed, melatonin (Mel) treated, 2.45 GHz microwave (MWs) exposed and MWs + Mel treated ( $n = 4$  in each group). All the experiments were performed during day time and the melatonin was applicated intraperitoneally (2 mg/kg) every morning at 08.00 AM to avoid its effects as neurotransmitter and/or neuromodulator (Drago et al. 2001). Several other researchers used the same dose of melatonin in their studies (Koc et al. 2003; Taysi et al. 2008; Sokolovic et al. 2008). Similarly sham exposed animals were injected with phosphate buffered saline (PBS) as control. All animals were housed in an air-conditioned room, where the temperature was maintained at 25–27 °C, with constant humidity (40–50%) and kept with constant 12-h light and 12-h dark schedule throughout the experiment. The animals were fed standardized normal diet (Tetragon Chemie Private Limited, Bangalore) and provided with water ad libitum. The amount of diet consumed and animal weight were recorded on a daily basis. The protocols for animal experimentation described in the present study were approved by the Institutional Animal Ethical Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals. All subsequent animal experiments were followed as per the “Guidelines for Animal Experimentation” of the University.

## 2.2 Exposure Chamber

Rats were placed in a Plexiglas cage ventilated with holes of 2 mm diameter on the walls. This was designed so that each animal could be lightly restrained in a fixed position with proper ventilation. Exposure was carried out in an anechoic chamber. All animals were facing horn antenna and the field relative to the long axis of the cages were vertically polarized. The horn antenna gain was 15 dBi calibrated. Anechoic chamber was lined with radar absorbing material (attenuation, 40 db) to minimize the reflection of scattered beam. Rats were exposed to 2.45 GHz radiation source. The temperature in the chamber was maintained around 25 °C throughout the experiment. The animals were exposed to radiation for 2 h per day for 35 days at 0.2 mW/cm<sup>2</sup> power density. The power density was measured within animal cage in the anechoic chamber. The whole body specific absorption rate (SAR) was estimated to be 0.14 W/kg following the method of Durney et al. (1984). For the measurement, power coupled through inner wire was detected by the detector crystal and measured by a power meter (Model-U2000 series USB power sensors, Agilent Technologies), which is a peak sensitive device. The microwave generated power through a series of power generators releasing power through horn antenna as indicated in Fig. 1. Similar exposure setup was used earlier by Chauhan et al. (2016). Each day, the cage was placed in the same position facing the horn antenna. Similar experiment was performed with sham exposed animals without energizing the system.



**Fig. 1** Schematic diagram of exposure setup with animal cage indicating individual animal's position. Microwave oven was opted here as radiating source (microwave generator) and the generated microwave power further taken into tunable probe and RF coaxial cable transmission line and then power was released through horn antenna. The horn antenna face have wide dimensions, where the cage was placed in the same line of the horn antenna face

### 2.3 Lipid Peroxidation (LPO) Assay

Lipid peroxidation was measured by the method of Buege and Aust (1978). One gm of the tissue was homogenized in 9 ml of 1.15% KCl. The tissue homogenate (0.8 ml) was mixed with 1.2 ml of trichloroacetic acid (TCA) (15% w/v), thio-barbituric acid (TBA) (0.375% w/v) and hydrochloric acid (HCl) (0.25 N) solutions (Himedia, Mumbai, Maharashtra, India), prepared in a 1:1:1 ratio. The mixture was heated in a boiling water bath for 30 min. Samples were centrifuged at 1000 g for 10 min. After centrifugation, the absorbance was recorded at 532 nm by using Ultra Violet-Vis Double Beam Spectrophotometer (Double Beam Spectrophotometer 2203, Systronics, Ahmedabad, Gujarat, India). A standard curve was prepared by using tetra-methoxy-propane (TMP: purchased from Himedia, Mumbai, Maharashtra, India). After comparison with a standard curve the LPO level was expressed in nmol/gm tissue.

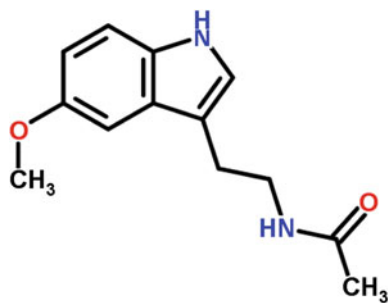
### 2.4 Reactive Oxygen Species (ROS)

0.1 g of whole brain tissue was homogenized with addition of 1:9 (W/V) phosphate buffered saline (0.1M  $\text{Na}_2\text{HPO}_4$ , 0.1M  $\text{KH}_2\text{PO}_4$ , 1.37M  $\text{NaCl}$ , 2.7mM  $\text{KCl}$ , pH 7.4). The homogenates were centrifuged at 14,000 rpm for 10 min at 4 °C (Eppendorf centrifuge 5418 R, Germany) and supernatant was collected for ROS measurement. ROS assay was performed (immediately after centrifugation) by employing DEPPD staining (Hayashi et al. 2007). ROS levels in sample was calculated from the calibration curve of  $\text{H}_2\text{O}_2$  and expressed as equivalent to levels of hydrogen peroxide (1 unit = 1.0 mg  $\text{H}_2\text{O}_2$ /l). Calibration curve for the standard solution was obtained by calculating slopes from optical density graph.

### 2.5 Preparation of Drug Enzymes 3D Structures

Ligand file of melatonin was downloaded in *mol* format (Fig. 2) from Chem Spider Chemical Database (Pence and Williams 2010). We have also converted it into *pdb* files. The crystal structure of AChE (PDB ID: 3LII) (Fig. 3) and BuChE (PDB ID: 1P0M) (Fig. 4) was obtained from Protein Data Bank. Published structures were edited to remove HETATM and water molecules, also further the ligands and enzymes were submitted for CHARMM (Brooks et al. 2009) energy minimization protocol using AMBER force field by Chimera tool Pettersen et al. (2004).

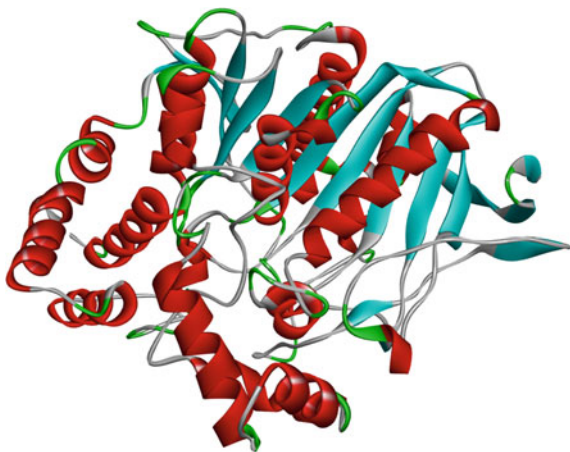
**Fig. 2** 2D structure of melatonin



**Fig. 3** Human  
Acetylcholinesterase (*AChE*)  
PDB ID-3LII



**Fig. 4** Human  
Butyrylcholinesterase (*BuChE*) PDB ID-1P0M



## 2.6 Molecular Interaction Analysis

The molecular interaction of AChE and BuChE enzymes with melatonin were analysed through computational docking studies.

Autodock 4.2 (Morris et al. 2009) and Cygwin interface was used to dock melatonin on binding site of the enzymes. Molecular docking methods followed by searching the best conformation of enzyme and melatonin complex on the basis of binding energy and inhibition constant. Kollman united charges and salvation parameters were added to the enzymes. Gasteiger charge was added to the melatonin compound. Grid box was set to cover the maximum part of enzymes and melatonin. The co-ordinates were set to  $60 \times 60 \times 60$  Å in X, Y and Z axis of a grid point. The default grid points, spacing was 0.375 Å. Lamarckian Genetic Algorithm (LGA) (Goodsell et al. 1996) was used for enzymes-ligands flexible docking calculations. The LGA parameters like population size, energy evaluations, mutation rate, crossover rate and step size were set to 150, 2,500,000, 27,000, 0.02, 0.8 and 0.2 Å, respectively. The LGA runs were set at 50 runs. The generated conformations of enzymes and melatonin were analyzed using Discovery Studio 4.5 molecular visualization software for the interactions and binding energy of the obtained docked complex structure (Ali et al. 2016).

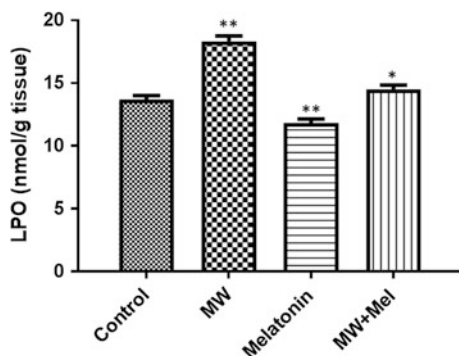
## 2.7 Statistical Analysis

As a balanced factorial design was used in this study, factorial ANOVA was performed with MW (or sham MW) and Mel. as fixed factors and replicate as random factor. The interactions MW \* Mel. (or sham MW \* Mel.) were included in the model. The general linear model procedure of SPSS (IBM-SPSS version 24) was used for the analysis.

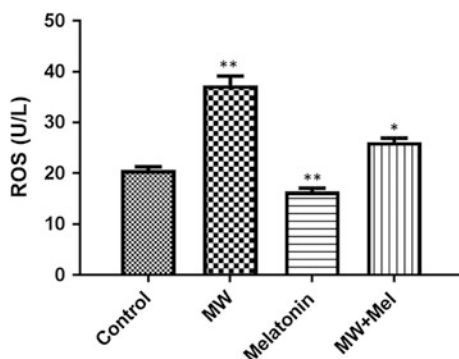
# 3 Results

## 3.1 Lipid Peroxidation

The increased level of LPO was recorded significantly ( $p < 0.05$ ) in the brain tissue of rats exposed to MWs as compared to control and Mel group (Fig. 5). While the melatonin treated group showed significant reduction ( $p < 0.05$ ) of LPO level in brain tissue of animals that were exposed with 2.45 GHz microwave radiation. This suggested MW-related decrease was most obvious in the groups exposed to melatonin (Mel), (Fig. 5), and the Mel \* MW interaction was statistically significant.



**Fig. 5** Lipid peroxidation level in whole brain exposed to 2.45 GHz microwave radiation. The microwave (MW) treatment was combined with melatonin (Mel.). The data shown are mean  $\pm$  SEM values. Factorial ANOVA was used for statistical testing; statistically significant effects for the two factors (MW and Mel.) and their interactions are reported below: Brain, after 35 days of exposure: MW  $p < 0.001$ ; Mel.  $p < 0.001$ ; and MW \* Mel  $p = 0.010$ . The significances for the MW effects are given in the figure: \* $p < 0.05$ ; \*\* $p < 0.001$



**Fig. 6** Reactive oxygen species level in whole brain exposed to 2.45 GHz microwave radiation. The microwave (MW) treatment was combined with melatonin (Mel.). The data shown are mean  $\pm$  SEM values. Factorial ANOVA was used for statistical testing; statistically significant effects for the two factors (MW and Mel.) and their interactions are reported below: Brain, after 35 days of exposure: MW  $p < 0.001$ ; Mel.  $p < 0.001$ ; and MW\*Mel  $p = 0.012$ . The significances for the MW effects are given in the figure: \* $p < 0.05$ ; \*\* $p < 0.001$

### 3.2 Reactive Oxygen Species (ROS)

The level of reactive oxygen species was recorded significantly higher ( $P < 0.001$ ) in the brain tissue of rats, exposed to microwave radiation as compared to sham exposed and Mel group (Fig. 6). Whereas, melatonin treatment in irradiated animals were showed significant reduction ( $p < 0.05$ ) in ROS level as compared to irradiated animals. This suggested MW-related decrease was most obvious in the groups exposed to melatonin (Mel), (Fig. 6), and the Mel\*MW interaction was statistically significant.



**Table 1** Docking results obtained from AChE and BuChE melatonin interaction complex

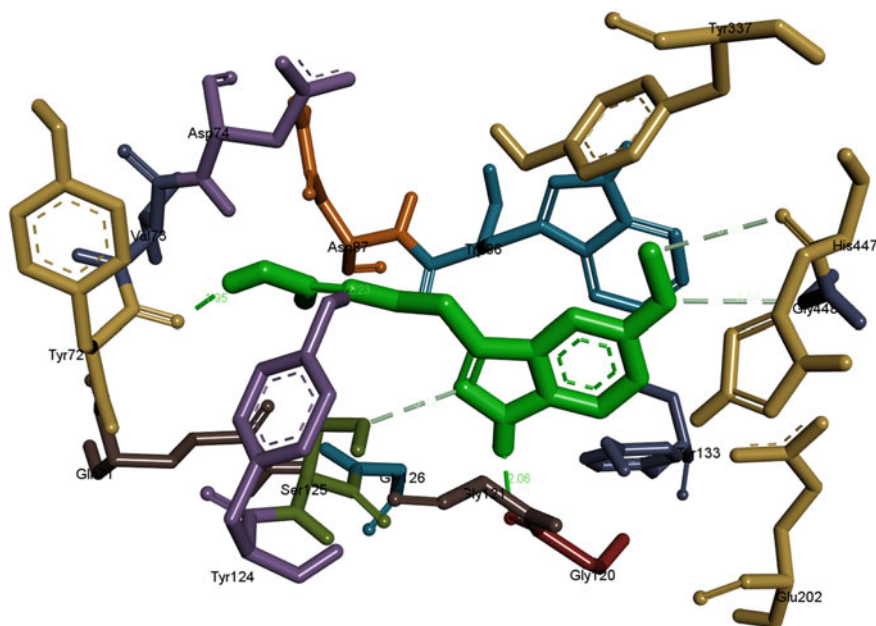
S. No	Protein-drug complex Name	H bonds	H bonds length	Final intermolecular energy (vdW + Hbond + desolv energy) (kcal/mol)	Inhibition constants (uM)	Residues involved in hydrophobic interaction
1.	AChE-melatonin	A:TYR124:HH—:UNK1:N3	2.23404	-9.01	3.11	Gln71, Tyr72, Val73, Asp74, Trp86, Asn87, Gly120, Gly121, Tyr124, Ser125, Gly126, Tyr133, Glu202, Tyr337, His447, Gly448
		:UNK1:H9—A:GLY120:O	2.061			
		:UNK1:H33—A:TYR72:O	1.95213			
		A:GLY448:CA—:UNK1:O16	3.54374			
		:UNK1:C7—A:SER125:OG	3.14395			
		:UNK1:C4—A:TRP86:O	3.48324			
		:UNK1:C17—A:HIS447:O	3.38297			
2.	BuChE-melatonin	A:HIS438:HE2—:UNK1:N3	2.19484	-7.89	20.46	Trp82, Gly115, Gly116, Gly117, Tyr128, Glu197, Ser198, Ala199, Trp231, Leu286, Ala328, Phe329, Phe398, Met437, His438, Gly439, Tyr440
		:UNK1:H9—A:GLU197:OE1	1.84661			
		:UNK1:H33—A:SER198:OG	1.9853			
		:UNK1:C17—A:HIS438:O	3.08519			

### 3.3 *In Silico Docking (AChE and BuChE)*

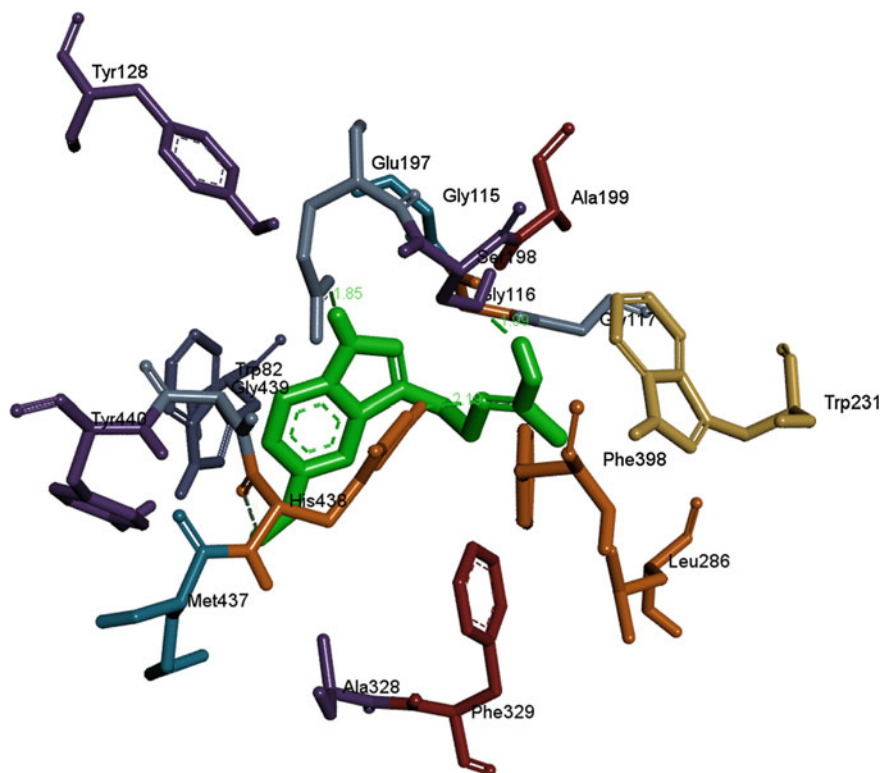
The in silico results obtained by docking analysis are documented in Table 1. The molecular docking results reveal that, drugs exhibited interactions with AChE and BuChE, and obtained binding energies are  $-9.01$  and  $-7.89$  kcal/mol respectively followed by inhibition constant i.e.  $3.11$  and  $20.46$   $\mu\text{M}$  respectively (Table 1).

The interaction reveals that Gln71, Tyr72, Val73, Asp74, Trp86, Asn87, Gly120, Gly121, Tyr124, Ser125, Gly126, Tyr133, Glu202, Tyr337, His447 and Gly448 amino acid residues (Fig. 7) are playing crucial role in the hydrophobic interaction with AChE while the amino acid residues Trp82, Gly115, Gly116, Gly117, Tyr128, Glu197, Ser198, Ala199, Trp231, Leu286, Ala328, Phe329, Phe398, Met437, His438, Gly439 and Tyr440 are interacted with BuChE (Fig. 8).

We have also seen seven hydrogen bonds interaction with AChE. On the other hand four hydrogen bonds interaction with BuChE (Table 1).



**Fig. 7** AChE Amino acids involved in hydrophobic interaction. Hydrogen bonds shown by green dotted lines



**Fig. 8** BuChE amino acids involved in hydrophobic interaction. Hydrogen bonds shown by *green dotted lines*

## 4 Discussion

In the experiment conducted to confirm the effects of melatonin, expected effects were observed: LPO and ROS levels were decreased. Similarly, decreased ROS was seen in the sperm cells after melatonin treatment (Meena et al. 2014). The study also demonstrated that exposure of microwave alone causes oxidative damage biochemically by increased levels of LPO and ROS. LPO triggers the loss of membrane integrity, causing increased cell permeability, enzyme inactivation, structural damage to DNA, and cell death (Halliwell 1994). The formation of free radicals significantly enhanced the ROS which indicates pathological changes in the brain tissue. There is evidence of free radical generation after RF-microwave exposures (Phillips et al. 2009; DeIullis et al. 2009; Kesari et al. 2012a, b). The increased level of LPO and ROS measured in microwaves exposed group is blocked by melatonin. This has been investigated by showing an interaction between microwave exposed group and melatonin treated group. Interestingly, the results show that melatonin has potential to decrease the increased level of LPO and

ROS after microwave exposure. Significant results from present study justify this statement. Although, melatonin acts as potent antioxidant and therefore is included in protection against oxidative stress (Meena et al. 2014). Moreover, 6-hydroxymelatonin (melatonin metabolite, which is excreted in urine) also shows an antioxidant potential, protecting the DNA molecules from oxidative damage, and reduces the level of hydroxyl radical (Lopez-Burillo et al. 2003; Qi et al. 2000).

The exact mechanism, by which melatonin controls cell death or damages are not entirely known, but however, it has been implied that mitochondria within the cells are targets for melatonin actions. Melatonin plays an effective role in regulating mitochondrial homeostasis (Castroviejo et al. 2011; Srinivasan et al. 2011). Melatonin is a powerful antioxidant that scavenges  $\cdot\text{OH}$  radicals as well as other ROS and RNS (Galano 2011). This article summarizes the several mechanisms through which melatonin can exert neuroprotective actions in neurodegenerative disorders. In case of brain disease to understand the mechanism of binding of melatonin to acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), melatonin was selected for in silico docking studies which were carried out using AutoDock 4.2, based on the Lamarckian genetic algorithm principle. AChE is a serine hydrolase whereas, BuChE is known as pseudocholinesterase or nonspecific ChE. These ChEs are highly efficient since they are able to cleave more than 10,000 molecules of ACh per second and produce acetate and choline rapidly (Choi et al. 2007). Its biological role is termination of impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter ACh to acetate and choline. AChE is mainly found at neuromuscular junctions and cholinergic brain synapses. The findings from this study shows a clear indication of brain disorder after exposure of microwaves. The damaging effects of MW radiation on the brain includes brain dysfunction and brain structural damage. The results obtained in this study (increased level of LPO and ROS) after microwave exposures may alter the levels of protective proteins or lipids and this reduction means a greater probability of Alzheimer's disease. AD is characterized by the loss of cholinergic innervations, reduction of choline acetyltransferase and enhanced acetylcholinesterase (AChE) activity (Rutten et al. 2002; Ansari and Khodagholi 2013). The progressive disturbance of cholinergic function is fundamentally close to the short-term memory loss seen in AD. Therefore, it is imperative to see the interaction of melatonin after microwave exposure in the brain as well as the elucidation of mechanism of action underlying the pharmacological and toxicological action of these agents for the purpose of rational drug design (Sussman et al. 1991).

Melatonin has been found to be highly protective against damage to macromolecules resulting from oxygen and nitrogen based reactants (Reiter et al. 2003). Melatonin prevents oxidative damage of biological membranes by preventing harmful lowering of the mitochondrial membrane potential. However, lowering may trigger mitochondrial transition pore opening and apoptosis cascade (Martin et al. 2000; Rodriguez et al. 2004; Winiarska et al. 2006). Friedman et al. (2007) showed that RF-EMW stimulate plasma membrane NADH oxidase in mammalian cells and cause production of ROS. ROS is the affecting factor, where RF-EMW may increase the level and induce formation of large aqueous pores on the cell

membrane. This phenomenon is called electroporation. Present study are in line with several other reports suggest that exposure of microwave radiations may induce oxidative stress by increasing ROS production, which may lead to DNA damage and genotoxic effects (Kumar et al. 2013; Shahin et al. 2013).

## 5 Conclusion

This study revealed that exposure of 2.45 GHz microwave frequency adversely affects the whole brain and cause oxidative damage. Also, data obtained from in silico analysis shows that the melatonin have a strong capability to interact with AChE and BuChE enzymes of the CNS. The biochemical elucidation of interaction patterns like binding energy, inhibition constants, hydrogen bonds and the residues involved in hydrophobic interactions suggests that melatonin could be used as potent free radical scavenger and antioxidant agent, melatonin for protecting cells and tissues from oxidative damage.

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