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Effect of mobile phone signal radiation on epigenetic modulation in the hippocampus of Wistar rat

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ABSTRACT

Exponential increase in mobile phone uses, given rise to public concern regarding the alleged deleterious health hazards as a consequence of prolonged exposure. In 2018, the U.S. National toxicology program reported, two year toxicological studies for potential health hazards from exposure to cell phone radiations. Epigenetic modulations play a critical regulatory role in many cellular functions and pathological conditions. In this study, we assessed the dose-dependent and frequency-dependent epigenetic modulation (DNA and Histone methylation) in the hippocampus of Wistar rats. A Total of 96 male Wistar rats were segregated into 12 groups exposed to 900 MHz, 1800 MHz and 2450 MHz RF-MW at a specific absorption rate (SAR) of 5.84×10^{-4} W/kg, 5.94×10^{-4} W/kg and 6.4×10^{-4} W/kg respectively for 2 h per day for 1-month, 3-month and 6-month periods. At the end of the exposure duration, animals were sacrificed to collect the hippocampus. Global hippocampal DNA methylation and histone methylation were estimated by ELISA. However, DNA methylating enzymes, DNA methyltransferase1 (DNMT1) and histone methylating enzymes euchromatic histone methylthransferase1 (EHMT1) expression was evaluated by real-time PCR, as well as further validated with Western blot. Alteration in epigenetic modulation was observed in the hippocampus. Global DNA methylation was decreased and histone methylation was increased in the hippocampus. We observed that microwave exposure led to significant epigenetic modulations in the hippocampus with increasing frequency and duration of exposure. Microwave exposure with increasing frequency and exposure duration brings significant ($p < 0.05$) epigenetic modulations which alters gene expression in the hippocampus.

1. Introduction

Electromagnetic radiations are the fourth-largest and most rapidly increasing, the anthropogenic source of pollution on the earth. Globally wireless communication systems have increased tremendously in the last decade, which principally uses radiofrequency microwaves. The United States, National Toxicology Program (NTP), U.S. Dept. of Health and Human Services conducted two-year toxicology studies in rats and mice to elucidate potential health hazards from exposure to radiofrequency (700–2700 MHz). NTP reported evidence of cell phone signal exposure with tumors in the heart, brain and adrenal glands (https://ntp.niehs.nih.gov/whatwestudy/topics/cellphones/index.html). Different experimental studies in animal model also reported for, radiofrequency microwave-induced oxidative stress, DNA damage, enhanced neuronal loss, altered neurotransmitters, increased blood-brain barrier permeability and cognitive impairment (Dasdag and Akdag, 2016; Dasdag et al., 2009; Deshmukh et al., 2013b; Maskey et al., 2010; Mausset-Bonnefont et al., 2004; Megha et al., 2015b; Nittby et al., 2009; Pall, 2018; Salford et al., 2003). However, microwave exposed DNA damage also reported in different tissues in rat as well in human being (Akdag et al. 2016, 2018; Bektas et al., 2020). Various epidemiological studies in human too reported about MW radiation-induced increased brain glucose metabolism (Volkow et al., 2011), brain physiology, attention, reaction time, working memory (Schmid et al., 2012), systemic immune response (Kimata, 2005), glioma risk (Carlberg and Hardell, 2017). However, few studies in humans have been reported

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about the insignificant effect of microwave radiation on human health (Elder et al., 2007; Hardell, 2017).

Hippocampus is a small curved part of the brain, located within the medial temporal lobe, and is well associated with learning, memory and spatial navigation. Higher polyunsaturated fatty acid content and metabolic rate as compared to other cells make neuronal cells more susceptible to molecular damage by different types of environmental stress as well as oxidative stress (Fritze et al., 1997; Hermann and Hossmann, 1997; Salim, 2017). Rich polyunsaturated fatty acid content makes neurons vulnerable to peroxidation due to the generation of ROS (Ferrante et al., 2017) and peroxidated fatty acids are well associated with neurodegeneration (Pratico, 2002). Mobile phone users constantly put their mobile in close contact with the head, therefore central nervous system (CNS) is the region primarily affected by MW radiation. ROS triggers oxidative stress in CNS (Salim, 2017) which could be a potent inducer of epigenetic modulation, responsible for cognitive dysfunctions.

Epigenetic modulations are the key regulators of gene expression without altering the genomic constitution. Intrinsic as well as extrinsic signals allow sustained changes in gene expression and allow an organism to adapt to its dynamic environment through modulated gene activity (Dauncey, 2012; Jaenisch and Bird, 2003; Mehler, 2008). DNA methyltransferase1 (DNMT1) transfers a methyl group to cytosine in genomic DNA, and responsible for the maintenance of methylation patterns (Chan et al., 2019). Whereas euchromatic histone methyltransferase1 (EHMT1) methylates histone H3 lysine-9 (H3K9), which brings transcriptional repression by modifying chromatin structure (Koemans et al., 2017). Disturbed methylation patterns of DNA and Histone associated with various pathological conditions including cancer, developmental abnormalities and cognitive functions (Chan et al., 2019; Koemans et al., 2017).

Increasing exposure to man-made electromagnetic radiation-induced changing environmental conditions is a threat to human health. Therefore, it is time to evaluate the impact of mobile phone signal radiation at genetic as well as epigenetic level. In an earlier study, we have reported about microwave-induced DNA damage (Deshmukh et al., 2016), reactive oxygen species (ROS) generation, oxidative stress (Deshmukh et al., 2013a) and ER-stress (Kumar et al., 2019) in rat brain. However, in neither study, we find any reports about microwave-induced epigenetic modulations in any experimental model, nor any study reported about the effect of microwave radiation on DNA/histone methylating enzymes (DNA methyltransferase1 and euchromatin histone methyltransferase1) expression in any experimental model. Therefore, the present study was designed to address the knowledge gap about microwave-induced epigenetic modifications and epigenotoxic nature of microwave, by evaluating the percentage of DNA/histone methylation and expression of DNA/histone methylating enzymes (DNMT1 and EHMT1) in the hippocampus following microwave exposure in male Wistar rat. In this study, we hypothesized that chronic microwave radiation may induce epigenetic changes in the form of altered DNA/histone methylation pattern, which disturb chromatin structure and transcription factors binding site in DNA, that could be responsible factor for microwave-induced carcinogenicity, reproductive toxicity, developmental abnormalities (Vornoli et al., 2019), and cognitive impairment (Deshmukh et al., 2015).

2. Methods

2.1. Experimental animal group

We obtained 96 male Wistar rats (100 ± 10 g) from the central animal house of the institute and randomly divided into 12 groups (Sham exposed, 900 MHz, 1800 MHz and 2450 MHz, each for one-month, three-month and six-month respectively), and animals were placed in galvanized wired cages. They were made familiarized to laboratory conditions for 7 days and kept under standard conditions (Humidity 40–50% and temperature 22 ± 2 °C) with 12-h light and dark cycle. Nutritionally adequate diet from Nutri-lab (Bengaluru, India) and water provided *ad-libitum*. Rats were divided into 12 groups as shown in supplementary table 1.

This study and protocol were approved by the Institutional Animal Ethical Committee, UCMS & GTB hospital (University of Delhi), Dilshad Garden, Delhi-110095 **(UCMS/IAEC/2016/093)**. Care of animals was undertaken as per guidelines suggested by the committee for the purpose of control and supervision of experiments on animals (CPCSEA), Govt. of India.

2.2. Microwave exposure system

Male Wistar rats were exposed to RF-MW under the gigahertz electromagnetic (GTEM) cell. The schematic diagram of the microwave exposure system with a signal generator and GTEM cell (Supplementary figure 1). RF-MW exposure system was designed with the help of the Centre for Applied Research in Electronics (CARE) at the Indian Institute of Technology, New Delhi, India. For uniformity of the electric field, the system was calibrated and experimentally validated before animal exposure by an E-Field probe (Rohde & Schwarz NRV-Z32, Germany). RF-MW exposure was operated in well-controlled temperature and lighting conditions.

Sham exposed animal groups were control groups, which were kept under the same conditions and duration of time without any exposure to RF-MW. Each group of rats was given whole body RF-MW exposure in GTEM cell (Amitec Electronics Ltd., India), kept 1 m away from the signal input port, at defined frequency of 900 MHz, 1800 MHz, and 2450 MHz, and SAR of 5.84×10^{-4} W/kg, 5.94×10^{-4} W/kg and 6.4×10^{-4} W/kg respectively by the established power balance method as suggested by Ardoino et al., (2005), as shown above in Table 1 (supplementary data) at power level 1 mW for 2 h per day for one-month, three-month and six-month. For Specific Absorbance Rate (SAR) measurement representative rats with 106 g average weight was used, and SAR was calculated by power balance method using following equation:

$$P_{abs}/rat = 1/n\ (P_{in} - P_{out} - P_{refl})$$

Where, P_{abs} = Radio frequency (RF) power in watt absorbed per animal, n = number of animals within the cell, P_{in} = input power (Watt), P_{out} = output power (Watt) and P_{refl} = reflected power (Watt).

2.3. Expression of DNA methyltransferases and histone methyltransferases

Immediately after completion of each specified duration (1-month, 3-month, and 6-month) for RF-MW exposure, animals were decapitated and the hippocampus was isolated from the brain. Total RNA was extracted from the hippocampus using TRIzol reagent (Life Technologies, USA) as per the manufacturer's given protocol. One microgram of total RNA was converted into cDNA with an iScript cDNA synthesis kit (Bio-Rad, USA) as suggested by the manufacturer. Further RT-PCR (qRT-PCR) was performed in 20 μl vol. with SsoFast Eva Green supermix (Bio-Rad, USA) on CFX Connect™ Real-Time PCR (Bio-Rad, USA) with primers listed in Table 1. Each sample was run in triplicate and the relative fold change of selected genes was calculated by the comparative $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001).

Table 1
Primer sequences used for qRT-PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
DNMT1	GAGGCACTGTCCGTCTTTGA	CTGATTGATTGGCCCCAGGT
EHMT1	TGGATTCCCTGGATCTCCGT	GCACCAAGAGTGGTGCTTTG
GAPDH	TGCCCCCATGTTTGTGATG	TGGTGGTGCAGGATGCATT

2.4. Western-blotting of ER-stress associated transcription factor

Total proteins were extracted from the hippocampus of the male Wistar rat by using TRIS-NaCl buffer, immediately after decapitation and western blots were performed as suggested by Mahmood and Yang, (2012). Briefly, samples with 50 ng of protein were loaded onto 10% polyacrylamide gel with 0.1% SDS and separated by electrophoresis at 100 V for 180 min. Proteins were then transferred onto PVDF membrane and blocked with 5% BSA for 1 h. Primary antibody against DNMT1 and EHMT1, protein at 1:1000 (Signal way antibody, USA) was added next and left overnight at 4 °C. After washing with PBST, membranes were incubated with secondary HRP conjugated antibodies for 2 h and after washing with PBST again, proteins were visualized with ECL reagents (Bio-Rad, USA) on chemidock (My ECL imager, Thermo, USA). The bands were analyzed with Kodak ID image analysis software. All band intensities were normalized to β-actin.

2.5. 5-Methylcytosine DNA ELISA

Genomic DNA was isolated from the hippocampus of the rat brain by using spin column method as per the manufacturer's protocol (Promega DNA isolation kit, USA). Global percentage DNA methylation was estimated by measuring 5-methylcytosine (5-mC), using a 5-mC DNA ELISA kit (Zymo Research, USA), as per manufacturer's protocol. Briefly, 100 ng of isolated genomic DNA along with positive and negative controls of double-stranded DNA (provided with the kit) was denatured and used to coat the wells of microtitre plate with given coating buffer. All standards and hippocampal DNA were assayed in duplicate. Anti-5-mC antibody and HRP conjugated secondary antibody used for colour development. Absorbance measured at 405 nm and the percentage of 5-mC DNA calculated by using a second-order regression equation.

2.6. Histone H3K9 methylation

Histone protein isolation and assessment of their methylation from the hippocampus of the rat brain were performed by using a Histone H3K9 methylation assay kit (Epigentek, EpiQuik Global Histone H3K9 Methylation Assay) as per manufacturer's protocol. Extracted histone protein of 200 ng/μl was used for the evaluation of histone methylation percentage as suggested by the manufacturer. The percentage of H3K9 methylation was measured by using the standard curve, which was plotted with positive and negative control run along with the extracted histone protein.

2.7. Statistical analysis

Statistical analysis was carried out at SPSS (IBM SPSS statistics version 25) and values were expressed as mean ± SD (standard deviation). The significance of difference among the group was determined by a one-way analysis of variance (ANOVA) followed by Pearson's correlations and Tukey's post hoc test. Statistical significance was accepted at $p < 0.05$.

3. Result

DNA methyltransferases1 (DNMT1) and euchromatic histone methyltransferase1 (EHMT1) gene expression were evaluated by quantitative real-time PCR, in all specified groups of rats in Table 1. DNA methyltransferases1 and euchromatic histone methyltransferase1 have a crucial role in DNA and histone methylation respectively. In the present study, the expression of the *Dnmt1* gene was decreased, however, the *Ehmt* gene was increased in microwave exposed rat groups, with respect to sham exposed rat groups (Fig. 1A and 2A).

To validate the gene expression pattern of qRT-PCR, the expression levels of DNMT1 and EHMT1 further analyzed by Western blot by using a respective antibody, anti-DNMT1 antibody and anti-EHMT1 antibody (Signalway antibody, USA) in the hippocampal cell lysates. The Western blot analysis also indicated that expression of DNMT1 decreased and EHMT1 increased, with increasing frequency and duration of exposure (Fig. 1B and C, Fig. 2B and C).

3.1. EHMT1 expression

Expression of *Ehmt1* mRNA was increased with increasing microwave exposure frequency as 1.17-fold in 900 MHz, 1.29-fold in 1800 MHz and 1.63-fold in the 2450 MHz exposure group when compared with the sham-exposed group (Fig. 1A). In post hoc analysis, the difference was found significant ($p < 0.05$) when the sham-exposed group was compared with 900 MHz, 1800 MHz, and 2450 MHz exposure groups. When we compared the 900 MHz with 2450 MHz and the 1800 MHz with the 2450 MHz exposure group, we again noticed a significant ($p < 0.05$) change. However, 900 MHz and 1800 MHz exposed groups did not show any significant differences between each other.

After three-month of exposure, significant ($p < 0.05$) upregulation of *Ehmt1* mRNA expression in the hippocampus was noticed. After the 3-month exposure gene expression increases, 1.32-fold in 900 MHz, 1.40-fold in 1800 MHz and 1.70-fold in 2450 MHz frequency exposed group when compared to sham-exposed group respectively (Fig. 1A). We also observed a higher fold change in the three-month exposure group at the respective frequency. In the post hoc test, a significant ($p < 0.05$) increase in fold change was observed when the sham-exposed group was compared with 1800 MHz and 2540 MHz exposure frequency. The 900 MHz group and 2450 MHz group show a significant difference, but no significant difference in fold change was observed when we compared 900 MHz with 1800 MHz and 1800 MHz with 2450 MHz.

Similarly, in the six-month exposure group, significant ($p < 0.05$) upregulation of *Ehmt1* mRNA expression in the hippocampus of rat brain was also observed with increasing microwave exposure frequency, 1.6-fold in 900 MHz, 1.75-fold in 1800 MHz and 2.32-fold increase in 2450 MHz microwave exposure group with respect to sham-exposed group respectively (Fig. 1A). In the six-month exposure group, we observed greater fold change with respect to the three-month and one-month exposure group at respective frequency. Post hoc test, shown significant ($p < 0.05$) increase in fold change was noticed with respect to the sham-exposed group. The significant difference of fold change was obtained when we compared 900 MHz frequency fold change with 2450 MHz frequency and 1800 MHz frequency fold change with the 2450 MHz frequency fold change. However, no significant difference was found when we compared 900 MHz fold change with 1800 MHz fold change.

The gene expression profile was validated with Western blot analysis of EHMT1 protein (Fig. 1B). Data were normalized with housekeeping protein β-actin shown in the bar diagram (Fig. 1C). In post hoc analysis all the changed expression was found significant ($p < 0.05$).

3.2. DNMT1 expression

Expression of *Dnmt1* mRNA after one month was 0.71-fold downregulated in 900 MHz, 0.57-fold downregulated in 1800 MHz and 0.39-fold downregulated in the 2450 MHz exposure group (Fig. 2A). Post hoc analysis was found significant ($p < 0.05$) when we compared 900 MHz with 2450 MHz and 1800 MHz with a 2450 MHz exposure group. However, 900 MHz and 1800 MHz exposed groups did not show significant downregulation when compared with each other.

Significant ($p < 0.05$) downregulation of *Dnmt1*mRNA expression in the hippocampus was noticed. Gene expression decreases with increasing microwave exposure frequency 0.67-fold in 900 MHz, 0.53-fold in 1800 MHz and 0.37-fold in 2450 MHz frequency exposed group when compared to sham-exposed group respectively (Fig. 2A). Following three-month exposure, we observed more downregulation in gene expression with respect to one-month exposure at respective frequency. Significant ($p < 0.05$) decrease in fold change was observed

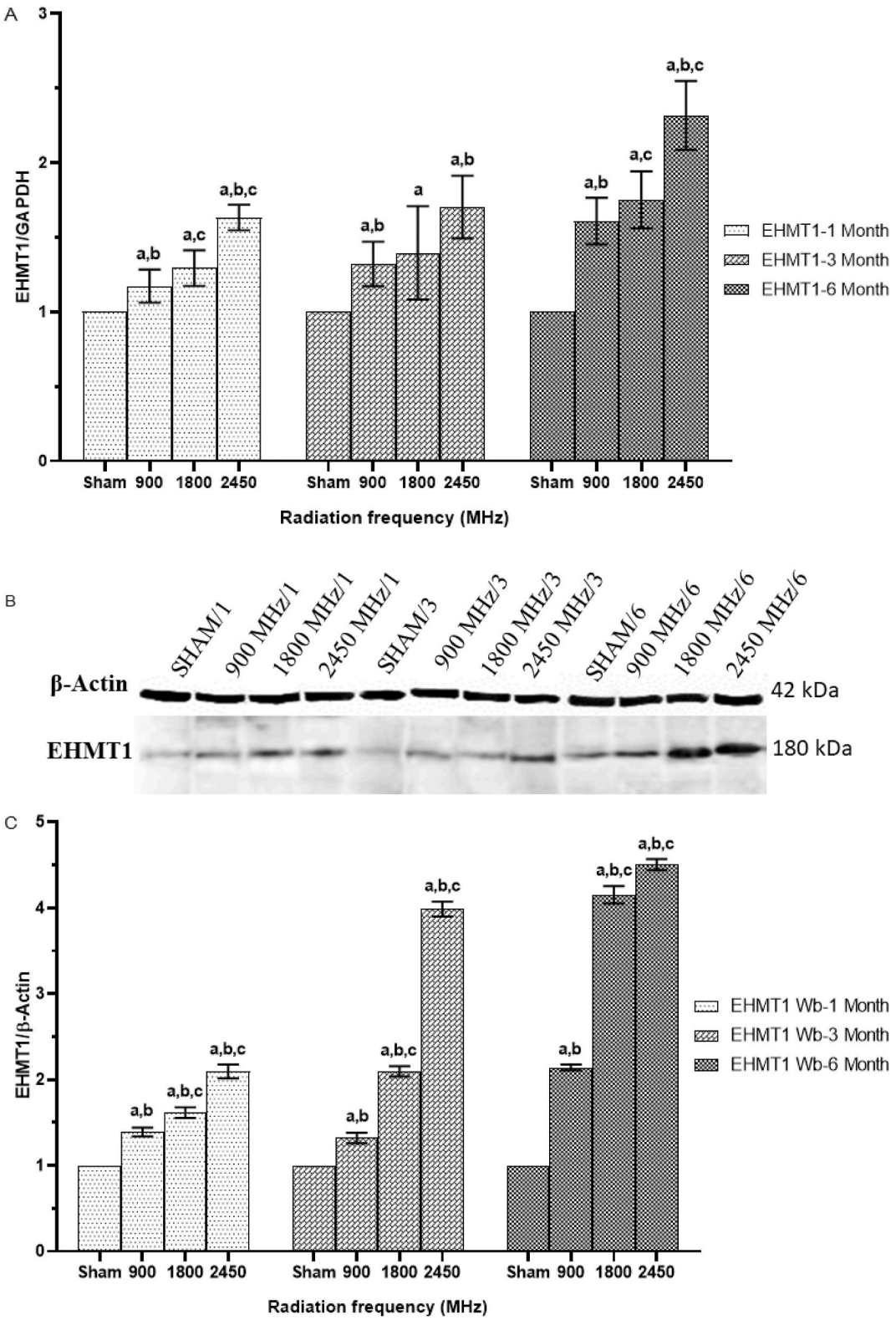
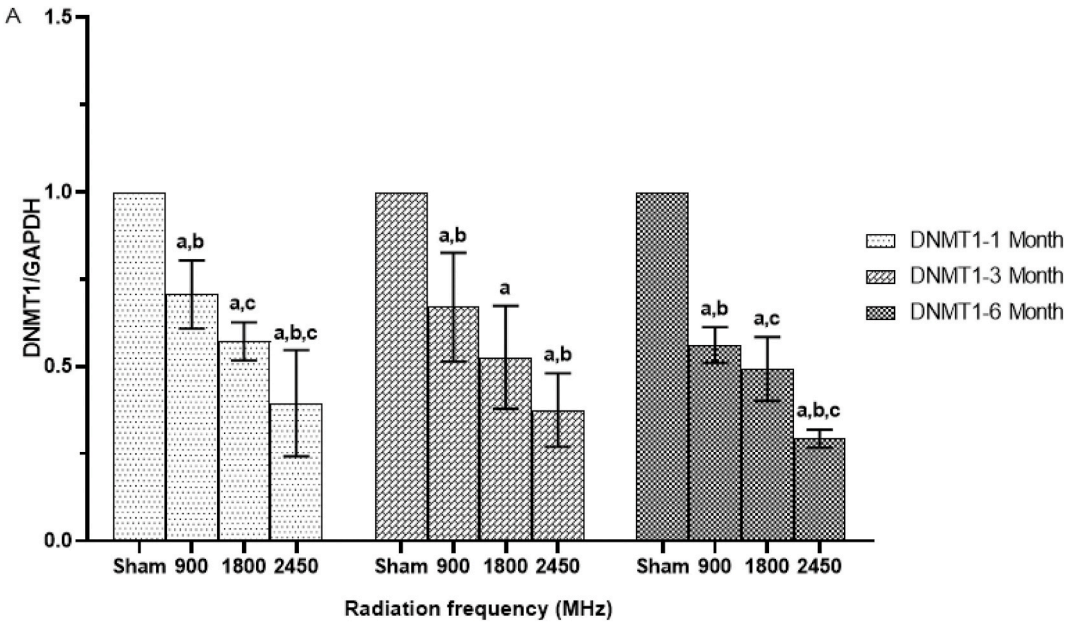
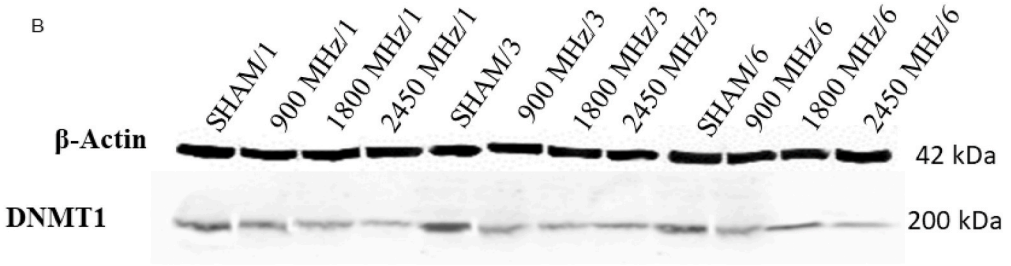
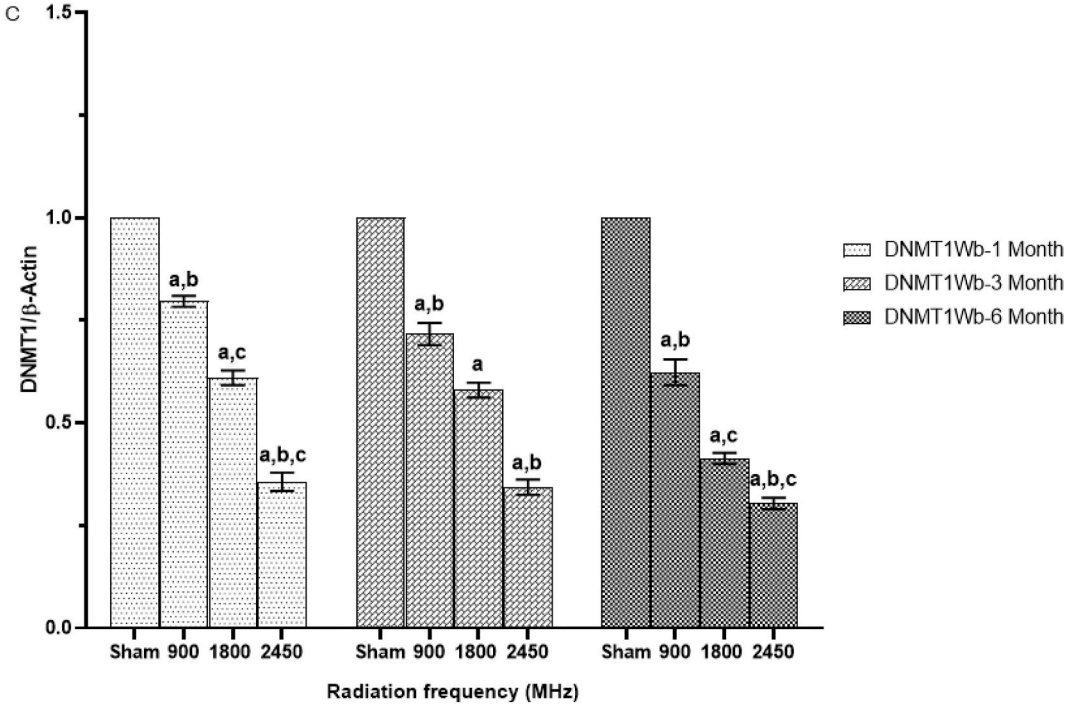


Fig. 1. (A) Effect of microwave exposure on *Ehmt1* gene expression in rat brain. The relative transcription levels of euchromatic histone methyltransferase1 (EHMT1) in the hippocampus of Wistar rat in response to radiofrequency microwave exposure for one-month, three-month and six-month. Total RNA was extracted from the hippocampus of sham-exposed and microwave exposed Wistar rats and was analyzed by quantitative real-time PCR to deduce the expression level of *Ehmt1* genes. The expressional value of the hippocampus of the microwave exposed Wistar rat were normalized to those of sham-exposed rat. The relative transcriptional values of the *Ehmt1* gene were calculated by normalizing to the GAPDH expression using $2^{-\Delta\Delta Ct}$ (n = 8) method and we found its increasing as with increasing frequency and exposure duration. (B) Western blot of Ehmt1 in the hippocampus of Wistar rat after one-month, three-month and six-month microwave exposure at 900, 1800 and 2450 MHz frequency. (C) Band intensity of Western blot was quantified by dosimetry, and the protein level was normalized relative to β-actin which was shown in the bar diagram. Each bar represents the mean value with ±SD of experiments in triplicates. Statistical significance value accepted in a two-tailed *t*-test, if $p < 0.05$. Respective p-value *a,b,c significantly different from respective control ($p < 0.05$) calculated by one way ANOVA followed by Tukey's test.*







(caption on next page)

Fig. 2. (A) Effect of microwave exposure on *Dnmt1* gene expression in rat brain. The relative transcription levels of DNA methylatransferase1 (DNMT1) in the hippocampus of Wistar rat in response to radiofrequency microwave exposure for one-month, three-month and six-month. Total RNA was extracted from the hippocampus of sham-exposed and microwave exposed Wistar rats and was analyzed by quantitative real-time PCR to deduce the expression level of *Dnmt1* genes. The expressional value of the hippocampus of the microwave exposed Wistar rat were normalized to those of sham-exposed rat. The relative transcriptional values of the *Dnmt1* gene were calculated by normalizing to the GAPDH expression using $2^{-\Delta\Delta Ct}$ (n = 8) method and we found its increasing as with increasing frequency and exposure duration. (B) Western blot of Dnmt1 in the hippocampus of Wistar rat after one-month, three-month and six-month microwave exposure at 900, 1800 and 2450 MHz frequency. (C) Band intensity of Western blot was quantified by dosimetry, and the protein level was normalized relative to β-actin which was shown in the bar diagram. Each bar represents the mean value with ±SD of experiments in triplicates. Statistical significance value accepted in a two-tailed *t*-test, if $p < 0.05$. The respective p-value, *a,b,c significantly different from respective control ($p < 0.05$) by one way ANOVA followed by Tukey's test.*

when we compared 900 MHz with 2450 MHz exposure group, however no significant decrease in fold change was noticed when we compared 900 MHz with 1800 MHz and 1800 MHz with 2450 MHz exposure group.

Similarly, in the six-month exposure group, significant ($p < 0.05$) downregulation of *Dnmt1*gene mRNA expression with increasing microwave exposure frequency in the hippocampus of rat brain was observed, 0.56-fold in 900 MHz, 0.49-fold in 1800 MHz and 0.30-fold downregulation in 2450 MHz microwave exposure with respect to sham-exposed group respectively (Fig. 2A). In the six-month exposure group, we noticed more downregulation in gene expression with respect to the three-month exposure group at respective frequency. In the post hoc test, a significant ($p < 0.05$) decrease in fold change was noted when we compared the 900 MHz frequency fold change with 2450 MHz frequency and 1800 MHz frequency fold change with 2450 MHz frequency fold change. However, no significant difference was found when we compared 900 MHz fold change with 1800 MHz fold change.

The expression pattern of the *Dnmt1*gene was validated with the Western blot of DNMT1 (Fig. 2B). In the post hoc test, significant ($p < 0.05$) downregulation was noticed in all groups when compared to the sham-exposed group. Data were normalized with housekeeping protein β-actin and shown in the bar diagram (Fig. 2C).

3.3. Histone (H3K9) methylation

Percentage methylation of histones was estimated after one-month, three-month and six-month of microwave exposure as shown in Fig. 3. After one month of microwave exposure, the percentage of methylated H3K9 was observed to be increasing with increasing frequency. Methylated H3K9 in the sham-exposed group was 4.15%, whereas it was 4.90% in 900 MHz, 6.43% in 1800 MHz and 7.40% in the 2450 MHz exposed group. In the post hoc test, a Significant ($p < 0.05$) increase in methylated H3K9 was observed in the post hoc test when we compared the sham-exposed group with 1800 MHz and 2450 MHz exposed group, but not with 900 MHz exposed group. A significant increase in methylated H3K9 was also noticed when we compared 900 MHz exposure group with 1800 MHz and 2450 MHz exposure groups. However, no significant increase was observed when compared 1800 MHz exposure group with a 2450 MHz exposure group.

Methylated H3K9 histone protein was 5.29% in the sham-exposed group, 7.70% in 900 MHz, 8.79% in 1800 MHz and 9.38% in the 2450 MHz following three-month MW exposure. Increased methylation percentage was also noticed with respect to one-month exposure at respective frequency. In the post hoc test, a significant ($p < 0.05$) increase in methylated H3K9 histone protein was observed when we compared sham with 900 MHz, 1800 MHz, and 2450 MHz. A significant increase was also noticed when compared to 900 MHz with 2450 MHz but not between 900 MHz and 1800 MHz. However, no significant difference was observed when we compared the 1800 MHz exposure group with the 2450 MHz exposure group.

An increase in methylated H3K9 histone protein was obtained in the hippocampus of microwave exposed Wistar rat brain as 5.69% in sham-exposed group, 8.67% in 900 MHz, 9.58% in 1800 MHz and 10.38% in 2450 MHz following six-month exposure. Increased methylation percentage was again noticed with respect to three-month exposure at respective frequency. Significant ($p < 0.05$) increase in methylated H3K9 histone protein was observed, when we compared the sham-exposed group with 900 MHz, 1800 MHz, and 2450 MHz exposure group. But when we compared 900 MHz with 1800 MHz and 2450 MHz and 1800 MHz with 2450 MHz, no significant difference was obtained.

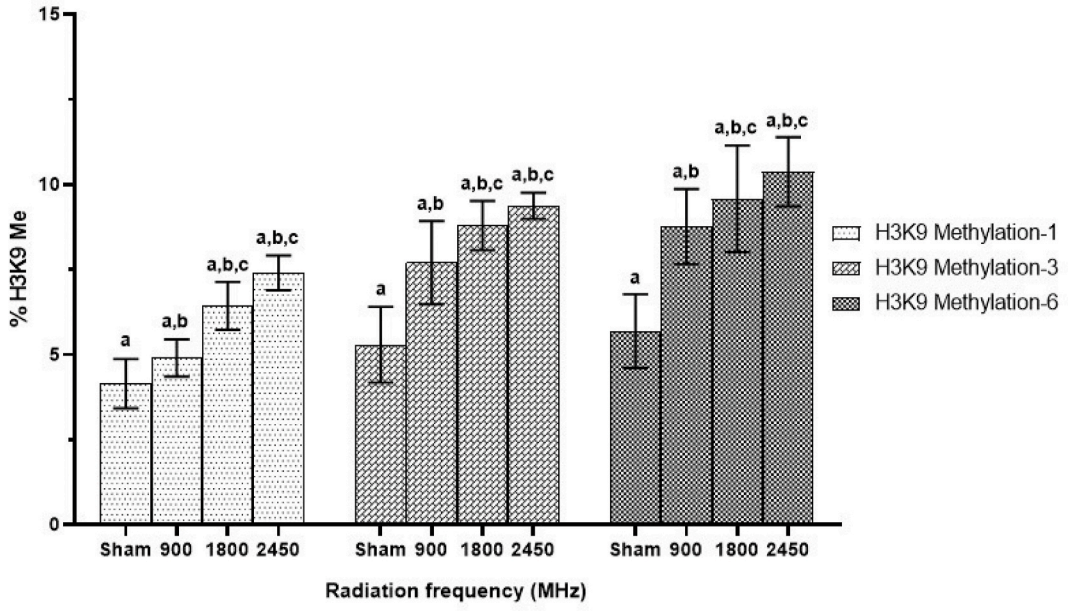


Fig. 3. Percentage Histone methylation (H3K9) in rat brain. *Values are expressed as mean ± SD (8 animals* per *group). The respective p-value, a,b,c significantly different from respective control ($p < 0.05$) by one way ANOVA followed by Tukey's test.*

3.4. 5-mC DNA methylation

Methylation of cytosine residue of DNA is well associated with the regulation of gene expression. Percentage methylation of DNA was evaluated as shown in Fig. 4. After one-month of exposure, the percentage methylation of 5-mC DNA was decreased with increasing frequency that is 17.23%, 16.81%, 15.04% and 12.96% in the sham-exposed group, 900 MHz groups, 1800 MHz group, and the 2450 MHz exposure group respectively. Significant ($p < 0.05$) decrease in methylation percentage was observed when the sham-exposed group was compared with 2450 MHz, but not when compared with 900 MHz and 1800 MHz. A significant decrease in methylation was observed, when we compared 900 MHz with the 2450 MHz exposure group but not with the 1800 MHz exposure group.

In three-month exposure group, a reduced percentage of methylated DNA was noticed with increasing microwave exposure frequency, 13.91% in the sham-exposed group, 12.69% in 900 MHz, 12.58% in 1800 MHz and 10.48% in 2450 MHz exposed group. Reduced methylation was also observed when compared with the one-month exposure group at respective frequency. In the post hoc test, a significant ($p < 0.05$) decrease in the percentage of methylated DNA was observed using post hoc test when compared to the sham-exposed group with 2450 MHz exposure group but not with 900 MHz and 1800 MHz exposure group. Further, a significant decrease in methylated DNA was also noticed when we compared 900 MHz with 2450 MHz and 1800 MHz with 2450 MHz exposure group.

In the six-month exposure group, a similar decreasing pattern of methylated DNA was noticed with increasing microwave exposure frequency. The sham-exposed group has 12.21% methylated DNA, whereas it is 11.27% in 900 MHz, 8.92 in 1800 MHz and 5.35% in the 2450 MHz exposed group in the hippocampus of Wistar rat. Reduced methylation was also observed when compared with the three-month exposure group at respective frequency. In the post hoc test, a significant ($p < 0.05$) decrease in methylated DNA was observed when we compared the sham-exposed group with 1800 MHz and 2450 MHz exposure groups. A significant decrease was also reported when compared to 900 MHz with 2450 MHz as well as 1800 MHz with a 2450 MHz exposure group.

4. Discussion

Mobile phone signal radiofrequency microwave (900, 1800 and 2450 MHz, 2 h per day for one, three and six month) induce epigenetic modulations in the hippocampus with significant increase in histone (H3K9) methylation, significant decrease in DNA methylation and significant fold change in transcriptional as well as translational level of DNA/histone methyltransferase (DNMT1/EHMT1) enzyme.

We are in constant exposure to electromagnetic radiation, notably under the blanket of artificial electromagnetic radiation, especially microwave radiation, emitted from wireless communication system (mobile phones, Wi-Fi/Bluetooth devices), and surveillance technologies (radar, security scanner) (Bandara and Carpenter, 2018). Due to the dramatic increase of wireless communication systems (cellular phones), the concern raised about the possible effects of mobile phone signals on human health (Myers, 2018). Various study group reported about microwave exposure induced alteration in blood, cord blood (Bektas et al., 2018), placenta (Bektas et al., 2020), ear canal hair follicle (Akdag et al., 2018),as well as in testes (Alkis et al., 2019a). Hippocampus is the primary region of the brain which regulates learning, memory, and behavior (Rubin et al., 2014). Studies reported the evidence in human model of neurodevelopmental or behavioral disorder in children (Divan et al., 2008), altered brain metabolism (Volkow et al., 2011), brain electrical activity (Schmid et al., 2012), DNA damage in peripheral blood lymphocytes (Zothansiama et al., 2017), risk of brain tumor (Carlberg and Hardell, 2017), fatigue, depression, and headaches (Yakymenko et al., 2011). However, microwave exposure affects neurodevelopment and behavior in mice (Aldad et al., 2012), oxidative stress, apoptosis of glial cells (Alkis et al., 2019b; Dasdag et al. 2004, 2009), amyloid protein, protein carbonyl (Dasdag et al., 2012), male fertility (Kesari et al., 2018), neuro inflammations (Megha et al., 2015a) cognitive functions, (Deshmukh et al., 2015; Kleinlogel et al., 2008), and micro RNA expression (Dasdag et al. 2015a, 2015b) in rats, but neither study has reported for epigenetic modulations with microwave exposure.

Our prior study reported that mobile phone signal exposure impairs cognitive functions (Deshmukh et al., 2015), heat shock protein modulation (Deshmukh et al., 2012), neurotransmitter alteration (Megha et al., 2015b), oxidative stress (Alkis et al., 2019b; Megha et al., 2012), and ER-stress in rat brain (Kumar et al., 2019), prompted us to speculate

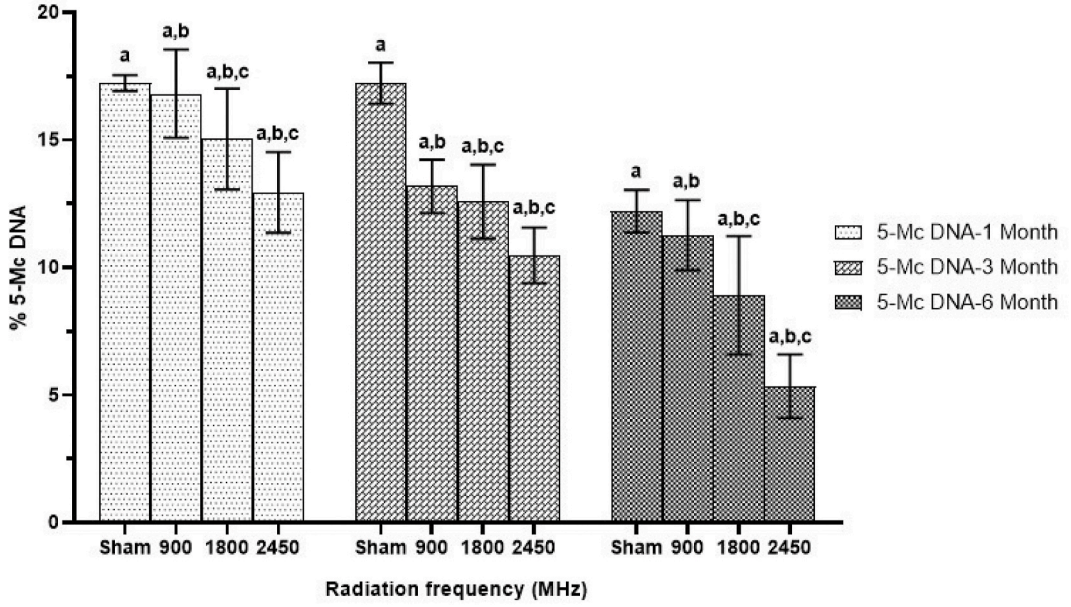


Fig. 4. Percentage methylation of 5-methyl cytosine DNA (5-mC-DNA) in rat brain. *Values are expressed as mean ± SD (8 animals per group).* Respective p-value *a,b,c significantly different from respective control ($p < 0.05$) calculated by one way ANOVA followed by Tukey's test.*

that above said changes in neuronal development and behavior crucially driven by epigenetic modulations. We explored our speculation in the present study by evaluating whether mobile phone signal exposure (900–2450 MHz) modulate DNA/histone methylating enzymes and DNA/histone methylation in the hippocampus of Wistar rat. Mobile phone radiofrequency microwave radiation led to significant fold change in euchromatic histone methyltransferase1 EHMT1 (Fig. 1), DNA methyltransferase1 DNMT1 (Fig. 2) enzyme as apparent by hypermethylation of histone, H3K9 (Fig. 3) and hypomethylation of DNA, 5-mC (Fig. 4) at selected mobile phone signal radiofrequency microwave.

We previously reported that radiofrequency microwave exposure of rat to 900–2450 MHz for one to six month exposure induces cognitive impairment. In this study, we extended our observations by elucidating whether radiofrequency microwave exposure induces epigenetic modulations with augmented levels of methyltransferase (DNMT1 and EHMT1) enzymes in the hippocampus compared to sham-exposed rats. Being hippocampus is crucially involved in learning, memory, and behavior, microwave-induced epigenetic modifications of the hippocampus more likely the causative factor.

Hippocampus is the vulnerable and sensitive target of mobile phone signal radiation which may deficits learning and memory (Zhao et al., 2012). However, no data available on the microwave exposure risk to epigenetic modulations. Cognitive functions including long-term/short-term memory are a unique feature of the healthy brain. Differential gene expression changes protein synthesis in memory-related regions of the hippocampus (Bailey et al., 2004). Altered synaptic properties propagate through persisting molecular changes which translate into changes in memory and memory recall processes. Epigenetic modulations (DNA/histone methylation) around gene promoters induce changes in gene expression thus causing cognitive and memory dysfunction (Barroso and Chevet, 2016; Ramos-Lopez et al., 2018). We observed increased H3K9 methylation and euchromatic histone methyltransferase1 enzyme with respect to sham exposed rat, similar observation is reported by Iacono et al., who showed increased H3K9 methylation associated with cognitive dysfunction in mice (Iacono et al., 2018). The ability of learning and memory depends on the transient translation of gene expression which influences synapse activity and connectivity of neurons. Histone methylation under the control of EHMT1 expression, which brings chromatin remodeling (Lagali et al., 2010). EHMT1 enzyme induces histone methylation at lysin residue, which modifies chromatin structure and disturbs gene regulatory networks that affect learning and memory (Koemans et al., 2017). Chromatin is a highly dynamic biomolecule which is readily modulated under the influence of internal or external stimuli. Epigenetic modulations bring reversible covalent modifications of histone or DNA. Epigenetic modulations of histone and DNA modulate DNA–histone interactions and the access of DNA replication and transcription complex, which functions as a transcription gatekeeper that brings cell-specific gene activation or repression (Ng et al., 2009; Parkel et al., 2013). H3K9 methylation is a repressive mark for gene activation (Hathaway et al., 2012) and depressive behavior in animal models as well as in humans (Tsankova et al., 2006). Jarome and Lubin (2013), reported the association of histone methylation in neurodegeneration in mouse brain due to chromatin structure disturbance and blockade of normal gene expression via disruption of transcription regulatory network (Graff et al., 2012; Jarome and Lubin, 2013).

Studies have reported that learning triggers change in DNA methylation in the hippocampus (Lubin et al., 2008). DNA methylation brings transcriptional activation in the adult central nervous system (Chahrour et al., 2008; Suzuki and Bird, 2008). In the present study, we observed decreased DNA methylation and DNMT1 enzyme in microwave exposed rats with respect to sham-exposed rats. We also observed decreasing DNA methylation with increasing frequency (900 MHz–2450 MHz) and with increasing duration of exposure (one-month to six-month exposure group). DNMT1 enzyme is the only maintenance methyltransferase enzyme that maintains and regulates cellular epigenome. It is vital for native chromatin structure as well as embryonic development and neuronal survival (Baets et al., 2015). DNA demethylation hampers the binding of the methyl-CpG binding protein, MeCP2 at the *Zif268* promoter. Binding of MeCP2 with methylated DNA and cyclic adenosine monophosphate response element binding-1 (CERB-1) protein, regulates the transcription of the gene (Chahrour et al., 2008). Lubin et al. reported that the cAMP response element-binding site residing around the *Zif268* DNA promoter region is methylated in adult hippocampal neurons. Learning induces DNA methylation which regulates the transcription of brain-derived neurotrophic factors (BDNF) (Lubin et al., 2011). Repression of DNA methylation via infusion of the DNA methyltransferase1 inhibitor in the hippocampus brings the differential regulation of BDNF variants and reduces the process of learning and memory (Lubin et al., 2008). DNA repair process also mediates active DNA demethylation in the adult hippocampus which involves enzymes removing the methyl group from 5-methylcytosine or removing the whole nucleotide (Kangaspeska et al., 2008; Ooi and Bestor, 2008). Growth arrest and DNA-damage inducible gene 45 alpha (GADD42A), a key regulator of DNA demethylation are also crucially involved in DNA repair, maintenance of genomic stability and cell cycle checkpoints. Several study reports support that DNA demethylation is actively dependent on daily activity and DNA demethylation imparts another level of complexity in the epigenetics of neurobiology (Lubin, 2011). Hence, it also opens a new window with a potential mechanism for the manipulation of DNA demethylation specifically in the restoration of cognitive functions where DNA methylation profile is altered.

Epigenetic modifications play a crucial role in the regulation of cognitive and learning-dependent synaptic plasticity (Gupta et al., 2010; Jiang et al., 2008; Lubin et al., 2011). However, epigenetic modulations are not the only mechanism but also influences several other mechanisms through chromatin structure regulation and transcription and translation machinery via ER-stress and unfolded protein response. DNA methylation in concert with histone methylation redirects the microenvironment of gene promoters and influences transcription of the genes in the hippocampus of the rat brain (Barrett and Wood, 2008; Graff and Mansuy, 2008).

5. Conclusion

In summary, 900–2450 MHz for 2 h daily for one-month, three-month and six-month exposure led to hypermethylation of histone (H3K9) protein by upregulating euchromatic histone methyltransferase1 enzyme, whereas hypomethylation of DNA (5-Mc) by down-regulating DNA methyltransferase1 enzyme with increasing microwave frequency as well as exposure duration of Wistar rat. Band 3 (1800 MHz) is widely used frequency band for communication. Higher frequency corresponds to higher energy, hence more adverse effect than 900 MHz frequency but lower than 2450 MHz, which we also observed in this study in the form of epigenetic modulations, gene expression as well as in protein expression in experimental Wistar rats. We found maximum bio-molecular alteration in six-month exposure group at 2450 MHz frequency, as it possesses maximum energy. Higher frequency and longer duration of exposure responsible for the larger changes in biomolecular characteristics. Hence 2450 MHz frequency for six month of exposure is the most effective frequency and duration to impart health hazards. The result may deliver insight into the pathway of cognitive impairment induced by mobile phone signal radiofrequency, which may be useful for assessment of mobile phone radiation risk in mental health and setting guidelines for policy makers.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2020.110297.

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