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Exposure to radio-frequency electromagnetic waves alters acetylcholinesterase gene expression, exploratory and motor coordination-linked behaviour in male rats

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ABSTRACT

Humans in modern society are exposed to an ever-increasing number of electromagnetic fields (EMFs) and some studies have demonstrated that these waves can alter brain function but the mechanism still remains unclear. Hence, this study sought to investigate the effect of 2.5 Ghz band radio-frequency electromagnetic waves (RF-EMF) exposure on cerebral cortex acetylcholinesterase (AChE) activity and their mRNA expression level as well as locomotor function and anxiety-linked behaviour in male rats. Animals were divided into four groups namely; group 1 was control (without exposure), group 2–4 were exposed to 2.5 Ghz radiofrequency waves from an installed WI-FI device for a period of 4, 6 and 8 weeks respectively. The results revealed that WiFi exposure caused a significant increase in anxiety level and affect locomotor function. Furthermore, there was a significant decrease in AChE activity with a concomitant increase in AChE mRNA expression level in WiFi exposed rats when compared with control. In conclusions, these data showed that long term exposure to WiFi may lead to adverse effects such as neurodegenerative diseases as observed by a significant alteration on AChE gene expression and some neurobehavioral parameters associated with brain damage.

1. Introduction

The use of wireless technologies such as Wireless Fidelity (Wi-Fi) communication devices have been growing tremendously over the past years in houses, workplaces, public areas, schools among others. However, rapid development of wireless technologies has steadily increased the environmental electromagnetic field (EMF) levels. Public and scientific awareness that was previously focused on the adverse health effects of EMF emitted from mobile phones has shifted to the biological hazards of wireless equipment such as Wi-Fi because the health effects of such equipment are still unclear [33]. The Council of Europe recommends restrictions on the use of mobile phones and internet access in all schools across the continent to protect young children from potentially harmful radiation [18,24].

Therefore, understanding the relationship between electromagnetic fields and health diseases such as neurological disorder is very important for public especially for young children whom utilize wireless internet very frequently during adolescent years. In addition, uncontrolled wireless internet usage can turn into a habit and may continue throughout ones life being unaware of potential harmful effects of electromagnetic fields [15,2].

Acetylcholine (ACh) is a neurotransmitter with an important role in many functions of both the peripheral and central nervous systems acting in the learning and memory processes as well as locomotor control and cerebral blood flow [8,19,11]. The proximal promoter of the AChE gene includes; among others, consensus motifs for the leukemia-associated factorAML1/Runx1 [17,22], and *c-fos*, a transcription factor known to regulate AChE gene expression under stress [17]. Exposure to radiofrequency can happen from a multitude of sources such as smart devices, phones, office gadgets connected to Wi-Fi, desktops, laptops etc which has been found to cause a wide range of biochemical and physiological dysfunctions such as anxiety, obesity, reduced cognitive function etc. It has been shown that the AChE activity is implicated in cell proliferation and neurite outgrowth [7]. AChE is

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expressed in brain tumors including meningiomas, astrocytomas andglioblastomas [26,4]. Research studies have revealed that AChE responds to various insults including oxidative stress, an important event that has been related to the pathogenesis and progression of a variety of central nervous system disorders [7]. Interestingly, AChE levels are found to be very low in all types of normal glia, but increased in astrocytic tumors [30,16]. This hinted the possibility that tumor-specific transcription factors regulate AChE gene expression in astrocytomas. Thus, this enzyme is a target for the emerging therapeutic strategies to treat neurological disorders such as Alzheimer's disease (AD), Parkison disease, hungtinton's disease etc [29].

Due to the significance of AChE in neurological disease and the health implications of WiFi exposure in humans, it is expedient to determine the effect of long-term exposure of this radiation on neurological function. Hence, this study sought to investigate the effect of 2.5 Ghz band radio-frequency electromagnetic waves (RF-EMF) exposure on cerebral cortex acetylcholinesterase (AChE) activity and their mRNA expression level as well as locomotor function and anxiety-linked behaviour in male rats.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals and reagents such as: $MgCl_2$, TRIzol Reagent, Taq DNA polymerase (Invitrogen), 5,5′-dithiobisnitrobenzoicacid, acetylthiocholine iodide were obtained from Sigma-Aldrich (USA), while others used were all of analytical grade. The water used was glass-distilled.

2.2. Animal care

Twenty-four (24) male albino rats (four weeks old and nearly of the same weight) were obtained from the animal breeding unit at College of Medicine, Afe-Babalola University. They were acclimatized for a period of two weeks before subjecting them to experimentation. The animals were provided with standard pellet diet (obtained from ABUAD farm) and were given water *ad libitum*. The handling and use of the animals were in accordance with NIH guildline for the care and use of Laboratory animals. This study was approved by the animal ethical committee of Afe-Babalola University, Ado-Ekiti, Nigeria.

2.3. Electromagnetic field exposure

A signal device, which emits Wi-Fi signals at approximately 2.5 GHz frequency band, was used to represent the exposure system. The design and methodology for exposure to radiation was according to [10]. The radiation-generating device was tested at the laboratory of the Physics and Electronics Engineering Department and was able to create electric field densities from 0.1–45.5 V/m while the maximum output power was 2 W. The electric field density was set at 11 V/m. Rats in the control and exposure groups were placed in a Plexiglas cage (55 × 32 × 20 cm). For the RF exposure group, a shield was used to ensure that no other EMF/RF exposure sources from external environment cause interferences. Rats were free to move with no restriction in the cage during the study. The rats in the control and exposure groups lived in the cage under normal circumstances. Rats in the exposure group were subject to 2.5 GHz RF radiation 24 h/d for 4, 6 and 8 weeks respectively. Rats in both groups were kept 50 cm far away from the antenna of the generator. The same experimental conditions were applied to the rats in the control group, except the irradiation. Electromagnetic power density and the electrical field inside the Plexiglas cage were measured by field probe EMR 300 (data not shown).

2.4. Experimental design

After acclimatization, the animals were divided into four groups (n = 6) where the first group (control group) had no exposure while groups 2–4 were exposed to 2.5 Ghz radiofrequency waves from an installed Wi-Fi device at intervals of 4, 6 and 8 weeks with free access to food and water *ad libitum*. After the treatment period, animals were fasted overnight and euthanized using light ether anesthesia. In this study, the cerebral cortex was isolated from the whole brain and analyzed for key enzyme of the cholinergic system.

2.5. Behavioral study

On the final day of the exposure, animals were subjected to neurobehavioral study to assess the locomotor activity and anxiety level of the experimental animals.

2.5.1. Open field test

The analysis of locomotor activity of rats was measured by the open-field test (OFT). The animals were placed in an open field container (4 × 4 × 40 cm dimensions), with its floor divided to 16 equal-sized squares. A video camera was placed on the top of the apparatus at 120 cm heights for video typing. Each animal was placed in the apparatus and after 5 min for habituation; its activity was recorded for 10 min. The types then were analyzed for locomotion (number of line crossing) by an independent trained observer without the knowledge of the experiment.

2.5.2. Rotarod test

The animals were placed on the rotating rod (Rotarod) at 34 rpm to measure passive rotation and time it takes for animal to fall off the rotating rod which is used as an indicator of motor coordination level in animal model.

2.6. Biochemical evaluation

2.6.1. Determination of acetylcholinesterase (AChE) activity

The AChE enzymatic assay was determined using a modification of the spectrophotometric method as previously described by [22]. The reaction medium (2 mL final volume) contained 100 mmol/L of K^+-phosphate buffer, pH 7.5, and 1 mmol/L of 5,5′-dithiobisnitrobenzoic acid. The method is based on the formation of the yellow anion, 5,5′dithio-bis-acid-nitrobenzoic, measured by absorbance at 412 nm during 2 min incubation at 25 °C. The enzyme (40–50 μg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mmol/L of acetylthiocholine iodide. All samples were run in triplicate, and enzyme activity was expressed in μmol./mg protein.

2.6.2. Real-time reverse transcription polymerase chain reaction (RT-qPCR)

The analysis of AChE mRNA expression was carried out by a two-step quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay. Total RNA of the cerebral cortices of experimental animals were extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. The total RNAs extracted were quantified using NanoDrop 2000 spectrophotometer and the ratio of OD28/OD280 of all extracted RNA samples was between 1.8 and 2.0. For reverse transcription (RT), first strand complementary DNA (cDNA) was synthesized from RNA by using a cDNA synthesis kit (Maxima H Minus First Strand cDNA Synthesis Kit) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction was performed in 20 μL reaction volumes containing 2 μL RT product (cDNAs) as template, 1X PCR buffer, 25 μM dNTPs, 0.2 μM of each primer (Table 1), 1.5 mM $MgCl_2$, 0.1X SYBR Green I (molecular probes), and 1U Taq DNA polymerase (Invitrogen).The thermal cycle was carried out using a Step One Plus real-time PCR system (Applied Biosystems, NY) according to the following protocol: activation of the Taq DNA polymerase at 95 °C for 5 min, followed by 40 cycles of 15 s at 95 °C, 15 s at 8 °C, and 25 s at 72 °C. Threshold and baselines were

Table 1
The sequences of oligonucleotide primers used for real-time RT-qPCR analysis.

Gene	Primer	Product size (bp)
AChE	F: CGCACCCCAGCCAGGAACTG R: GCCTCCGTGGGCATGCACAT	466
β-actin	F: AGCAAGAGAGGCATCCTGAC R: GTGGTACGACCAGAGGCATA.	268

manually determined using the StepOne Software version 2.0 (Applied Biosystems,NY). SYBR fluorescence was analyzed by StepOne software version 2.0 (Applied Biosystems, NY), and the CT (cycle threshold) value for each sample was calculated and recorded using $2^{-\Delta\Delta CT}$. The mRNA levels of the AChE were normalized against β-actin which serves as the endogenous reference genes. Specific β-actin primers were used for the internal control to normalize the sample amounts.

2.6.3. Protein content

The protein concentration in the tissue of the animals was assayed as described by Randox kits using serum albumin as standard.

2.7. Statistical analysis

All data were expressed as mean ± standard error of mean (S.E.M.). The statistical analysis used was one-way ANOVA, followed by the post hoc Tukey's test, where $p < 0.05$ was considered to represent a significant difference.

3. Results

3.1. Rotarod test (Anxiety index investigation)

The motor coordination level was monitored using Rotarod test and the result is presented in Table 2. According to Table 2, exposure to Radio frequency-electromagnetic wave (RF-EMW) caused a significant increase in the level of coordination as observed by a reduction in the latency of fall duration when compared with the control rats.

3.2. Open field test (exploratory index investigation)

The analysis of locomotor activity of rats using open field test inRadio frequency-electromagnetic wave (RF-EMW) exposed rats is presented in Table 3. The result revealed that, control had the highest exploratory activity, however, exposure to Radio frequency-electromagnetic wave (RF-EMW) caused a significant decrease in the locomotor activity as revealed by the lines crossing frequency when compared with the control rats.

Table 2
The latency of fall duration and number of passive rotation in control and Radio frequency-electromagnetic wave (RF-EMW) exposed rats using rotarod test.

Grouping	Latency of fall (s)
Group 1	11.92 ± 3.31[a]
Group 2	6.41 ± 1.42[b]
Group 3	3.98 ± 0.36[c]
Group 4	3.89 ± 0.36[c]

Values represent mean ± SEM (n = 6).
Values with the same alphabets along the column and not significantly different while different alphabet along the column are statistically significant ($P < 0.05$).
Key:
Group 1: Rats without exposure to Radio frequency-electromagnetic wave (RF-EMW).
Group 2: Rats exposed to Radio frequency-electromagnetic wave (RF-EMW) for four (4) weeks.
Group 3: Rats exposed to Radio frequency-electromagnetic wave (RF-EMW) for six (6) weeks.
Group 4: Rats exposed to Radio frequency-electromagnetic wave (RF-EMW) for eight (8) weeks.

Table 3
Line crossing frequency pattern as revealed by open field test result of control and Radio frequency-electromagnetic wave (RF-EMW) exposed rats.

Grouping	No of lines crossed
Group 1	92 ± 11.92[a]
Group 2	74 ± 10.40[b]
Group 3	50 ± 2.76 [c]
Group 4	15 ± 3.95 [d]

Values represent mean ± SEM (n = 6).
Values with the same alphabets along the column and not significantly different while different alphabet along the column are statistically significant ($P < 0.05$).
Key:
Group 1: Rats without exposure to Radio frequency-electromagnetic wave (RF-EMW).
Group 2: Rats exposed to Radio frequency-electromagnetic wave (RF-EMW) for four (4) weeks.
Group 3: Rats exposed to Radio frequency-electromagnetic wave (RF-EMW) for six (6) weeks.
Group 4: Rats exposed to Radio frequency-electromagnetic wave (RF-EMW) for eight (8) weeks.

3.3. Acetylcholinesterase (AChE) activity in exposed rats

The cerebral cortex acetylcholinesterase (AChE) activity was monitored in control and WiFi exposed rats. As presented in Fig. 1, there was a significant ($P < 0.05$) decrease in AChE activity in exposed rats when compared with the control rats.

3.4. AChE gene expression level in exposed rats

There was a significant ($P < 0.05$) increase in AChE mRNA expression level in rats exposed to RF-EMW when compared to the control rats as presented in Fig. 2. Although no significant difference was observed for 4 weeks expose rats when compared with control.

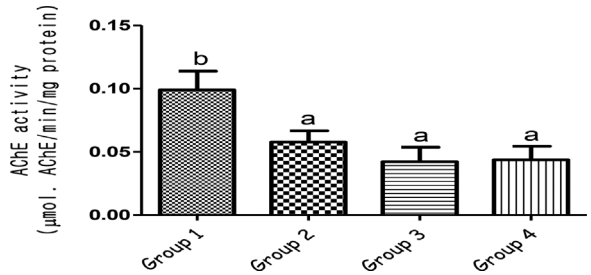

Fig. 1. Effects of 2.5 Ghz Radio-frequency electromagnetic waves (RF-EMW) exposure on the acetylcholinesterase (AChE) activities in cerebral cortex of rats.
Each bar represent mean ± SEM (n = 6).
Bars with the same alphabets are not significantly different at $P < 0.05$.
Key:
Group 1: Rats without exposure to Radio frequency-electromagnetic wave (RF-EMW)
Group 2: Rats exposed to Radio frequency-electromagnetic wave (RF-EMW) for four (4) weeks
Group 3: Rats exposed to Radio frequency-electromagnetic wave (RF-EMW) for six (6) weeks
Group 4: Rats exposed to Radio frequency-electromagnetic wave (RF-EMW) for eight (8) weeks

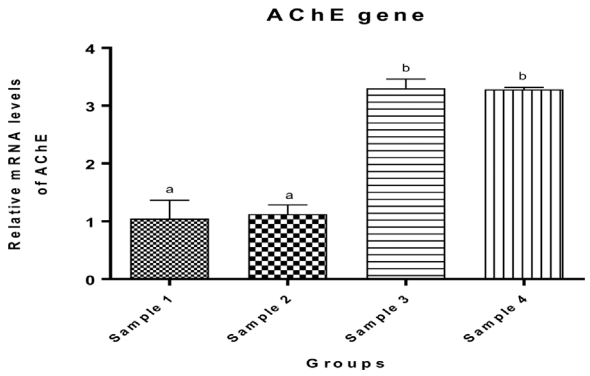

Fig. 2. Effect of 2.5 Ghz Radio-frequency electromagnetic waves (RF-EMW) exposure on relative mRNA levels of AChE in rats.
Each bar represent mean ± SEM (n = 6).
Bars with the same alphabets are not significantly different at P < 0.05.
Key:
Sample 1: Rats without exposure to Radio frequency-electromagnetic wave (RF-EMW)
Sample 2: Rats exposed to Radio frequency-electromagnetic wave (RF-EMW) for four (4) weeks
Sample 3: Rats exposed to Radio frequency-electromagnetic wave (RF-EMW) for six (6) weeks
Sample 4: Rats exposed to Radio frequency-electromagnetic wave (RF-EMW) for eight (8) weeks

4. Discussion

Given the wide use of wireless technologies in everyday life, whether RF-EMF exposure affects brain development is a major concern. During brain development, any environmental stimuli that influence cholinergic neurotransmission may disturb learning and memory process. In the present study, WiFi exposure alters some neuro-behavioral function associated with neurological diseases. As presented in both Tables 1 and 2, WiFi exposure affect motor coordination and alters exploratory motor function in exposed rats when compared to control. This is in agreement with previous studies where high radiofrequency electromagnetic radiation exposure induces cognitive impairment and stress-related behaviours in rats [13,25,34]. This effect has been linked to the ability of radiowaves to cross the blood brain barrier (BBB) due to their selective permeability and ionization properties [31]. This is an indication that electromagnetic field exposure could result in neurodegeneration.

Electromagnetic field exposure has been reported to change protein activity both inside and/or outside the cell [20,21]. The biological effects of electromagnetic fields may lead to exposure and alternation in biological molecules structure [1]. Whenever these changes over pass from the range of normal variations, or physiological compensatory mechanisms, can alter cell behaviours,

Previous studies have shown a strong relationship between radiation and cholinergic system [8,11]. Acetylcholine (ACh) is a neurotransmitter of the cholinergic system and plays a vital role in many functions of both the peripheral and central nervous systems acting in the learning and memory processes as well as locomotor control and cerebral blood flow [8,19,11]. This neurotransmitter (ACh) is usually degraded in the synaptic cleft by acetylcholinesterase (AChE), an enzyme that convert acetylcholine to inactive form, choline and acetate. The inhibition of this enzyme has been implicated in neurodegenerative diseases. As presented in Fig. 1, WiFi exposure inhibited AChE activity when compared with control. This will result in over-activation of ACh in the CNS and thus lead to neurotoxicity in exposed rats. This finding is in agreement with Mahdavi et al. (2014), where ACh neurotransmitter is alter as a result of electromagnetic radiation (1 and 5 Hz radiation) exposure in male rats.

Furthermore, in this study, WiFi exposure activates AChE gene expression level when compared with control (Fig. 2). It has been reported that AChE expression in normal brain is regulated by transcription factors such as *c-fos* and *HNF*3α/β which are shown to control neuronal AChE expression under psychological and chemical stress conditions [17,28]. The observed alteration of AChE mRNA expression levels in exposed rats is in line with previous studies which ascertains cholinergic/inflammatory responses and molecular damage in the brain cells as stimulated by the microwave exposure [12]. Also, AChE levels have been found to be very low in all types of normal glia but increased in astrocytic tumors [16]. Hence, the link between radio frequency exposures to various brain tumorigenesis. However, there is need for the proper understanding of the pathogenesis via investigations of genes and proteins involved in brain tumors before conclusions can be drawn. In addition, a significant decrease in AChE enzymatic activity was observed here (Fig. 1), this result does not corroborates with AChE gene expression where an increase was observed. This implies that post-translational modification may be involve in the regulation of AChE synthesis [6].

Oxidative stress alters the neurotransmission and neuronal function. It is important to note that enzymes such as AChE are significant components of the biological membranes and, thus, can be important targets of oxidation of membrane caused by radiation exposure [9]. These results allow us to infer that, these alterations may explain the changes in the activities of this enzyme, its mRNA levels in the brain tissues and, consequently, impairment of anxiety index observed in exposed rats used in this study.

5. Conclusion

In conclusion, these results improve our understanding of the potential adverse effects of RF-EMF exposure on developing rat brain and suggest some possible mechanisms of RF-EMF neurotoxicity via alteration of AChE activity and gene expression level.

Conflict of interest

The authors declare no conflict of interest on the work whatsoever.

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