Zagazig University Medical Journal
www.zumj.journals.ekb.eg
https://doi.org/10.21608/zumj.2024.340955.3713 Volume 31, Issue 2, FEB. 2025, Supplement Issue
Manuscript ID ZUMJ-2412-3713
DOI 10.21608/zumj.2024.340955.3713

ORIGINAL ARTICLE

Effects of Vitamin D on the Cognitive Functions in Rats Exposed to Wi-Fi Radiation: Role of Autophagy and CREB Gene

Eman R. Abozaid, Abeer A Khalefa*, Nawal K. Gerges, Hend M Eissa
Medical Physiology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt

Corresponding author:
Eman R. Abozaid

E-mail
eman.zaher2005@gmail.com

Submit Date 03-12-2024
Revise Date 17-12-2024
Accept Date 22-12-2024

ABSTRACT

Background Exposure to electromagnetic radiation (EMR) is closely associated with neurobehavioral disorders. Vitamin D (Vit.D) has neuroprotective functions. This work aimed to clarify the effect of Vit. D3 on the cognition of rats exposed to wireless fidelity (Wi-Fi) and explore the possible underlying mechanisms.

Background Exposure to electromagnetic radiation (EMR) is closely associated with neurobehavioral disorders. Vitamin D (Vit.D) has neuroprotective functions. This work aimed to clarify the effect of Vit. D3 on the cognition of rats exposed to wireless fidelity (Wi-Fi) and explore the possible underlying mechanisms.

Method Forty male albino rats were divided into 4 equal groups: normal control (NC), NC+Vit.D, Wi-Fi, and Wi-Fi+Vit.D. The rats in NC+Vit.D and in Wi-Fi+Vit.D groups were treated with Vit.D3 (500 IU/kg) orally once daily for 40 days, the Wi-Fi and Wi-Fi +Vit.D groups were subjected to EMR of 2.45 GHz for two hours per day for 40 days. Rat neurobehavior tests were done then isolation of hippocampus was performed to assay hippocambal tumor necrosis factor-α (TNF-α), superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), and malondialdehyde (MDA). apoptosis-related gene (BAX and BCL2), cyclic AMP-response element binding protein (CREB), autophagy genes of LC3 and P62 expressions were assessed. Immunohistochemistry for caspase-3 and histopathology of the rat hippocampus were examined.

Results Wi-Fi exposure induced anxiety and memory dysfunction in rats associated with histological signs of neuronal degeneration in their hippocampus, Treatment with Vit.D significantly improved cognitive dysfunction and decreased neuronal damage. The Wi-Fi group showed significantly higher MDA, TNF-α levels, caspase-3 immune reactions, and *BAX*, *P62*, and *BAX/BCL-2* ratio expression. However; a significant decrease in GSH, SOD, CAT, and expression of BCL2, *CREB*, and *LC3*. Treatment with Vit.D significantly reversed the parameters mentioned above.

Conclusions Administering Vit.D3 can protect against cognitive impairment associated with Wi-Fi radiation exposure.

Keywords: EMR; Neurobehavior disorders; Vitamin D.

INTRODUCTION

The usage of mobile phones and wireless fidelity (Wi-Fi) devices has increased worldwide recently, with potential health hazards. [1, 2]. Several devices can emit radiation with a 2.45 GHz or 5 GHz frequency range, such as wireless routers and access points, old smartphones/tablets, Bluetooth devices. The biological effects of electromagnetic radiation (EMR) can be classified as thermal and non-thermal. Thermal effects are caused by an energy transfer, resulting in a rise in temperature. However, there is no significant thermal effect on the brain or other organs in the body related to Wi-Fi exposure [3]. While the non-thermal effects are attributed to EM field interactions with neural function, which can induce oxidative stress, neurotransmitter levels alteration, and disrupt cellular communication[4].

Data suggest that the EMR exposure results in cognitive dysfunction and memory deficits [5]. The hippocampus is the brain part that controls cognition and behavioral functions; it is vulnerable to EMR exposure [6]. In the hippocampus, the CA3 region has more extensive neural connection than other hippocampal regions, with a specific role in memory processes, and is highly susceptible to neurodegeneration [7].

Exposure to 2.45 GHz Wi-Fi has been suggested to induce oxidative stress in biological systems [8].This increases the production of reactive oxygen species (ROS) in cells. Elevated ROS can damage cell components like proteins, DNA, and lipids. As previously described, EMR mediated oxidative stress in the heart and gonads, with subsequent inflammation and apoptosis [9, 10].

ROS are the main mediators proposed to mediate the non-thermal effects of EMR, specifically on the nervous system. During such stresses, there are compensatory cellular protective mechanisms; autophagy is a major protective proteolytic system that faces cellular stresses. It can deal with any intracellular damage through sequestration of abnormal proteins and dysfunctional organelles. It was reported that autophagy was involved in rat hippocampal neuronal damage following radiation exposure [11, 12].

Furthermore, targeting neurotrophic protein is a possible mechanism related to the harmful effect of EMR on the brain. A study indicated that EMR affects the cyclic AMP-response element binding protein (*CREB*) gene expression in the rat hippocampus [13]. *CREB* is considered an important transcription protein, modulating the expression of other genes necessary for the integrity and proliferation of neurons in addition to the consolidation of memory [14]. Interestingly, Seok et al. [15] observed a possible link between *CREB* and autophagy gene expression in the hepatic cells of mice.

Vitamin D (Vit.D) is considered as a neuroprotective steroid with antioxidant and anti-inflammatory properties, in addition to its role in calcium-phosphate homeostasis and bone metabolism [16]. It was observed that Vit.D receptors and the enzymes contributing to its metabolism are widely distributed in the nervous tissue. It was evidenced that Vit.D is associated with cognitive preservation, increasing attention and memory functions, and inhibiting neural deterioration in models of Alzheimer's disease, multiple sclerosis and lipopolysacharide-induced inflammation [17], interestingly. a link between Vit.D intake and autophagy enhancement in retonone-treated rats was observed [19]. Moreover, Nadimi et al. [20] observed that Vit.D has increased CREB phosphorylation in the hippocampus in diabetic rats. However, the effect of CREB gene or its molecular pathway are not clear .

Although Vit.D was observed to be neuroprotective in different neurodegenrative models, however the role of Vit.D on cognition during the exposure to EMR is still unclear and need more clarification. So, in this study, we aimed to clarify the effect of Wi-Fi exposure on rat cognition and the possible ameliorative effect of Vit. D administration on cognitive functions in rats exposed to Wi-Fi radiation and to study the possible underlying mechanisms including hippocampal apoptosis, inflammation and oxidative states, autophagy, and CREB involvement.

2 Methods

2.1 Experimental animals

Forty male albino rats; 160–180 g weight, 8-week-old , were housed in plastic cages (size 50x50x60 cm) , 5 rats per cage. kept at 20-24 ºC with a 12-hour dark-light cycle; free access to chow and water was allowed. Acclimatization to the animal behavior lab for one week before the study was done [21].The experimental protocol was approved by the Zagazig University institutional animal care unit committee (ZU-IACUC; Sharkia; Egypt) with approval number (ZU-IACUC/3/F/39/2021).

2.2 Animal grouping and study design

Rats were randomly divided into 4 equal groups: Normal control group (NC) (n=10) Rats were subjected to a Wi-Fi router, but the device was turned off. NC+Vit.D group (n=10) Rats were treated with Vit.D3 with 500 IU/kg by oral gavage once daily for 40 days [22] and subjected to a Wi-Fi router, but the device was turned off. Wi-Fi group (n=10) Rats were subjected to a non-ionizing EMR of 2.45 GHz for two hours per day, from 12:00 to 14:00 for 40 days [23]. Wi-Fi +Vit.D group (n=10) Rats were subjected to Wi-Fi radiation and treated with Vit.D using the methods mentioned above.

2.3 Wi-Fi setup exposure

Animals were exposed to EMR from a centrally positioned Wi-Fi device (about 25 cm from the rat cages), ensuring adequate space and environmental control for temperature and humidity, the head and body of animals were subjected to microwave radiation with a power density of 0.016 mW/cm2 and a specific absorption rate of 0.017 W/kg for 2 hours/day. Manually, the modem was switched off and on at constant time daily. Measure the Wi-Fi signal strength (mW/m²) at the location where the rats were housed using a local Wi-Fi signal

strength meter. The Wi-Fi signals were picked up directly by a specific access point for indoor use. It supports wireless networking speeds of up to 150 Mbps with the public frequency 2.45 GHz for two hours/day over 40 days [23].

2.4 Vit.D supplementation

Vit.D3 Cholecalciferol (Vidrop; Medical Union Pharmaceuticals, Ismailia, Egypt) was used; it was diluted in corn oil and was given daily by oral gavage (500 IU/kg/day) to rats in the NC+Vit.D and Wi-Fi + Vit.D groups [22]. At the exact time, NC and Wi-Fi-exposed rats were given oral corn oil by gavage for 4o days.

2.5 Neurobehavioral studies

Neurobehavioral tests were conducted at the last week of the study, it began at the day 33 of the study and continued for one week .The following tests were done:

2.5.1 Open field test (OFT).

It tested anxiety. The open field maze was used; it consisted of one big square (its side is 1 meter long); its floor was divided into smaller 25 squares (20 cm side length). The test was done according to Briones-Aranda et al. [24]. The squares number that was entered by each rat, rears number, latency to move and to rear, and the numbers of fecal boli were assessed.

2.5.2 Modified T-maze test

It tested working memory and used a modified spontaneous alternation T-maze. A manual T-maze, which has a central part with a guillotine door at each goal arm, was used. The test was done according to Wu et al. [25]. For all rats, one sample trial and five choice runs were allowed per day for two days, with a total of 12 trials for each rat with a total of 10 possible alternations. The percentage or proportion of correct choices (alternations) per rat was calculated as follows:

$$\frac{\text{Number of correct choices (Alternations)}}{\text{Total possible alternations}} \times 100$$

2.5.3 Modified Barnes Maze test

It assessed spatial learning in rats. A circular apparatus 122 cm with a wide table having forty random holes. One target hole that leads to the escape box. Habituation was done for all rats before the test. Then three target sites were tested, differing in their distance from the center. Rats were allowed for 3 trials per day to reach the target. After the acquisition trial, a probe test was done by removing the escape box. For each trial, the number of errors and the latency to reach the escape box were recorded according to Morel et al. [26].

2.6 Hippocampal dissection and tissue preparation

After 40 days from the beginning of the study, the rats were sacrificed by decapitation under urethane injection (1.2 g/kg IP) [27], the brain was dissected and rinsed in ice-cold saline, and the hemisphere was cut to bihalfs. To expose the hippocampus, the olfactory bulb and the frontal cortex were cut, then the ventral side of the brain and the midbrain were dissected. The hippocampus was extracted and divided into two parts. One part was prepared for tissue homogenate for biochemical and gene analysis; this first part of the hippocampal specimens was homogenized with ice-cold phosphate-buffered saline. Then, centrifuged at 4,000–6,000 RPM for twenty minutes, the supernatant was obtained [28]. The second part was immediately placed in formalin 10% for histopathology and immunohistochemical study of the CA3 region.

2.7 Hippocampal oxidative and inflammatory markers assay

Assessment of malondialdehyde (MDA) and the tumor necrosis factor-α (TNF-α) levels in the hippocampus by using enzyme-linked immunosorbent assays (ELISA) (catalog numbers MBS727531 and CSB-E11987, respectively, Egyptian Company for Biotechnology, Cairo, Egypt). Reduced glutathione (GSH) levels, superoxide dismutase (SOD), and catalase (CAT) activities in the hippocampus were assessed by ELISA kits (catalog numbers CSB-E12144r, CSB-E08555r, and MBS2600683, respectively; Biodiagnostic Company for Biotechnology, Dokki, Giza, Egypt), all procedures were performed according to the manufacturer's protocols.

2.8 Gene expression analysis

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) was used for analysis of hippocampal *BAX*, *BCL2*, *P62*, microtubule-associated protein light chain 3-II (*LC3-II*), and *CREB* mRNA expressions. RNAs were extracted from hippocampal tissue homogenate using GENEzolTM Reagent. The RNAs were measured by nanodrop spectrophotometry (ND 1000-NanoDrop®). Reverse transcriptions were done by the TOPscriptTM cDNA Synthesis Kit (Enzynomics). Mx3005P RT-PCR Systems (Agilent Stratagene, USA) were used for the RT-PCR. In the PCR cycling, the samples were run in triplicates. The primers of oligonucleotide were obtained from Sangon Biotech (Beijing, China). The levels of the target gene expressions were neutralized with the mRNA expressions on the housekeeping genes, *GAPDH*. Data were presented as fold changes in comparison to control groups according to the $2^{-\Delta\Delta CT}$ method (Table 1).

2.9 Histopathological study

The second part of the hippocampal specimens was immediately placed in formalin 10%, then dehydrated by alcohol, and embedded in paraffin, with multiple sectioning for five µm in thickness, followed by Hematoxylin-Eosin staining [29].The intact neurons were defined as round-shaped cells with an intact cytoplasmic membrane, without any nuclear condensation or distorted aspect. The degenerated cells were defined with small and shrunken appearance, with condensed small darkly stained nucleus. The pyramidal cells in the hippocampal CA3 region were examined at high magnification (400×).

2.10 Immunohistochemistry

After being placed in formalin, the hippocampal sample was put in paraffin, and 5-µm sections were prepared for immunohistochemical study [30]. The sections were incubated with 3% H2O2 for 5 minutes, followed by phosphate-buffered saline for 5 minutes. The sections were then treated with primary antibodies for 1 day at 4 °C and thereafter incubated with a secondary antibody for 10 minutes. The rabbit polyclonal antibodies against rat brain active caspase-3 (ab2302; Abcam, Cambridge, USA) were used. Slides were photographed by an electric light microscope (Leica ICC50 W) at the Image Analysis Unit of the pathology department.

2.11 Morphometric analysis

The percentages of surviving cells in H&E section of CA3 region and the percentage area of caspase-3 positive cells were measured by Automated Cell Counting with ImageJ/Fiji (NIH, USA) software program. Quantitative data was evaluated in 8 non-overlapped high power random fields for same hippocampal CA3 area (magnification 400).

STATISTICAL ANALYSIS

SPSS version 20 for windows (SPSS Inc, Chicago, IL, USA) was used for data analysis. Comparisons were obtained with one-way ANOVA with post hoc analysis using Tukey test. Modified Barnez maze test was evaluated using repeated measures two- way anova followed by post hoc analysis using Tukey test. Data were expressed as mean ±SD. The level of significance in all experiments was considered $p < 0.05$.

RESULTS

3.1 Open field test (OFT)

Wi-Fi exposure led to a marked decline in the numbering of rears and squares that were entered by each rat, with a marked increase in latency to rear and to move and fecal boli number compared to controls ($p< 0.001$). However, Wi-Fi-exposed rats treated with Vit.D showed a significantly higher number of rears and squares that were entered by each rat ($p<0.05$), associated with a marked decrease in latency to move and to rear and fecal boli numbering compared to those in the Wi-Fi group ($p < 0.001$) (Figure 1).

3.2 Modified T-maze and Barnes maze test

Wi-Fi exposure led to a significant reduction in the score of the T maze in comparison to controls ($p < 0.001$), implying working memory deficits. However, Wi-Fi-exposed rats treated with Vit.D showed significantly higher scores than those in the Wi-Fi group ($p < 0.001$) (Figure 1). In the Barnes maze test, the rats in the Wi-Fi group spent more time and displayed more error numbers to reach the escape tunnel in both acquisition trials and probe trials compared to the control groups ($p < 0.001$). However, Wi-Fi-exposed rats treated with Vit.D showed a significant decline in duration and number of errors in reaching the escape tunnel compared to Wi-Fi-exposed rats ($p < 0.001$) (Figure 2).

3.3 Hippocampus oxidative and inflammatory markers

Wi-Fi exposure significantly decreased the activities of SOD, CAT, and GSH levels in the hippocampus ($p < 0.001$), while significantly increasing the pro-oxidant "MDA" and the inflammatory marker "TNF-α" levels compared to controls ($p < 0.01$). However, Wi-Fi-exposed rats treated with Vit.D showed a significant increase in the activities of SOD ($p< 0.05$) and CAT and GSH levels ($p < 0.001$) while significantly decreasing the MDA and TNF-α levels in comparison to Wi-Fi-exposed rats ($p < 0.001$) (Figure 3).

3.4 Hippocampal *Bax, BCL2, LC3-II, P62* and *CREB* mRNA expression

The proapoptotic BAX expressions were increased, while the anti-apoptotic *BCL-2* expressions were decreased by exposure to Wi-Fi compared to control groups, resulting in an increased *BAX/BCL-2* ratio. Wi-Fi-exposed rats treated with Vit.D showed a significant decrease in *BAX* expressions with an increase in *BCL-2* expressions, resulting in a reduction of *BAX/BCL-2* ratio compared to Wi-Fi-exposed rats ($p < 0.001$) (Figure 4).

The expressions of autophagy markers "*LC3-II"* in the hippocampus were significantly downregulated, while the expression of "*P62*" was upregulated considerably in Wi-Fi-exposed rats compared to controls. However, Wi-Fi-exposed rats treated with Vit.D showed a significant increase in expression *LC3-II* ($p < 0.001$) and drop in *P62* expressions when compared to Wi-Fi-exposed rats ($p < 0.001$) (Figure 4).

CREB expression significantly decreased by exposure to Wi-Fi radiation compared to controls ($p < 0.001$). At the same time, its expression was

significantly elevated in Wi-Fi-exposed rats treated with Vit. D compared to the untreated Wi-Fi-exposed ($p < 0.001$) (Figure 4).

3.5 Histopathology

H&E staining of the hippocampal CA3 region showed small shrunken neuronal cells with pyknotic dark-stained nuclei in the Wi-Fi group, and the percentage of healthy neurons was markedly decreased when compared with the controls ($P < 0.05$). However, Vit.D administration significantly increased the percentage of intact neurons when compared to Wi-Fi-exposed neurons ($P < 0.05$) (Figure 5).

3.6 Immunohistochemistry

Immunohistochemistry for caspase-3-positive cells was assessed. The percentage area of caspase-3 active cells has increased markedly in the Wi-Fi group when compared with control rats ($p < 0.001$) (Figure 6). Vit.D significantly decreased the percentage area of caspase-3 immune-positive cells when compared to untreated Wi-Fi-exposed rats ($p < 0.001$).

Table 1. Primer sequences for rRT-PCR analysis of all targeted gene expression

Reverse Primer	Forward Primer	Gene	bp	Accession NO
R: 5′ CAAACATGTCAGCTGCCACAC 3′	F: 5′ CGAATTGGCGATGAACTGGA 3′	*BAX*	109	NM_017059.2
R: 5′ CTGAGCAGCGTCTTCAGAGACA 3′	F: 5′ GACTGAGTACCTGAACCGGCATC 3′	*BCL-2*	135	NM_016993.1
R: 5′ ACACAGTTTTCCCATGCCCA 3′	F: 5′ GAAATGGTCACCCCACGAGT 3′	*LC-3*	147	NM_012823.2
R: CCAAGGGTCCACCTGAACAA 3′	F: 5′ GGAAGCTGAAACATGGGCAC 3′	*P62*	183	NM_181550.2
R: 5′ ACGCCATAACAACTCCAGGG 3′:	F: 5′ ACTCAGCCGGGTACTACCAT 3′	*CREB*	166	NM_134443.2
R: 5′ TACGGCCAAATCCGTTCACA 3′	F: 5′ GCATCTTCTTGTGCAGTGCC 3′	*GAPDH*	74	NM_017008.4

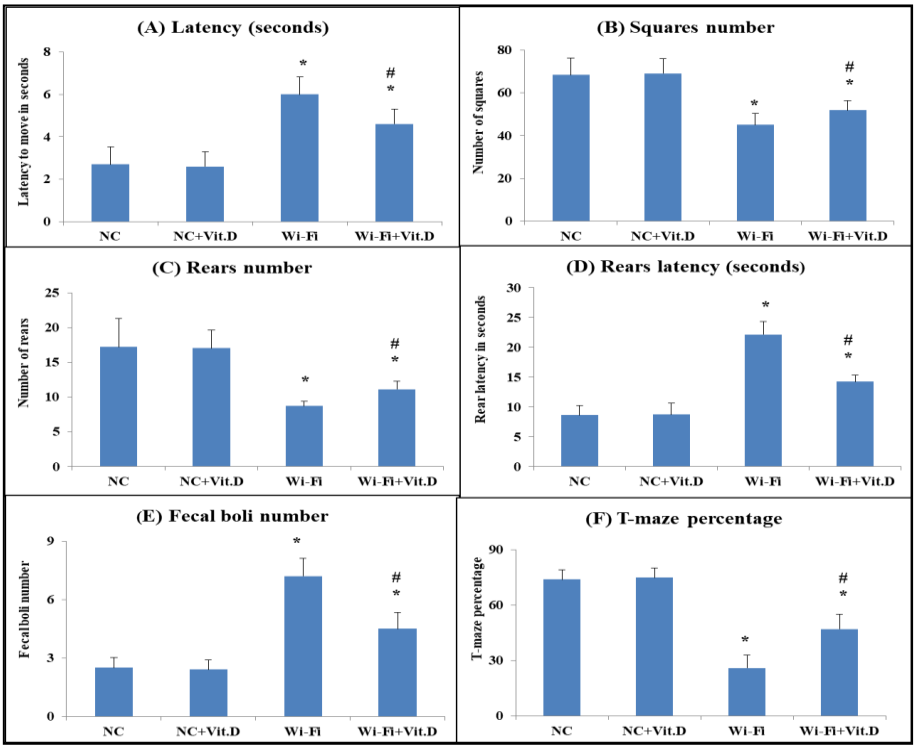

Figure 1. (A) Latency to move, (B) Number of squares, (C) Number of rears, (D) Rear latency in seconds, (E) Number of fecal boli, and (F) T-maze percentage. Data are evaluated by One-way ANOVA and presented as Mean ± SD, p-value < 0.05. *: significant versus Normal control (NC), Normal control with vitamin D (NC+Vit.D), #: significant versus WiFi group.

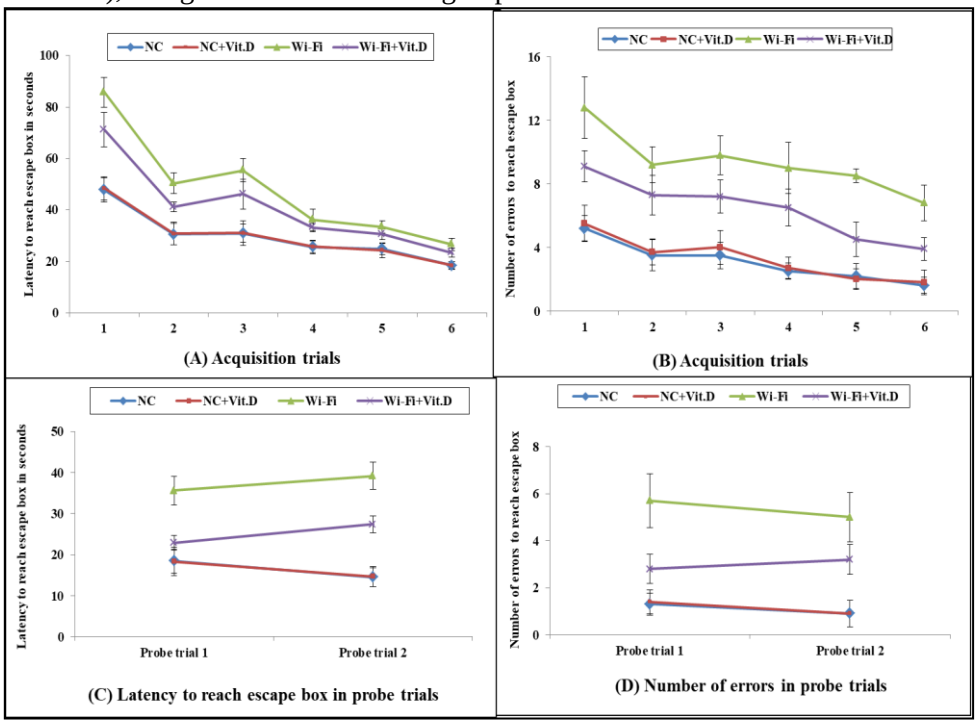

Figure 2. Modified Barnes maze (latency and number of errors to reach the escape box in the studied groups in Acquisition trials (AT) and probe trials (PT) for testing memory in Normal control (NC), Normal control with vitamin D (NC+Vit.D), Wi-Fi and Wi-Fi +Vit.D groups. Data are expressed as Mean ± SD, repeated measures two-way ANOVA, and p-value <0.05.

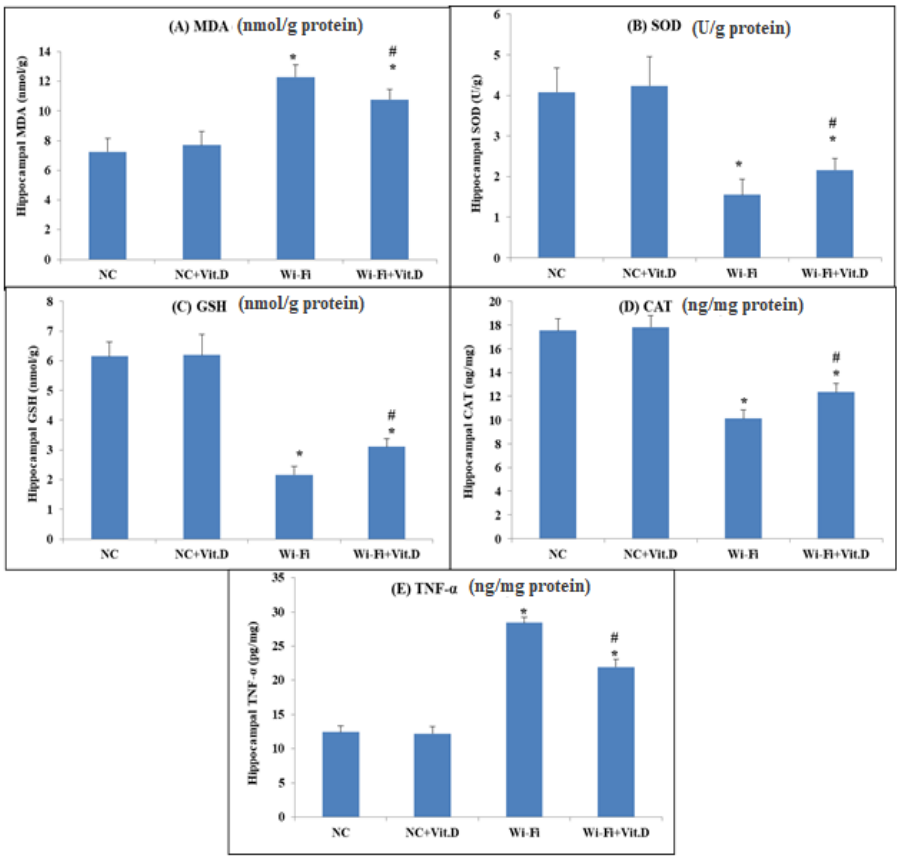

Figure 3. (A) Hippocampal malondialdehyde (MDA) nmol/g, (B) Hippocampal Superoxide dismutase (SOD) U/g, (C) Hippocampal reduced glutathione (GSH) nmol/g, (D) Hippocampal catalase (CAT) ng/mg, and (E) Hippocampal tumor necrosis factor-α (TNF-α) pg/mg. Data are evaluated by one-way ANOVA and presented as mean ± SD, p-value < 0.05. *: significant versus normal control (NC), normal control with vitamin D (NC+Vit.D), #: significant versus WiFi group.

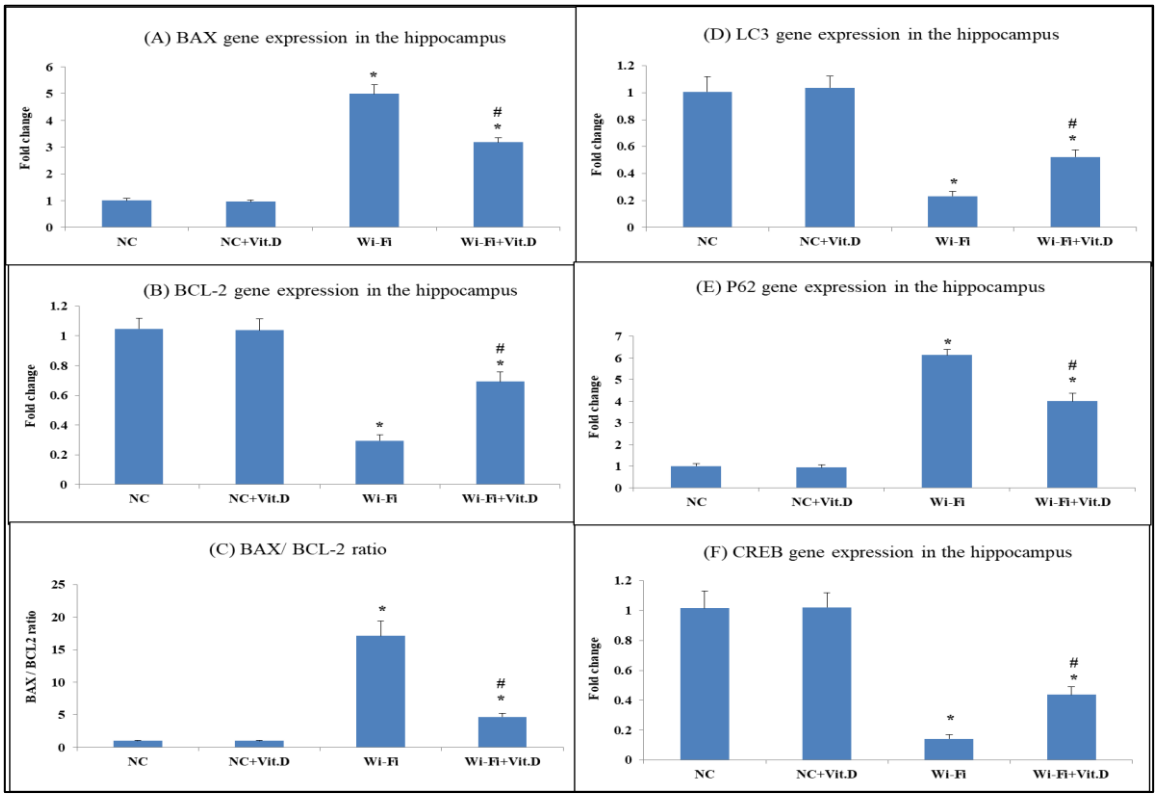

Figure 4. A) *BAX* gene expressions in the hippocampus, B) *BCL-2* gene expressions in the hippocampus, C) *BAX/ BCL-2* ratio, D) Microtubule-associated protein light chain 3-II (*LC3-II*) gene expressions in the hippocampus, E) *P62* gene expressions in the hippocampus, and F) cyclic AMP-response element binding protein (*CREB*) gene expressions in the hippocampus. Data are evaluated by one-way ANOVA and presented as mean ± SD, p-value < 0.05. *: significant versus normal control (NC), normal control with vitamin D (NC+Vit.D), #: significant versus WiFi group.

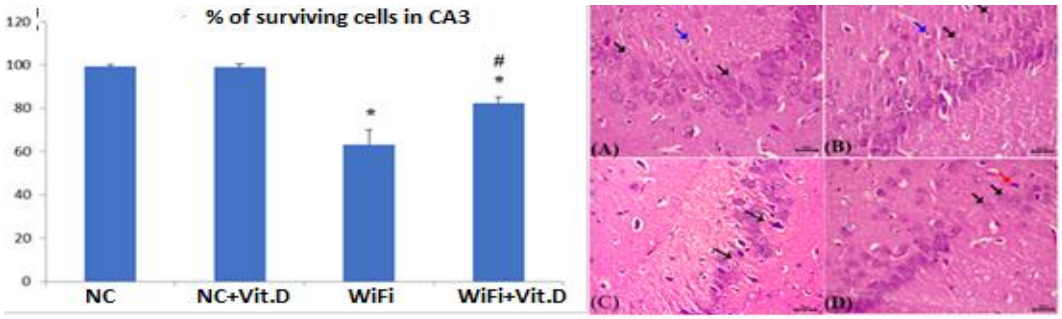

Figure 5. Hematoxylin and eosin staining of a section of the hippocampus of CA3 in all studied groups, including (A) normal control (NC), (B) normal control with Vit.D (NC+Vit.D), (C) WiFi and (D) WiFi +Vit.D. NC and NC+Vit.D groups show normal pyramidal cells with vesicular nuclei and small central nucleoli and oligodendrocyte (blue arrow). WiFi group shows degeneration of nuclei with dark stained nuclei (black arrows) and perineuronal edema (arrowhead). WiFi +Vit.D group shows improvement with a increase in the percentage number of surviving. normal pyramidal cells with vesicular nuclei (black arrows), with decrease in number of the degenerated cells (red arrow). Data are evaluated by one-way ANOVA and presented as mean ± SD, p-value <0.05. *: significant versus NC, NC+Vit.D, #: significant versus WiFi group, Scale bar= 20 µm, x400.

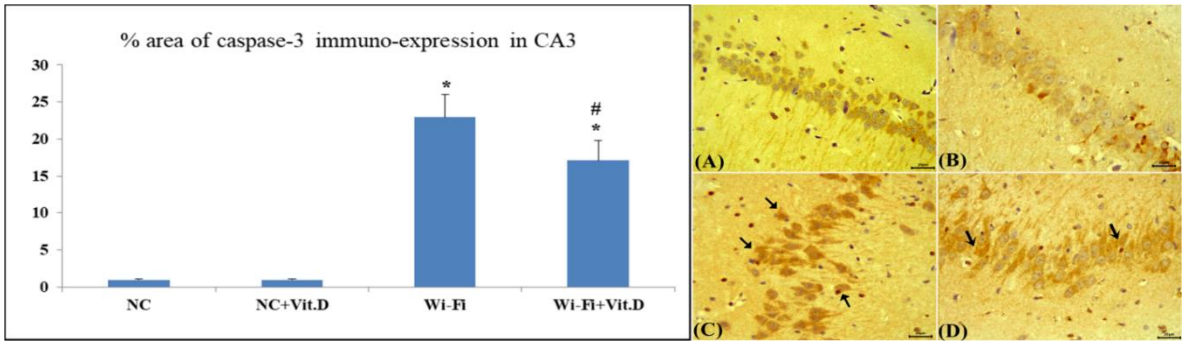

Figure 6. Immunohistochemical staining for Caspase-3 positive cells within the CA3 region in all studied groups, including (A) normal control (NC), (B) normal control with Vitamin D (NC+Vit.D), (C) WiFi and (D) WiFi +Vit.D. The bar chart shows the statistical analysis of the percentage area of caspase-3 immune-positive cells among all studied groups. Black arrow signifying the expression of Caspase-3 positive cells. Data are evaluated by one-way ANOVA and presented as mean ± SD, p-value < 0. 05.. *: significant versus NC and NC+Vit.D groups, #: significant versus WiFi group, Scale bar= 20 μm, x400.

DISCUSSION

EM field can affect cognitive function and memory [4]. The hippocampus is the brain area concerned with cognition and is highly vulnerable to stress [6]. So, we conducted this study to evaluate the effect of Wi-Fi exposure as a source of EMR on rat cognitive function and to study the modulating effect of Vit.D on rat cognition during the exposure to Wi-Fi radiation and to evaluate the involvement of different possible mechanisms, including hippocampal apoptosis and the associated inflammation and oxidative states, autophagy, and CREB involvement.

OFT was used to test anxiety-like behavior. The present study revealed that Wi-Fi exposure significantly increases anxiety-like behavior. The modified T Maze test and Barnes Maze protocol were used to test working memory, spatial memory, and learning, respectively. Wi-Fi exposure led to a significant decline in the T maze score, and the rats spent a significantly longer time in the barnez maze with a higher number of errors to reach the escape box compared to controls, indicating memory and learning deterioration. Shahin et al. [6] found memory impairment, and Tarsaei et al. [31] observed anxiety-like behavior following exposure to EMR in rats. Although Cobb et al. [32] didn't observe any memory changes with low-level EMR exposure. They expose rats to 2.45 GHz fields for 10 days, we suppose the short duration of exposure of the rats in the previously mentioned study may not be enough to affect the memory, as the memory disturbance may be related to the duration of exposure rather than levels of radiation.

Oxidative stresses can explain the non-thermal effect of EMR on the neurons [4]. Our study observed significant high MDA and TNF-α with a significant decrease in hippocampal protective antioxidant SOD, CAT activities, and GSH levels in the Wi-Fi group. In the same line, Mojez et al. [33] showed that chronic exposure to EMR markedly reduced the total antioxidant capacity, resulting in cell death.

In the rats exposed to Wi-Fi, ROS levels in the brain exceeded the neuronal cell antioxidant capacity, so oxidative stress and inflammation occurred, which was evidenced by the increase in the level of MDA and TNF -α respectively. MDA resulted from a reaction between ROS and polyunsaturated fatty acids in the lipid bilayer of the cell membrane. Also, the reduced antioxidant activities after Wi-Fi exposure reflect the impairment of the cell's ability to neutralize ROS, leading to oxidative stress, inflammation, which could lead to apoptosis of neurons.

Apoptosis, which is important for maintaining tissue homeostasis [34], was accused of mediating the hazardous effect of EMR exposure. In this issue, an in vitro study has observed the induction of caspase-dependent (intrinsic) apoptotic pathways in relation to EMR exposure [35]. However, also caspase-independent (extrinsic) apoptotic pathways have been activated following EMR exposure in rat neuronal cultures [36].

The pro-apoptotic BAX protein, when activated by signals from Wi-Fi, translocates to the mitochondrial membrane, promoting cytochrome c release, which further activates the caspase family, this is known as caspase dependent apoptotic pathway [37]. Caspase-3 activation is an essential step in the apoptosis cascade, as it causes DNA fragmentation and the breakdown of cellular components, leading to cell death. However, the anti-apoptotic action of *BCL-2* inactivates caspase-3, thereby promoting cell survival. *BAX* to *BCL* -2 ratio is critical in determining the susceptibility of a cell to apoptosis [38].

Given that, the current study revealed a significant high proapoptotic BAX gene expression and apoptotic marker "caspase-3" immunoreactivity. However, the anti-apoptotic BCL-2 gene expressions were significantly decreased in the hippocampus of the Wi-Fi exposed rats with significant increase in the ratio of *BAX* to *BCL-2* when compared to the controls.

As previously described, EMR can produce an increase in Ca^{2+} signaling secondary to the activation of voltage-gated Ca^{2+} channels; this elevates calcium/calmodulin-dependent nitric oxide synthesis, so elevates NO and superoxides inside cells [40]. . The increased ROS/NO reacts with each other to form peroxynitrite, which further breaks down to become free radicals, producing protein and lipid carbonylation of both neuronal and glial cells of the hippocampus, and results in inflammation and further apoptosis, which resembles the extrinsic apoptotic pathway [41]. Based on that, in the current study, the noted high level of hippocampal TNF-α in Wi-Fi-exposed rats,, which is an important marker of inflammation, indicates the involvement of extrinsic apoptotic pathways as well.

Our work proved that treatment with Vit.D in Wi-Fi-exposed rats significantly reversed the disturbance of the neurobehavioral function, decreased anxiety, and improved memory and learning compared to the untreated Wi-Fi group. Also, Vit.D that was administered to Wi-Fi-exposed rats also resulted in a significant recovery of oxidative stress and inflammation compared to the Wi-Fi group. Moreover, Vit.D significantly decreased apoptotic BAX gene and caspase-3 expressions and increased the antiapoptotic BCL-2 gene, so decreased the ratio of *BAX* to *BCL-2*. These results approved a role for Vit.D in modulating both the caspase-dependent and caspase-independent apoptotic pathways.

Bakhtiari-Dovvombaygi et al. [42] demonstrated that Vit.D improved chronic mild stress. Also, it decreased the anxiety-like profiles and enhanced the memory and learning activities of old ovariectomized rats and limited TNF-α and interleukin-6 in the hippocampus [43]. Moreover, in humans; a relationship between abnormally low levels of Vit. D and the high incidence of dementia was observed [44]. Vit. D limited the oxidative stresses through the upregulation of antioxidant enzymes [45].

Autophagy maintains cell homeostasis by protecting cells from environmental stresses. However, the dysfunction of autophagy causes aggregation of abnormal proteins, leading to a variety of neurodegenerative diseases [46]. Autophagy flux can be evaluated by the quantitation of *LC3-II*, moreover, *LC3-II* can interact with *P62* to help the degradation of abnormal protein aggregates [47].

There are conflicting reports about the relation of EMR with autophagy. A study supports the idea that EMR is positively involved in activating autophagy [48]. Another one suggests that no EMR effects on autophagy [49].

Our results support the involvement of autophagy as an additional mechanism mediating the EMR neuronal injury, and we evidenced that the exposure to Wi-Fi radiation induced a significant drop in the expression of the *LC3-II* gene with a significant rise in expression of the *P62* gene in the rat hippocampus compared with controls, suggesting disturbance of autophagy. However, we observed the inducing effect of Vit.D treatment on *LC3* expression levels and decreased *P62* expression when compared to the untreated Wi-Fi groups. Agreeing with us, Magdy et al. [19] observed that Vit.D increased *LC3-II* expression and decreased *P62* expression in retonone-treated rats.

Interestingly, Vit.D modulates autophagy at several levels through different mechanisms, including the regulation of intracellular calcium levels and its downstream pathways [50]. Tavera-Mendoza et al. [51] reported that the Vit.D receptors acts as a master transcriptional regulator of autophagy. They observed that Vit.D induces autophagy in the normal mammary gland through up-regulation of the LC3 protein level accompanied by increased autolysosome volume. On contrary to these findings, Golpasandi et al. [52] indicated that Vit.D reduced the excessive autophagy in rats with type 2 DM. This discrepancy suggests a role for Vit.D in regulating disturbed autophagy rather than promoting or suppressing autophagy.

In another point of view, targeting the neurotrophic proteins may be related to the hazardous effect of EMR exposure. *CREB* is reported to be closely integrated in memory and learning activities. *CREB* regulates neuronal proliferation and differentiation, as well as learning and memory functions. The active form, phosphorylated *CREB* (*pCREB*), plays a role in neurogenesis and hippocampal neuroprotection [53]. The current study observed a significant decrease in expressions of the *CREB* gene in the hippocampal tissue of rats that were exposed to Wi-Fi radiation in comparison to the controls. While a significant rise in the expressions of the *CREB* gene with Vit.D treatment was observed.

Interestingly, Tan et al. [54] observed a decrease in spatial memory functions associated with downregulation of p-*CREB* after six hours of microwave exposure. On the contrary, Kumar et al. [55] observed no effect of exposure to 2.45 GHz EMR on the expression of *CREB* in the hippocampus. This different *CREB* expression pattern might result from the activation of diverse signal pathways, which included PKA, MAP kinases, and other Ca^{2+}/calmodulin kinases, these pathways can be affected by the dose and duration of microwave exposure in the hippocampus.

Nadimi et al. [20] observed that administration of Vit.D in animals with type 1 DM has increased *CREB* phosphorylation in the hippocampus. In contrast, in a study on chronic Vit.D effects on mice, Kouba et al. [56] observed no effects on the p-CREB/*CREB* ratio in the hippocampus, however; both studies didn't investigate the gene expression of *CREB*.

Notably, our study shined a light on the inducing effect of Vit.D on *CREB* gene expressions in the hippocampus, which may add another mechanism explaining the positive effect of Vit.D on cognition in our model.

Moreover, there is a link between *CREB* and autophagy was observed, as it was previously described that *CREB* upregulates autophagy genes in hepatic cells [15]. CREB also indirectly modulates the transcription of genes linked to autophagy and lipid degradation. [57], this suggests that the role of CREB in autophagy can affect the molecular basis and extends to metabolic regulations. Based on these studies, we supposed that the possible regulation of hippocampal autophagy-related genes under the effect of *CREB* may be considered an additional factor that mediate Vit.D effects on autophage and oxidative stress. However, the pathways and molecular mechanisms behind this relation still need to be clarified further.

Finally, hippocampus histopathology of the CA3 region confirmed abnormal arrangement and signs of death of neurons with a significant reduction in the number of normal viable neurons in Wi-Fi-exposed rats. However, Vit.D significantly improved the histopathological findings. Our biochemical and histological findings reflect the changes in the hippocampus of the Wi-Fi-exposed rats and Vit.D-treated rats, which also explain the findings of neurobehavioral tests.

Some limitations were noted; first evaluation of Vit.D levels should be considered in similar models, second; studying the effect of EMR on Vit. D receptors distribution in hippocampus still need evaluation. And third: different pathways of CREP such as mitogen-activated protein kinases (MAPK), and the Ca+2-calmodulin-dependent protein kinase pathways need more clarification with their possible involvement in Vit. D mechanism for cognition improvement.

CONCLUSIONS

Wi-Fi radiation exposure produced cognition disturbance that reflected oxidative stress and an inflammatory state in hippocampal neurons. It activated both extrinsic and intrinsic apoptotic pathways. In addition, autophagy and *CREB* were involved in this effect. Vit.D improved the neurobehavioral tests, It enhanced autophagy and *CREB* expression, and thus, it helped the hippocampal neurogenesis via the suppression of the neuroinflammatory and apoptotic processes induced by the Wi-Fi radiation exposure. Vit.D protected rats against the cognitive impairment associated with the Wi-Fi radiation exposure. But there is a need for further studies before clinical application. Also, further additional work is needed to find out possible molecular mechanisms that lie behind the relation between autophagy and oxidative stress under Wi-Fi exposure and the possible relationship between CREB and the autophagy pathway in the hippocampus.

CONFLICT OF INTEREST.

There is no conflict of interest.

FINANCIAL DISCLOSURE

No funding was provided for this research

AUTHOR CONTRIBUTIONS.

Eman R. Abozaid, and Abeer A Khalefa; methodology, Hend M Eissa, Nawal K. Gerges, Eman R. Abozaid, and Abeer A Khalefa; software validation and formal analysis, Eman R. Abozaid1and Abeer A Khalefa; investigation, and data curation, Hend M Eissa1writing and editing, Eman R. Abozaid and Abeer A Khalefa supervision. All authors discussed the results and contributed to the final manuscript.

ACKNOWLEDGMENTS.

The authors extend their appreciation to the members of physiology animal behavior laboratory and member of pathology department, faculty of medicine. Zagazig university.

REFERENCES

1. Varghese R, Majumdar A, Kumar G, Shukla A. Rats exposed to 2.45 GHz of non-ionizing radiation exhibit behavioral changes with increased brain expression of apoptotic caspase 3. Pathophysiol 2018; 25(1):19-30.

2. Regalbuto E, Anselmo A, De Sanctis S, Franchini V, Lista F, Benvenuto M, et al. Human

fibroblasts in vitro exposed to 2.45 GHz continuous and pulsed wave signals: evaluation of biological effects with a multimethodological approach. Int J Mol Sci. 2020; 21(19):7069.
3. Williams. J. Biological effects of microwaves. Thermal and non-thermal mechanisms. 2016; (4): 7: 1-49.
4. Farahani K, Mischel PS, Black KL, De Salles AA, Anzai Y, Lufkin RB. Hyperacute thermal lesions: MR imaging evaluation of development in the brain. Radiology. 1995; 196(2) 517-20.
5. Hao Y, Liu W, Xu Z, Jin X, Ye Y, Yu C, et al. High-power electromagnetic pulse exposure of healthy mice: assessment of effects on mice cognitions, neuronal activities, and hippocampal structures. Front Cell Neurosci 2022; 16:898164.
6. Gökçek-Saraç Ç, Akçay G, Karakurt S, Ateş K, Özen Ş, Derin N. Possible effects of different doses of 2.1 GHz electromagnetic radiation on learning, and hippocampal levels of cholinergic biomarkers in Wistar rats. Electromagnetic Biol Med 2021; 40(1):179-90.
7. Cherubini E, Miles R. The CA3 region of the hippocampus: how is it? What is it for? How does it do it? Front Cell Neurosci. 2015; 5: 9:19.
8. Shahin S, Banerjee S, Singh SP, Chaturvedi CM. 2.45 GHz microwave radiation impairs learning and spatial memory via oxidative/nitrosative stress induced p53-dependent/independent hippocampal apoptosis: molecular basis and underlying mechanism. Toxicol Sci. 2015; 148(2):380-99.
9. Jafari M, Masoudi E, Sotoudeh N, Hosseini S F. Effects of Heat and Wi-Fi (2.4 GHz) Exposure on Rat Cardiovascular System. Health Scope. 2022; 11, e120282.
10. Jaffar FHF, Osman K, Ismail NH, Chin KY, Ibrahim SF. Adverse Effects of Wi-Fi Radiation on Male Reproductive System: A Systematic Review. Tohoku J Exp Med. 2019; 248,169-79.
11. Zhang Z, Zhang L, Zhou L, Lei Y, Zhang Y, Huang C. Redox signaling and unfolded protein response coordinate cell fate decisions under ER stress. Redox Biol 2019; 25:101047.
12. El-Kafoury BM, Abdel-Hady EA, El Bakly W, Elayat WM, Hamam GG, Abd El Rahman et al. Lipoic acid inhibits cognitive impairment induced by multiple cell phones in young male rats: role of Sirt1 and Atg7 pathway. Sci Rep. 2023; 13(1):18486.
13. Gökçek-Saraç Ç, Er H, Kencebay Manas C, Kantar Gok D, Özen Ş, Derin N. Effects of acute and chronic exposure to both 900 MHz and 2100 MHz electromagnetic radiation on glutamate receptor signaling pathway. Int J Radiat Biol.2017; 93(9):980-9.
14. Wang IF, Wang Y, Yang YH, Huang GJ, Tsai KJ, Shen CJ. Activation of a hippocampal CREB-pCREB-miRNA-MEF2 axis modulates individual variation of spatial learning and memory capability. Cell Rep. 2021; 36(5):109477.
15. Seok S, Fu T, Choi SE, Li Y, Zhu R, Kumar S, Sun X, et al. Transcriptional regulation of autophagy by an FXR-CREB axis. Nature. 2014; 516(7529):108-11.
16. Cui C, Xu P, Li G, Qiao Y, Han W, Geng C et al. Vitamin D receptor activation regulates microglia polarization and oxidative stress in spontaneously hypertensive rats and angiotensin II-exposed microglial cells: Role of renin-angiotensin system. Redox Biol. 2019; 26:101295.
17. Bao Z, Wang X, Li Y, Feng F. Vitamin D alleviates cognitive dysfunction by activating the VDR/ERK1/2 signaling pathway in an Alzheimer's disease mouse model. Neuroimmunomodulation 2021; 27(4):178-85.
18. Mokhtari-Zaer A, Hosseini M, Salmani H, Arab Z, Zareian P. Vitamin D3 attenuates lipopolysaccharide-induced cognitive impairment in rats by inhibiting inflammation and oxidative stress. Life Sci 2020; 253:117703.
19. Magdy A, Farrag EA, Hamed SM, Abdallah Z, El Nashar EM, Alghamdi MA et al. Neuroprotective and therapeutic effects of calcitriol in rotenone-induced Parkinson's disease rat model. Front Cell Neurosci 2022; 16:967813.
20. Nadimi H, Djazayery A, Javanbakht MH, Dehpour A, Ghaedi E, Derakhshanian H, et al. Effect of vitamin D supplementation on CREB-TrkB-BDNF pathway in the hippocampus of diabetic rats. Iran J Basic Med Sci 2020; 23(1):117.
21. Gui Y, Silha JV, Murphy LJ Sexual dimorphism and regulation of resistin, adiponectin, and leptin expression in the mouse. Obes Res 2004; 12(9):1481-91.
22. Hajiluian G, Nameni G, Shahabi P, Mesgari-Abbasi M, Sadigh-Eteghad S, Farhangi MA. Vitamin D administration, cognitive function, BBB permeability and neuroinflammatory factors in high-fat diet-induced obese rats. Int J obes 2017; 41(4):639-44.
23. Karimi N, Bayat M, Haghani M, Saadi HF, Ghazipour GR. 2.45 GHz microwave radiation impairs learning, memory, and hippocampal synaptic plasticity in the rat. Toxicol Indust Healt 2018; 34(12):873-83.
24. Briones-Aranda A, Castellanos-Pérez M, Vega-Villa VM, Picazo. Impact of exposure to environmental enrichment on the anxiety-like behavior of ovariectomized mice. Iran J Psychiat 2020; 15(1):88.

25. Wu CYC, Lerner FM, Couto E Silva A, Possoit HE, Hsieh TH, Neumann JT, et al. Utilizing the Modified T-Maze to Assess Functional Memory Outcomes After Cardiac Arrest. J Vis Exp. 2018; 5 (131):56694.
26. Morel GR, Andersen T, Pardo J, Zuccolilli GO, Cambiaggi VL, Hereñú CB, et al. Cognitive impairment and morphological changes in the dorsal hippocampus of very old female rats. Neurosci 2015; 303:189-99.
27. Field KJ, Lang CM. Hazards of urethane (ethyl carbamate): a review of the literature. Lab Animals 1988; 22(3):255-62.
28. Delrobaei F, Fatemi I, Shamsizadeh A, Allahtavakoli M. Ascorbic acid attenuates cognitive impairment and brain oxidative stress in ovariectomized mice. Pharmacol Rep 2019; 71(1):133-8.
29. Suvarna SK, Layton C, Bancroft JD. Bancroft's Theory and Practice of Histological Techniques 8th Edition. Elsevier Health Sci. 2018; ISBN: 9780702068645
30. Liu J, Wang A, Li L, Huang Y, Xue P, Hao A. Oxidative stress mediates hippocampal neuron death in rats after lithium–pilocarpine-induced status epilepticus. Seizure 2010; 19(3):165-72.
31. Tarsaei M, Peyrovan ZS, Mahdavi SM, Modarresi Chahardehi A, Vafaee R, Haidari MH. Effects of 2.45 GHz Non-Ionizing Radiation on Anxiety-Like Behavior, Gene Expression, and Corticosterone Level in Male Rats. J Lasers Med Sci. 2022; Dec 4;13:e56.
32. Cobb BL, Jauchem JR, Adair ER. Radial arm maze performance of rats following repeated low level microwave radiation exposure. Bioelectromagnetics 2004; 25:49–57
33. Mojez MR, Gaeini AA, Choobineh S, Sheykhlouvand M. Hippocampal oxidative stress induced by radiofrequency electromagnetic radiation and the neuroprotective effects of aerobic exercise in rats: a randomized control trial. J Physi Activ Healt 2021; 18(12):1532-8.
34. Culmsee C, Landshamer S. Molecular insights into mechanisms of the cell death program: role in the progression of neurodegenerative disorders. Curr Alzheimer Res 2006; 3(4):269-83.
35. Narayanan SN, Mohapatra N, John P, Nalini K, Kumar RS, Nayak SB, Bhat PG. Radiofrequency electromagnetic radiation exposure effects on amygdala morphology, place preference behavior and brain caspase-3 activity in rats. Environ Toxicol Pharmacol 2018; 58:220-9.
36. Joubert V, Bourthoumieu S, Leveque P, Yardin C. Apoptosis is induced by radiofrequency fields through the caspase-independent mitochondrial pathway in cortical neurons. Radiat Res 2008; 169(1):38-45.
37. Le, DA, Wu, Y, Huang, Z, Matsushita, K, Plesnila, N, Augustinack, JC et al. Caspase activation and neuroprotection in caspase-3 deficient mice after in vivo cerebral ischemia and in vitro oxygen glucose deprivation. Proceedings of the National Academy of Sciences. 2002; 99(23), 15188-93.
38.. Lossi, L, Castagna, C, Merighi, A. Caspase-3 mediated cell death in the normal development of the mammalian cerebellum. International journal of molecular sciences 2018; 19(12), 3999.
39. Gupta SK, Mesharam MK, Krishnamurthy S. Electromagnetic radiation 2450 MHz exposure causes cognition deficit with mitochondrial dysfunction and activation of intrinsic pathway of apoptosis in rats. J Biosci 2018; 43:263-76.
40. Pill AA. Electromagnetic fields instantaneously modulate nitric oxide signaling in challenged biological system Biochem. Biophys. Res. Commun 2012; 28 (426): 330-3.
41. Shahin S, Banerjee S, Swarup V, Singh SP, Chaturvedi CM. From the cover: 2.45-GHz microwave radiation impairs hippocampal learning and spatial memory: Involvement of local stress mechanism-induced suppression of iGluR/ERK/CREB signaling. Toxicol Sci 2018; 161(2):349-74.
42. Bakhtiari-Dovvombaygi H, Izadi S, Zare Moghaddam M, Hashemzehi M, Hosseini M, Azhdari-Zarmehri H, Dinpanah H, Beheshti F. Beneficial effects of vitamin D on anxiety and depression-like behaviors induced by unpredictable chronic mild stress by suppression of brain oxidative stress and neuroinflammation in rats. Naunyn-Schmiedeberg's Archives of Pharmacol 2021; 394(4):655-67.
43. Babaei P, Damirchi A, Hoseini Z, Hoseini R. Co-treatment of vitamin D supplementation and aerobic training improves memory deficit in ovariectomized rat. Int J Neurosci 2020; 130(6):595-600.
44. Jayedi A, Rashidy-Pour A, Shab-Bidar S. Vitamin D status and risk of dementia and Alzheimer's disease: A meta-analysis of dose-response. Nutr Neurosci 2019; 22(11):750-9.
45. Paprocki J, Sutkowy P, Piechocki J, Woźniak A. Association between Vitamin D Supplements, Oxidative Stress Biomarkers, and Hyperbaric Therapy in Patients with Sudden Sensorineural Hearing Loss. Oxid Med Cell Longev. 2021 Mar 10;2021:8895323.
46. Cai Q, Ganesan D Regulation of neuronal autophagy and the implications in

neurodegenerative diseases. Neurobiol Dis 2022; 162:105582.
47. Pankiv, S, Clausen, T. H, Lamark, T, Brech, A, Bruun, J. A, Outzen, H, & Johansen, T. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. Journal of biological chemistry 2007; 282(33): 24131-45.
48. Hao YH, Li ZH, Peng RY. Effects of electromagnetic radiation on autophagy and its regulation. Biomed Environ Sci 2018; 31(1):57-65.
49. Zuo WQ, Hu YJ, Yang Y, Zhao XY, Zhang YY, Kong W, Kong WJ. Sensitivity of spiral ganglion neurons to damage caused by mobile phone electromagnetic radiation will increase in lipopolysaccharide-induced inflammation in vitro model. J Neuroinflammation 2015; 12, 105.
50. Abdel-Mohsen, M A, El-Braky, AAA, Ghazal, AAER, & Shamseya, M M. Autophagy, apoptosis, vitamin D, and vitamin D receptor in hepatocellular carcinoma associated with hepatitis C virus. Medicine 2018; 97(12).
51. Tavera-Mendoza, L. E, Westerling, T, Libby, E, Marusyk, A, Cato, L, Cassani, R. et al. Vitamin D receptor regulates autophagy in the normal mammary gland and in luminal breast cancer cells. Proceedings of the National Academy of Sciences 2017; 114(11), 2186-94.
52. Golpasandi, H, Rahimi, M. R, Ahmadi, S, Łubkowska, B, & Cięszczyk, P. Effects of Vitamin D3 Supplementation and Aerobic Training on Autophagy Signaling Proteins in a Rat Model Type 2 Diabetes Induced by High-Fat Diet and Streptozotocin. Nutrients 2023; 15(18), 4024.
53. Jiang T, Wang XQ, Ding C, Du XL. Genistein attenuates isoflurane-induced neurotoxicity and improves impaired spatial learning and memory by regulating cAMP/CREB and BDNF-TrkB-PI3K/Akt signaling. The Korean J Physiol Pharmacol 2017; 21(6):579.
54. Tan X, Gao L, Cai X, Zhang M, Huang D, Dang Q, Bao L. Vitamin D3 alleviates cognitive impairment through regulating inflammatory stress in db/db mice. Food Sci Nutr 2021; 9(9):4803-14.
55. Kumar, M, Singh, SP, Chaturvedi, CM. Effect of 2.45 GHz Pulsed Microwave Radiation on Hippocampus Mediated Behaviour: in a Specific Relation to the Expression of Rac1, Cdc42 and pCREB. Anxiety 2017; 6(10).
56. Kouba BR, Torrá ACNC, Camargo A, Rodrigues ALS. The antidepressant-like effect elicited by vitamin D3 is associated with BDNF/TrkB-related synaptic protein synthesis. Metab Brain Dis 2023; 38(2):601-11.
57. Di Malta C , Cinque L, Settembre C. Transcriptional Regulation of Autophagy: Mechanisms and Diseases. JOURNAL=Frontiers in Cell and Developmental Biology. 2019; 7 URL=https://www.frontiersin.org/journals/cell-and-developmental biology/articles/10.3389/fcell.2019.00114.

Citation

Abozaid, E., khalefa, A., Gergis, N., Eissa, H. Effects of Vitamin D on The Cognitive Functions in Rats Exposed to Wi-Fi Radiation: Role of Autophagy and CREB Gene. *Zagazig University Medical Journal*, 2025; (682-694): -. doi: 10.21608/zumj.2024.340955.3713