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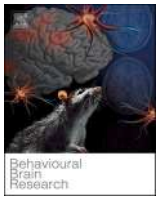

Research article

Possible effects of radiofrequency electromagnetic radiation on contextual fear conditioning, hippocampal perivascular space, apoptosis and adrenal gland microarchitecture in rats

Sareesh Naduvil Narayanan [a,*,1,2], Raju Suresh Kumar [b], Naveen Kumar [c], Pavithra Prabhakar [d], Satheesha Badagabettu Nayak [e], Perumunda Gopalakrishna Bhat [f]

[a] Department of Physiology, Melaka Manipal Medical College, Manipal Academy of Higher Education, Manipal 576104, India
[b] Department of Basic Sciences, College of Science and Health Professions, King Saud Bin Abdulaziz University for Health Sciences (KSAU-HS), National Guard Health Affairs (NGHA), P. O. Box 9515, Jeddah 21423, Kingdom of Saudi Arabia
[c] Department of Anatomy, Ras Al Khaimah College of Medical Sciences, Ras Al Khaimah Medical & Health Sciences University, PO Box. 11172, Ras Al Khaimah, United Arab Emirates
[d] Department of Pathology, Melaka Manipal Medical College, Manipal Academy of Higher Education, Manipal 576104, India
[e] Division of Anatomy, Department of Basic Medical Sciences, Manipal Academy of Higher Education, Manipal- 576104, India
[f] Division of Biotechnology, School of Life Sciences, Manipal Academy of Higher Education, Manipal 576104, India

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ABSTRACT

Whilst the world sees the tremendous growth of mobile phone technology, radiofrequency electromagnetic radiation (RF-EMR) induced possible health effects have emerged as a topic of recent day debate. The current study is designed to test the hypothesis that chronic 900 MHz radiation exposure would potentially dysregulate the stress response system (HPA axis) in vivo, via, its non-thermal mechanisms, leading to alterations in the microarchitecture of the adrenal gland, vulnerable brain regions such as the hippocampus which may results in altered behaviours in rats. Male albino Wistar rats aged four weeks, weighing 50–60 g were subjected to 900 MHz radiation from a mobile phone for four weeks at a rate of one hour per day. On the 29th day, animals from the control, sham exposed and RF-EMR exposed groups were tested for contextual fear conditioning. They were later euthanized to study hippocampal and adrenal gland cytoarchitecture. Bright and dark compartment transitions in the avoidance box were considerably elevated in the RF-EMR exposed group and they exhibited a significant decrease in the latency to enter the dark compartment during the contextual fear conditioning test. Apoptosis was apparent in the CA3 region and perivascular space was significantly increased in the hippocampus of the radiation-exposed group. In addition to lymphocytic infiltrates, congested sinusoids, apoptotic-like changes were evident in the zona fasciculata of the adrenal gland. However, the cytoarchitecture of the adrenal medulla was comparable in all three groups. Chronic RF-EMR exposure caused changes in contextual fear conditioning, enlargement of hippocampal perivascular space, apparent CA3 apoptosis, and apoptotic-like changes in the zona fasciculata of the adrenal gland in rats.

1. Introduction

Man-made radiofrequency electromagnetic radiation (RF-EMR) is ubiquitous in the modern world and life without the technologies that use this radiation is unimaginable. Although RF-EMR has been utilized in several modern technologies; its predominant application is evident over the globe in the telecommunication sector such as the mobile phone industry. Over three decades, mobile phone technology has influenced all facets of human life. Recently deployed 5 G technology utilizes the new sub-6 GHz band in addition to lower RF-EMR frequencies (< 2

* Corresponding author.
E-mail addresses: sareeshnn@yahoo.co.in, snaduvil@uclan.ac.uk (S.N. Narayanan).
[1] **Present address:** Department of Physiology, School of Medicine and Dentistry, AUC-UK Track, University of Central Lancashire, Preston, PR1 2HE, United Kingdom
[2] ORCID: https://orcid.org/0000-0002-2980-2352

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GHz). While the world sees the tremendous growth of this technology, the health effects of RF-EMR emerged as a topic of recent day debate. Numerous research suggest the possible biological effects of various frequency bands, which are used in telecommunication technology [1–3] and several reports have addressed the impact of this technology on the brain [4–8].

Although some reports are available on the effects of RF-EMR on contextual fear conditioning and associated changes in the brain, their outcomes are contradictory. According to Bouji et al. [9] exposure to 900 MHz radiation to 22–24 months old rats for one month showed no changes in memory, behaviour or movements during old age. Aldad et al. [10] have demonstrated that radiation exposure during pregnancy can induce significant memory impairment, and behaviour in mice. One-time exposure to 900 MHz radiation increased neurotransmitter levels in the olfactory bulb and enhanced contextual emotional memory in rats [9]. It was also reported that 900 MHz radiation exposure during the gestational period, can affect the emotional learning of the offspring [11]. According to one of the previous studies, mobile phone exposure does not change the passive avoidance behaviour of adult rats [12]. However, Singh et al. [13] have found that exposure to 1966.1 MHz for 16 weeks only slightly alters the contextual fear memory in rats. Ahmadi et al. [14] have found that 28 days of cellphone exposure can impair the inhibitory avoidance (IA) memory performance in rats. It was also reported in one of the earlier studies, one-month exposure to 900 MHz RF-EMR from a mobile phone in vibratory mode had led to altered fear learning and memory and this was associated with changes in hippocampal microarchitecture in rats [15]. It is pertinent to understand the RF-EMR effects alone in a similar experimental scenario but in the absence of vibration and its effects on fear learning and memory, changes in the microarchitecture of the hippocampus and possible effects on the final organ of the hypothalamic-pituitary and adrenal gland (HPA) axis- the adrenal glands, which were evaluated in the current study.

The hippocampus is a part of the hippocampal formation buried within the temporal lobe. It is a component of the limbic system responsible for a wide variety of functions, which primarily control the behaviour of an organism. In particular, it plays a selective role in the formation of episodic memory and inhibition of certain behaviours [16]. Therefore, rodents with hippocampal damage demonstrate difficulty in learning to inhibit responses that have been previously taught. In addition, hippocampal lesions are often associated with anxiety-like behaviour in rodents [17]. The hippocampus is vulnerable to RF-EMR and damage to the hippocampal neurons usually leads to learning and memory deficits [2]. Although, there is evidence in the literature for the vulnerability of the hippocampus to RF-EMR, apoptotic cell death per se in the hippocampus due to RF-EMR is controversial. A study observed increased apoptotic protein levels and decreased anti-apoptotic protein levels after the RF-EMR exposure [18]. Also, both granule and pyramidal cell count was found to be decreased following long-term RF-EMR exposure. Maskey et al. [19] have reported TUNEL-positive cells in the hippocampus of mice chronically exposed to RF-EMR. However, another report by Kim et al. [20] demonstrates that RF-EMR decreases apoptosis in mice. A study by Joubert et al. [21] reported that there was no apoptosis among the rats exposed to GSM mobile fields. These contradictory observations demand further verification of the apoptosis-inducing effects of RF-EMR.

Perivascular spaces (PVS) or Virchow-Robin spaces are small fluid-filled spaces surrounding small arterioles, capillaries and venules in the brain. Although significant uncertainty and controversy continue to exist surrounding its anatomy and function, the enlarged PVS seen in ageing brains [22] and patients with neurodegenerative diseases make it an ideal candidate for studies including the RF-EMR effect on PVS morphology in various brain regions.

The RF-EMR effects on adrenal gland microarchitecture and markers of adrenal functions are scanty and available reports demonstrate significant contradictions in their outcomes. According to a recent report, exposure to RF-EMR for 1–2 months had induced marked morphological and hormonal changes in the adrenal gland [23]. Sangün et al. [24] reported that in humans, RF-EMR has an endocrine effect on the thyroid and adrenal glands. Additionally, multiple studies on animal models demonstrate that several stress responses are initiated during and following the RF-EMR exposure period [25–27]. These effects are primarily mediated by the hypothalamic-pituitary and adrenal gland (HPA-axis). It is reported that significantly high cortisol levels will be detected by the hypothalamus and hippocampus, resulting in a shutdown of the stress response by decreasing the ACTH levels. A report demonstrates the presence of apoptosis in the zona fasciculata and reticularis of the adrenal cortex of rats 3 days after hypophysectomy and this was largely prevented by ACTH replacement [27] indicating the link between ACTH and apoptosis in the adrenal cortex.

Therefore, the current study is designed to test the hypothesis that chronic 900 MHz radiation exposure would potentially dysregulate the stress response system (HPA axis) in vivo, via, its non-thermal mechanisms leading to alterations in the microarchitecture of the adrenal gland, vulnerable brain regions such as the hippocampus which may results in altered behaviours in rats. The specific objective is to evaluate the effects of chronic 900 MHz radiation RF-EMR exposure on contextual fear conditioning, hippocampal perivascular space, apoptosis and adrenal microarchitecture in rats.

2. Materials and methods

2.1. Research animals

Male albino rats (Wistar strain) aged four weeks (weighing 50–60 g) were used in this study. They were kept in plastic cages measuring 41 cm × 28 cm × 14 cm (3 rats per cage). Rats were provided with food (*ad-libitum*) and water. They were maintained in a day-night cycle (12:12 h) in an air-conditioned room at the Central Animal Research Facility (CARF). Permission to conduct this study was obtained from the Animal Ethics Committee of the Manipal Academy of Higher Education.

2.2. Study design and experimental groups

Rats were divided randomly into three groups ($n = 12$ per group). Control: these rats were kept in the home cage for 28 days. Sham exposed group: This group was exposed to a switched-off mobile phone for four weeks. RF-EMR exposed group: This group was subjected to 900 MHz from a mobile phone (in silent mode, for 1 hr per day) for 4 weeks. On the 29th day, rats were subjected to contextual fear conditioning. They were later euthanized to study hippocampal perivascular space (PVS), hippocampal apoptosis by TUNEL staining, and adrenal gland cytoarchitecture using H&E staining.

2.3. RF-EMR exposure and power dosimetry

The radiation exposure of the animals was conducted as per one of the earlier publications [28]. Briefly, RF-EMR exposed group rats were exposed to 900 MHz from a mobile phone (power level 2 W and SAR 1.15 W/kg). During exposure, the mobile phone was kept in the centre of the home cage inside another separate wire mesh cage. The phone was activated continuously through unattended calls (50 calls/hr) for 1 hr per day for 4 weeks. An indigenously made autodialer unit was used for this purpose and this could dial four phones at a time. To determine the power density a spectrum analyzer was used (SPECTRAN HF-2025E with MCS Real-Time Spectrum Analyzer Software, Aaronia AG, Germany). The peak power density recorded was 146.6 $\mu W/cm^2$.

2.3.1. Analysis of fear memory

The method described by Bures et al. [29] was followed to perform contextual fear conditioning. Panlab passive avoidance box controlled by ShutAvoid (version 1.8.2) software was used for this test. The

experiments consisted of 3 stages, 1st stage: exploration trial, 2nd stage: aversive stimulation & learning trial 3rd stage: memory retention test. The exploration phase is composed of three trials. While performing this test, rats were placed in the bright compartment facing away from the entrance to the dark compartment. The animal behaviour was recorded for 3 minutes in each of the trials. The time taken to enter the dark compartment, time spent in both the dark and bright compartments, the number of crossings between bright and dark compartments and the number of defecation boli were recorded. All the activities were monitored using a PC (installed with ShutAvoid version 1.8.2 software) connected to the passive avoidance box. After this, the animal was placed again in the bright compartment. Once, the rat entered the dark compartment the sliding door was lowered and an electric shock (0.5 mA for 5 seconds duration) was given to the animal. The animal was then kept back in the home cage. Memory retention was carried out 24 hours after the aversive stimulation test. During this test, the rats were placed in the centre of the brighter chamber for three minutes and their behaviour was monitored. All parameters mentioned above were recorded for each animal.

2.3.2. Analysis of hippocampal perivascular space (PVS)

Cervical dislocation, method was used to kill the rats. Dissected brains were fixed with formalin and embedded in paraffin. Five micron-thick paraffin sections were obtained and stained with Hematoxylin and Eosin stains following the standard protocol. The perivascular spaces, confined to the stratum lacunosum-moleculare were selected for further analysis. From each section, images of 8 perivascular spaces were captured (under 40 × magnification) using a bright field dual-headed microscope (Olympus BX-43, Japan) connected to a DP 21 camera (Olympus, Japan). A total of 48 perivascular spaces were imaged from each animal and in a group, a total of 288 perivascular spaces were evaluated. CellSens imaging software (version 1.6, Olympus, Japan) was used to measure the area and diameter of each PVS. The area of each perivascular space (in μm^2) was calculated by subtracting blood vessel area from total area. A trained, independent person took the measurements to avoid researcher bias.

2.3.3. Analysis of hippocampal apoptosis

The TUNEL staining was carried out to determine the programmed cell death (apoptosis) in the hippocampal CA3 region. The staining of brain sections (5μm thickness) were carried out based on available literature [7]. The stained tissues were assessed qualitatively through Olympus BX 51 fluorescence microscope attached with an AndoriQ EMCCD camera. Photomicrographs of these sections (10 × magnification) were captured using Olympus BX51 fluorescence microscope and Andors' EMCCD camera attached to a PC installed with AndoriQ (version 2.2.2) software. TUNEL-positive cells were counted manually from these images on three successive frontal sections in a 500 μm length of dorsal hippocampus CA3 region from each rat. Mean positive cells of the three sections were obtained as total apoptotic cells per rat. From each group, three brains were used for this procedure. Apoptotic cell count is reported as mean ± SE per group.

2.3.4. Analysis of adrenal gland microarchitecture

Adrenal glands were processed for routine histological staining using H&E and reticulin stains. An expert pathologist conducted qualitative analysis of these sections. Photomicrographs of gland microarchitecture were obtained using a bright-field dual-headed microscope (Olympus BX-43, Japan) connected to a DP 21 camera (Olympus, Japan), which is attached to a PC installed with CellSens (version 1.6, Olympus, Japan) imaging software.

2.4. Statistical analysis

The data is represented as Mean ± SE. Differences between the means of various experimental groups in different assays were analyzed by one-way ANOVA and post hoc Tukey's test for multiple comparisons. A 'p' value ≤ 0.05 was considered statistically significant at a 5 % significance level. GraphPad Prism (version 5.01) software (San Diego, CA, USA) was used for data analysis.

3. Results

3.1. Analysis of fear memory

3.1.1. Habituation session

Analysis of bright and dark compartment transitions of rats during the habituation phase revealed that RF-EMR exposed rats were slightly anxious (or hyper-active) when compared with other groups. When their activity was evaluated in the apparatus for 3 minutes, RF-EMR exposed rats made more crossings between dark and bright compartments than control and sham-operated rats did (Fig. 1A; F-2.11, $p = 0.14$). More bright-to-dark compartment transitions were observed in the RF-EMR group. However, these were not statistically significant. The number of defecation boli count was also higher with the RF-EMR group than the others. (Fig. 1B; F-7.46, $p = 0.002$). These indirectly point to the fidgety or anxiety-like behaviour of RF-EMR exposed rats.

3.1.2. Fear memory retention session

3.1.2.1. Latency to enter the dark compartment. In the memory retention test, the rats exposed to RF-EMR crossed to the dark compartment faster than the control and sham-operated rats. The mean latency to enter the dark compartment was 170.4 ± 9.39 seconds in the control and 150.50 ± 15.46 seconds in the sham exposed group. This was significantly reduced in the RF-EMR group (73.29 ± 22.83 seconds) (Fig. 1C; F-7.77, $p = 0.002$).

3.1.2.2. Number of bright and dark compartment transitions. The crossings between the bright and dark compartments were observed to be significantly increased in the RF-EMR group when tested 24 hours after the aversive stimulation, compared to other groups (Figs. 1D, F-4.3, $p = 0.02$).

3.1.2.3. Time spent in the dark compartment. Analysis of this parameter indicated that RF-EMR exposed rats spent more time in the dark compartment (from which the rats received electric shock a day prior) than the other two groups. The mean values were 30.32 ± 13.09, and 28.02 ± 15.33 seconds in the control, and sham exposed groups respectively, but it was increased to 51.66 ± 15.43 seconds in the RF-EMR exposed group (Fig. 1E, F-0.79; $p = 0.46$).

3.1.2.4. Defecation index. The defecation index was found to be similar in all the experimental groups. There was no statistically significant difference observed in the defecation boli count among the three groups (Fig. 1F, F-0.12, $p = 0.88$).

3.1.2.5. Analysis of perivascular space (PVS). The area comprising blood vessels and perivascular space (total area in μm^2) was slightly elevated in RF-EMR exposed rats (1086.00 ± 84.55) than the control group (711.60 ± 33.37) but not with the sham exposed group (944.50 ± 108.30) (Fig. 2A; F-5.35, $p = 0.01$). There was an increase in the blood vessel area (μm^2), in the RF-EMR group (205.50 ± 19.71) when compared with the control (116.70 ± 12.60) and sham (140.00 ± 19.74) groups (Figs. 2B, F-6.78, $p = 0.008$). Perivascular space area, which was obtained by subtracting blood vessel area from the total area was significantly elevated in the RF-EMR group (880.10 ± 74.80; Fig. 2F) compared to control (594.70 ± 29.10; Fig. 2 D) but not with sham exposed (803.90 ± 90.35; Fig. 2 E) group (Figs. 2C, F-4.48, $p = 0.02$).

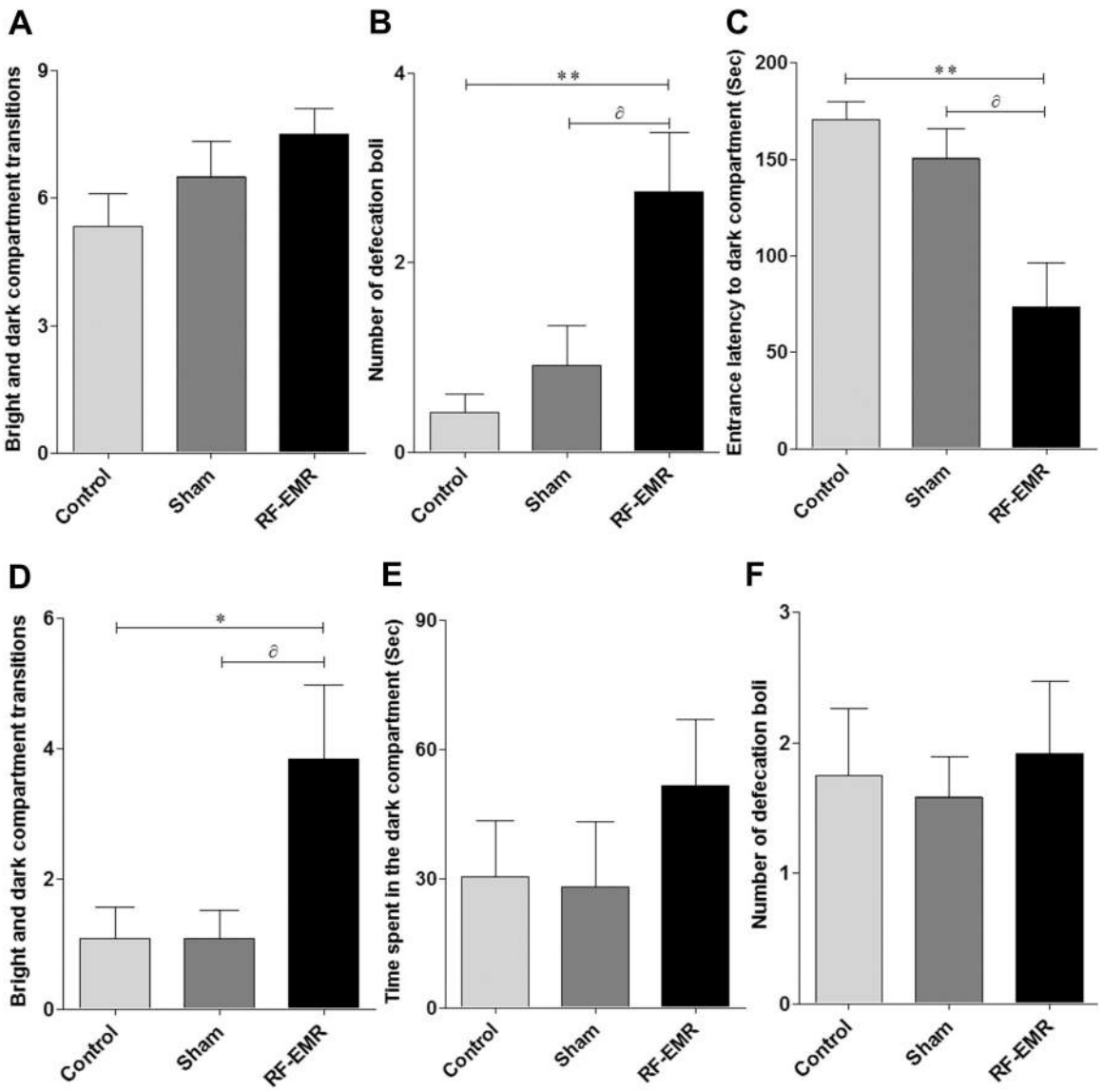

Fig. 1. RF-EMR effects on contextual fear conditioning. Bright and dark compartment transitions (A) and defecation index (B) during the habituation phase; Latency to enter the dark compartment during memory retention (C), bright and dark compartment transitions during memory retention (D), time spent in the dark compartment during memory retention (E) and defecation index during memory retention (F) in different groups. *$p < 0.05$; ∂$p < 0.05$.

3.1.2.6. Analysis of hippocampal apoptosis. Qualitative analysis of the hippocampal CA3 region of various experimental groups for the TUNEL-positive cells revealed the presence of several positive cells in the CA3 region of the RF-EMR exposed group (Fig. 3F) compared to the control (Fig. 3D) and sham exposed groups (Fig. 3E). However, quantitative analysis revealed that although the TUNEL-positive cell count was high in RF-EMR exposed animals, this increase was not statistically significant (Fig. 3G; F-1.84; $p = 0.23$). The cell density in the CA3 region was found to be decreased in the RF-EMR exposed group (Fig. 3C).

3.1.2.7. Analysis of adrenal gland microarchitecture. Morphological analysis of adrenal tissue sections from control and sham exposed animals showed no abnormal findings in the adrenal parenchyma. Sections showed an intact capsule, adrenal cortex (AC) and adrenal medulla (AM), (Fig. 4A, B, C) with cells separated by a rich network of sinusoidal capillaries in all three groups. Specifically, the adrenal cortex showed thin subcapsular zona glomerulosa (ZG) composed of small clusters and trabeculae of cells with moderate eosinophilic cytoplasm and central nucleus in control (Fig. 4A, D) and sham exposed animals (Fig. 4B, E). No significant morphological alterations were noted in the zona glomerulosa of the RF-EMR group (Fig. 4C, F) compared to other groups. Adrenal gland sections stained with reticulin stain in control (Fig. 4G), sham exposed (Fig. 4H) and RF-EMR exposed (Fig. 4I) animals also appeared normal.

The innermost zona reticularis (ZR) revealed unevenly arranged cells with granular eosinophilic cytoplasm in control (Fig. 4A), sham exposed (Fig. 4B) and RF-EMR exposed (Fig. 4C) animals. No significant morphological changes were detected among the three experimental groups in this region.

The middle thick zona fasciculata (ZF) in control (Fig. 4D) and sham exposed (Fig. 4E) animals demonstrated straight cords of round to polyhedral cells with distinct cell membranes, cytoplasm with numerous tiny lipid vacuoles and central nucleus. However, analysis of ZF under higher magnification in RF-EMR exposed animals revealed morphological changes which include focal apoptotic cells characterized by smaller cell size, densely eosinophilic cytoplasm and condensed pyknotic nucleus (Fig. 5C). Additionally, in the same region, scattered lymphocytic infiltrates (Fig. 5D) and foci of sinusoidal dilatation and congestion were observed in RF-EMR exposed animals (Fig. 5E) compared to control (Fig. 5A) and sham exposed animals (Fig. 5B).

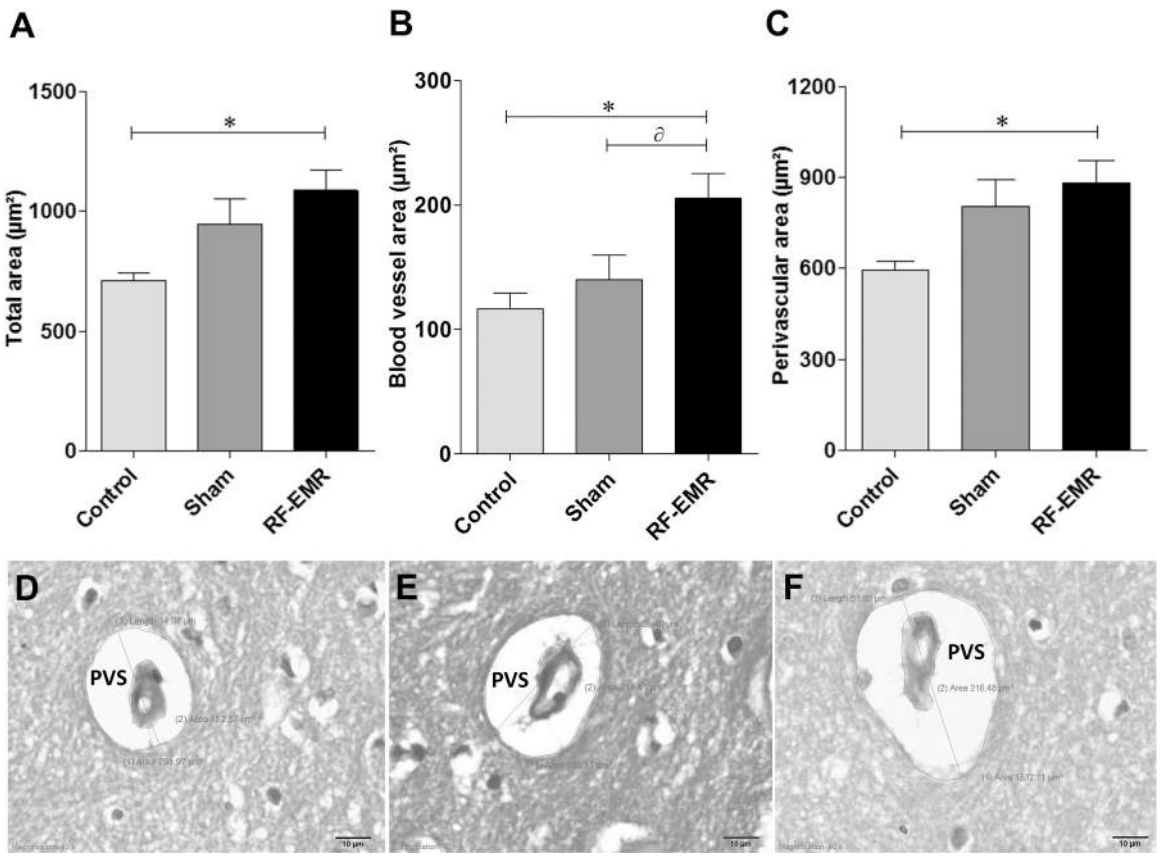

Fig. 2. RF-EMR effects on vascular and perivascular areas. Total area (A), blood vessel area (B) and perivascular area (C) measurements, and representative images of perivascular space from control (D), sham exposed (E), and RF-EMR exposed (F) animals. Note; PVS: perivascular space. Magnification 400 ×, Scare bar- 10 µm in panels D, E and F (H&E). $*p < 0.05$; $\partial < 0.05$

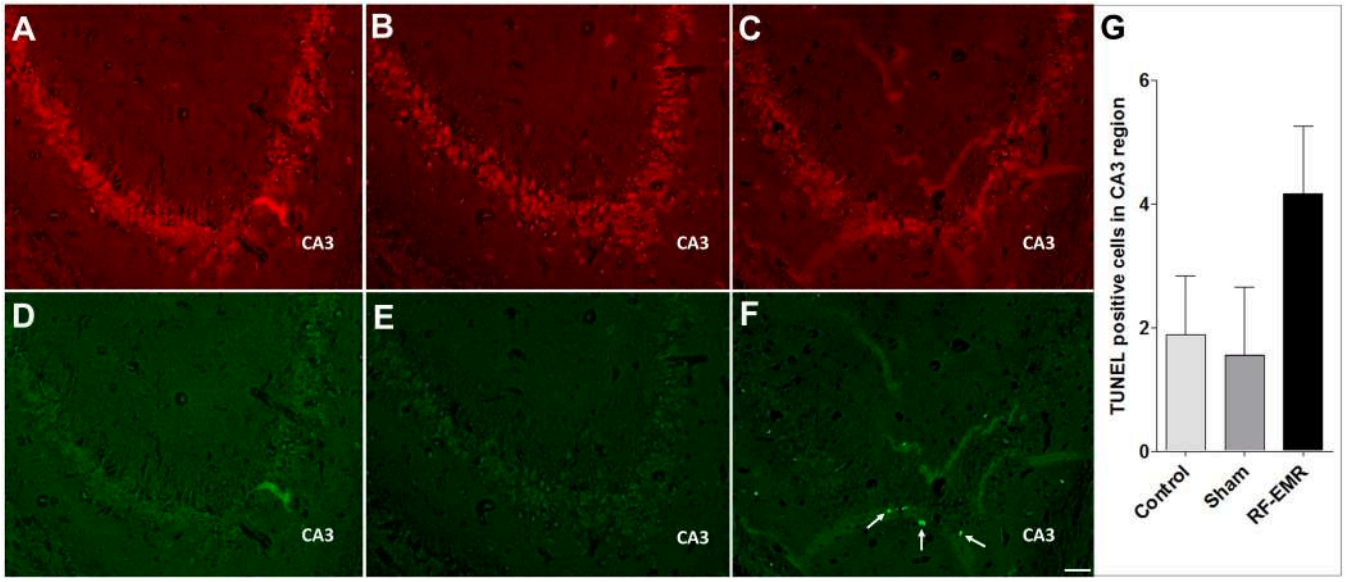

Fig. 3. RF-EMR effects on hippocampal apoptosis. Representative photomicrographs of hippocampal CA3 region from control (A and D), sham exposed (B and E) and RF-EMR exposed (C and F) rats stained with propidium iodide (A, B and C) and TUNEL (D, E, and F), and quantification of TUNEL positive cells in the hippocampal CA3 region of different groups (G). Note; TUNEL positive cells are indicated by white arrows representing DNA strand breaks. Magnification 100 ×, Scale bar- 50 µm.

The adrenal medulla is observed to be composed of nests and cords of chromaffin cells with basophilic granular cytoplasm separated by prominent vasculature in control (Fig. 6A), sham exposed (Fig. 6 B) and RF-EMR exposed (Fig. 6C) animals. The RF-EMR exposure did not significantly alter the adrenal medullary cytoarchitecture in rats.

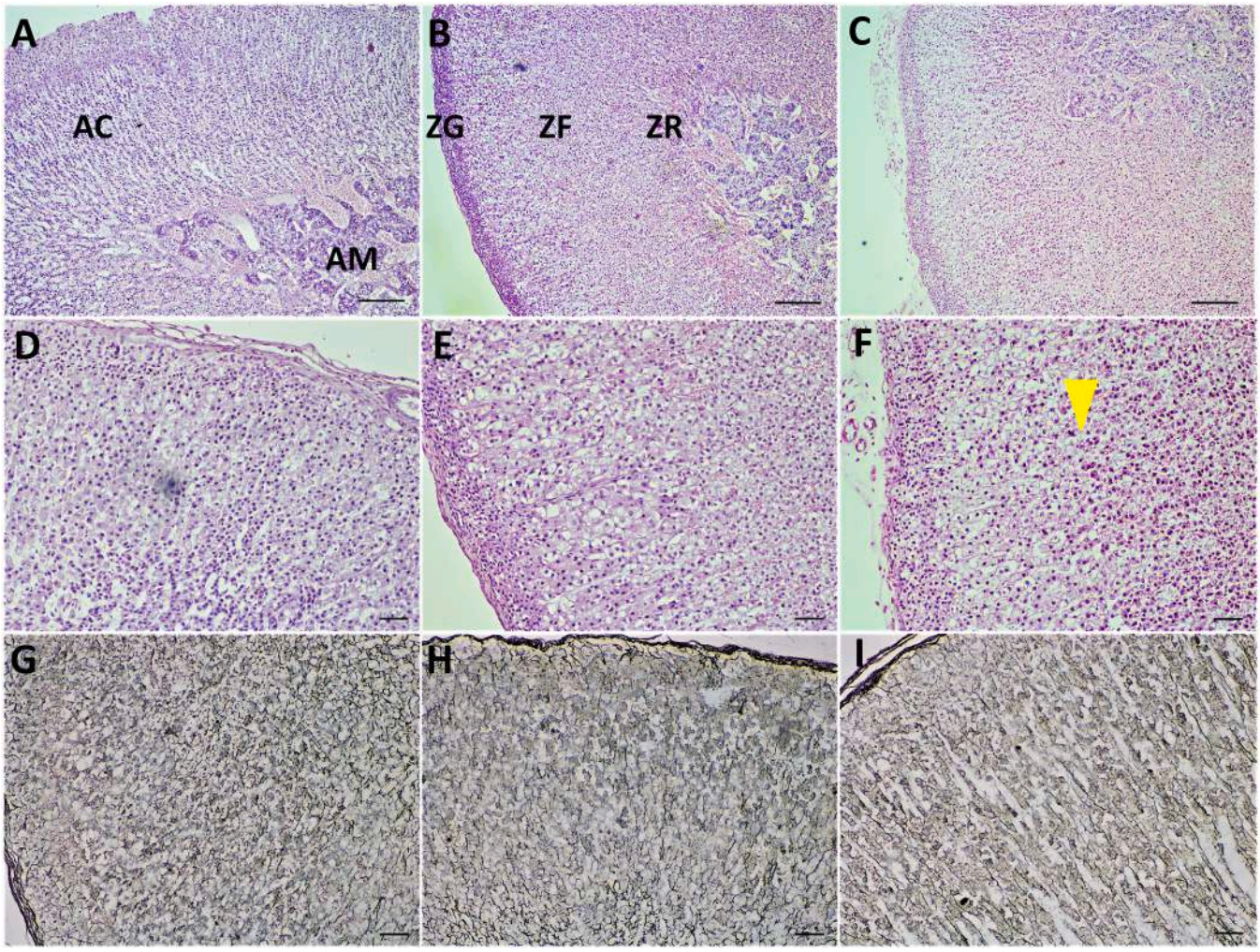

Fig. 4. RF-EMR effects on adrenal gland microarchitecture. Representative photomicrographs of adrenal parenchyma in the control (A, D- H&E; G- Reticulin), sham exposed (B, E- H&E; H- Reticulin) and RF-EMR exposed animals (C, F- H&E; I- Reticulin). The scattered lymphocytes in the ZF of the test animal is indicated by yellow arrow (F). Note; AC: Adrenal cortex, AM: Adrenal medulla, ZG: Zona glomerulosa, ZF: Zona fasciculata, Zona reticularis. Magnification 40 ×, Scale bar- 200 μm in panels A, B, & C; Magnification 100 ×, Scale bar- 50 μm in panels D, E and F; Magnification 100 ×, Scale bar- 50 μm in panels G, H and I.

4. Discussion

Mounting evidence suggests the potential impact of RF-EMR on various organs without having the capacity to "break molecular bonds" [5,23,30–32]. The current study results demonstrate that RF-EMR exposure for four weeks can induce a modification in emotional memory which is regulated by the amygdala and hippocampus. It is evident from the results that, the RF-EMR exposed animals were hyperactive and demonstrated anxiety-like behaviour as demonstrated by their multiple light-dark compartment transitions. Moreover, during the memory retention test after 24 hours, they demonstrated this behaviour in addition to decreased memory consolidation. This needs to be discussed in view of the multiple changes that could be occurring in various parts of the brain such as the amygdala and hippocampus under RF-EMR exposure. The hippocampus is a critical brain region, predominantly involved in regulating avoidance behaviour in rats. Changes in this brain region could induce a change in this behaviour. The imperative role of the hippocampus in contextual and trace tone fear conditioning has been extensively reported [33,34]. Consolidation of the inhibitory avoidance learning process is reported to be reduced when there are pre and post-session lesions of the hippocampus CA1/CA3 fields [35]. One of the other major observations in the current study is the presence of hippocampal apoptosis in the CA3 region of radiation-exposed rats compared to controls. Although apoptotic cells were observed to be more in the CA3 subfield of the hippocampus in RF-EMR exposed animals, this difference compared to controls was not significant as stated in the results section. This result is only partially aligned with the earlier published report where they observed Bax/Bcl2 mRNA expression in mice hippocampus in a duration-dependent manner [36]. One of the reasons for the difference between the current study and this report [36] is the difference in the radiation dose used or duration of exposures employed in these studies. The intragroup counting data variation could be another reason for the insignificant observation, which is possibly due to a smaller sample size used for the apoptotic assay, which is one of the limitations of this study. Evidence also suggests that the basolateral amygdala has an important role in modulating fear learning and consolidation [37]. RF-EMR exposure for 21 days as described in this study was reported to alter the structural integrity of basolateral amygdala [7], which could also contribute to the altered behaviour seen in RF-EMR exposed animals.

There is inconsistency among various research reports about the effect of radiofrequency electromagnetic radiation on emotional learning and memory in rats. In one of the earlier studies, RF-EMR exposure (900 MHz) of pregnant rats during their entire gestational period affected the behaviour and learning of their young ones [11]. In contrast, in the studies of Keles et al. [12] there was no change in the passive avoidance behaviour of rats exposed to RF-EMR. However, in another study [15], one-month exposure to RF-EMR caused changes in

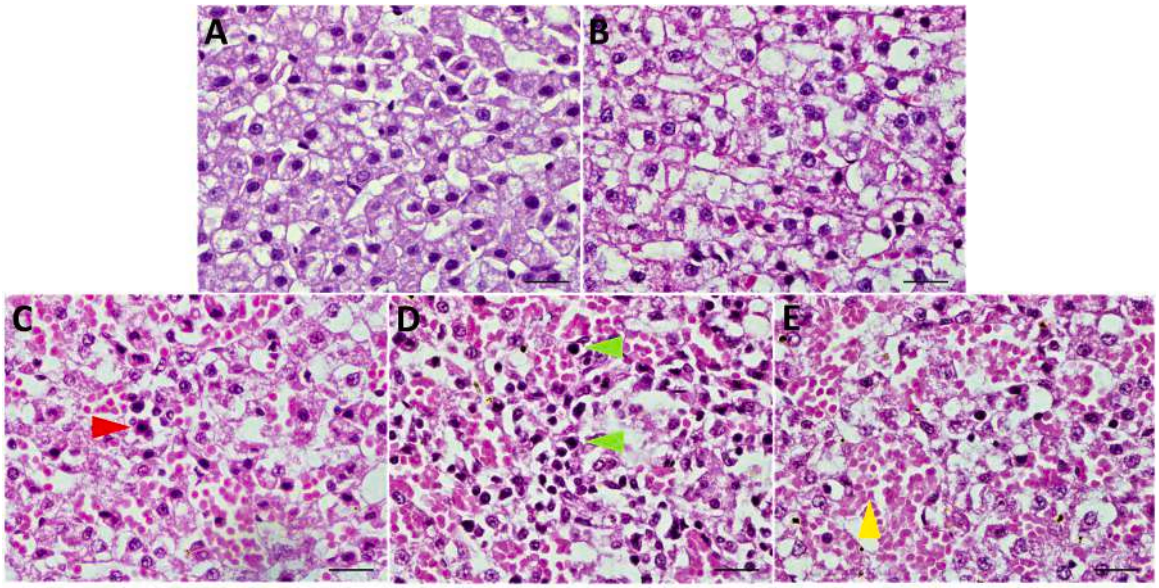

Fig. 5. RF-EMR effects on zona fasciculata. Representative photomicrographs of zona fasciculata showing polyhedral cells with foamy vacuolated cytoplasm in control (A), sham exposed (B) animals, and zona fasciculata from RF-EMR exposed animals showing apoptosis (C; indicated by red arrowhead), lymphocytic infiltrates (D; indicated by green arrowhead) and congested sinusoids (E; indicated by yellow arrowhead). Magnification 400 ×, Scale bar- 20 μm in panels A, B, C, D, and E (H&E).

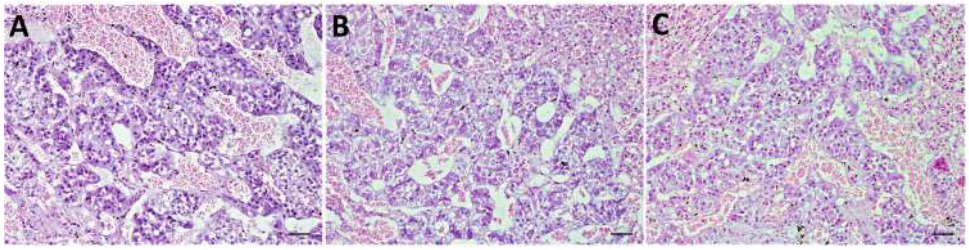

Fig. 6. RF-EMR effects on adrenal medulla. Representative photomicrographs of adrenal medulla showing chromaffin cells with abundant granular cytoplasm separated by prominent vasculature, in control (A), sham exposed (B) and RF-EMR exposed animals (C). Magnification 100 ×, Scale bar- 50 μm in panels A, B, and C (H&E).

avoidance behaviour and morphology hippocampus of rats. In a study by Ahmadi et al. [14], one-month exposure of rats to similar radiation had altered the inhibitory avoidance memory. In a recent study, young adult male rats when exposed to RF-EMR for four months continuously (2 h/day), experienced oxidative stress in the hippocampus, elevated levels of circulatory IL-1beta, IL-6, and TNF-alpha along with increased levels of stress hormones in rats along with certain changes in fear memory [13]. There could be multiple reasons for the altered fear memory in rats. This may be due to RF-EMR exposure-induced imbalance in brain neurotransmitters [14], changes in neuronal architecture in the brain regions [38], imbalance in oxidant and antioxidant status [39,40], or changes in cell survival in various brain regions [41,42]. Results of the current study demonstrate that perivascular space and cell survival in the hippocampus were altered in RF-EMR exposed rats. Although hippocampal cell survival is related to altered contextual fear conditioning [15,43], there are still conflicting reports on enlarged hippocampal perivascular space and associated changes in cognition [44–46]. Hippocampal perivascular space was enlarged in the RF-EMR group, which indicates that certain key changes could be going on at the microarchitecture level of these regions due to the RF-EMR exposure. Several etiopathogenesis have been suggested for the development of enlarged perivascular space. 1) Impairment of interstitial fluid circulation, 2) Spiral elongation of arteries, 3) Brain atrophy and/or perivascular myelin loss, and 4) Immune cell accumulation in the perivascular space [47]. However, it will be too early to attribute a possible cause for the observed change in the perivascular space of RF-EMR exposed rats. Further studies are warranted to determine the cellular mechanisms attributed to increasing the PVS in RF-EMR exposed animals and its potential impact on other forms of memory and cognitive functions. Recent reports demonstrate evidence for the association of an enlarged PVS with impaired cognitive function [47].

Modifications in the adrenal gland morphology are the other important findings of the current study. The adrenal cortical cells in the zona fasciculata synthesize glucocorticoids (cortisol) in response to adrenocorticotropin hormone (ACTH) from the pituitary gland. Cortisol has a protective role during stressful conditions. Activation of the HPA axis is an essential part of stress adaptation and survival. Multiple studies have proven that RF-EMR may cause chronic stress in animals [48,49]. Shahabi et al. [23] reported the presence of vacuoles in the brain of rats exposed to RF-EMR. In the same study, 8 weeks of exposure to RF-EMR induced significant changes in the adrenal gland morphology and increased the size and number of brain vacuoles. Current results suggest that RF-EMR may act as a chronic stressor, which causes inflammation and cell death in the zona fasciculata possibly due to fewer levels of ACTH [27] caused by suppression of the HPA-axis via the hippocampus and hypothalamus resulting adrenal weakness. This possibly further decreases body's innate ability to cope with stress by completely dysregulating the stress response system. The body then becomes increasingly susceptible to inflammatory responses and generalized oxidative stress responses, leading to pathophysiological

changes such as altered cognition, cell death and morphological changes in multiple organs, including the hippocampus, as observed in RF-EMR exposed animals. Although, we only have some indication about this vicious cycle currently, further studies will provide a clearer picture of these altered processes and the body's innate mechanisms to withstand the harmful effects of RF-EMR. Analyzing the corticosterone (or any other stress markers) levels in the blood at various timelines of this study would have been a useful parameter to support the argument mentioned above. Still, this assay couldn't be done due to the following reasons. Blood withdrawal after the experimental period (on the 29th day) requires restraining of animals and it is an invasive process which might alter the animal's behaviour before the avoidance test. Moreover, additional stress imposed on animals before the avoidance test might be a confounding factor when the actual test is done. Although this is another limitation of the study, to overcome this, more permanent changes, such as structural alterations in the adrenal gland following RF-EMR exposure have been reported.

It is evident that a potentially detrimental effect of RF-EMR exposure on animal behaviour does exist due to the non-thermal effects of RF-EMR. However, one should be careful while extrapolating this data into humans because of differences in the mode and nature of the RF-EMR exposure in humans. It is worth noting that the above-mentioned changes were observed in adolescent rats suggesting that future studies need to be planned and systematically carried out on these vulnerable populations to categorically understand the neuro-behavioural and psychological effects of RF-EMR on humans.

5. Conclusion

Chronic RF-EMR exposure in rats induced significant alterations in fear memory which was associated with enlarged perivascular space and apparent apoptosis in the hippocampus. Along with this, evidence of lymphocytic infiltration, congested sinusoids and apoptotic-like changes were observed in the adrenal gland. However, further studies are required to unravel the possible molecular mechanisms by which the above effects are induced in rats following RF-EMR exposure.

Ethical approval

All procedures performed in this study are approved by the Institutional Animal Ethics Committee (IAEC/KMC/36/2009–2010).

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CRediT authorship contribution statement

Sareesh Narayanan: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Raju Kumar:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Naveen Kumar:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation. **Pavithra Prabhakar:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation. **Satheesha Nayak:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation. **Perumunda Bhat:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation.

Declaration of Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SNN conceived the idea, designed, executed the study, analyzed/interpreted the data and wrote the first original draft of the manuscript. RSK participated in behavioural data generation and contributed to editing the article. NK and PP performed histological procedures and interpreted histological data. SNB supervised the project, contributed to editing and preparing the final manuscript. PGB co-supervised the project, contributed to editing the article and approved the final manuscript. All authors have read and approved the final manuscript.

Consent to participate

Not applicable

Consent to publish

Not applicable

Data Availability

Data will be made available on request.

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