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ORIGINAL ARTICLE



Effects of 1800 MHz RF-EMF exposure on DNA damage and cellular functions in primary cultured neurogenic cells

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ABSTRACT

Purpose: To systematically evaluate the effects of 1800 MHz radiofrequency electromagnetic fields (RF-EMF) exposure on DNA damage and cellular functions in primary cultured neurogenic cells.

Materials and methods: The primary cultured astrocytes, microglia and cortical neurons were exposed to RF-EMF at a SAR of 4.0 W/kg. The DNA damage was evaluated by γ H2AX foci formation assay. The secretions of pro-inflammatory cytokines (TNF- α , IL-6 and IL-1 β) in astrocytes and microglia, microglial phagocytic activity and neuronal development were examined by enzyme-linked immunosorbent assay, phagocytosis assay and immunofluorescent staining on microtubule-associated protein tau, microtubule-associated protein 2, postsynaptic density 95 and gephyrin, respectively.

Results: RF-EMF exposure did not significantly induce γ H2AX foci formation in three primary cultured neurogenic cells. Furthermore, RF-EMF exposure did not significantly affect the secretion of cytokines in astrocytes and microglia, and the morphological indicators of dendrites or synapses of cortical neurons. However, the exposure significantly reduced the phagocytic activity of microglia and inhibited the axon branch length and branch number of cortical neurons.

Conclusions: Our data demonstrated that exposure to RF-EMF did not elicit DNA damage but inhibited the phagocytic ability of microglia and the axon branch length and branch number of cortical neurons.

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KEYWORDS

RF-EMF; primary cultured neurogenic cells; DNA damage; cellular functions

Introduction

In recent decades, the developing mobile communication technologies are widely used in daily life, which has raised considerable concerns about possible health effects induced by radiofrequency electromagnetic fields (RF-EMF), e.g. emitted by mobile phone. In 2011, the International Agency for Research on Cancer (IARC) has classified RF-EMF as a possible human carcinogen (group 2B) (IARC working group et al. 2011), based mainly on the limited epidemiological evidence of the association between mobile phone using and an increased risk for brain cancer from the Interphone study (Interphone Study Group 2010) and the Hardell group (Hardell et al. 2011).

Compare to other organs, the brain is frequently exposed and in very close proximity to the mobile phone. In addition to brain cancer, some epidemiological studies have suggested a possible association between RF-EMF exposure and risk of other nervous system diseases, including headaches, changes in sleep patterns, cognitive abilities in children (Redmayne et al. 2013; Eyvazlou et al. 2016; Redmayne et al. 2016), however, others indicated lack of any association (Malek et al. 2015; Calvente et al. 2016). Previous *in vivo* studies have focused on the effects of RF-EMF exposure on

behavior (Daniels et al. 2009; Klose et al. 2014), cognitive functions (Ammari et al. 2008; Hao et al. 2013; Deshmukh et al. 2015), brain electrical activity (Marino et al. 2003; Lipping et al. 2009; Vorobyov et al. 2010) and neurogenesis (Odaci et al. 2008; Bas et al. 2009; Ragbetli et al. 2009; Ragbetli et al. 2010), but the findings were inconclusive and lack of mechanism explanations (Cucurachi et al. 2013). Regarding *in vitro* studies, the researchers have investigated the effects of RF-EMF exposure on genotoxicity (Wang X et al. 2015; Zuo et al. 2015) and cellular functions, including cellular behaviors (Merola et al. 2006; Joubert et al. 2007), gene/protein expression (Zhao R et al. 2007; Zhao TY et al. 2007), calcium homeostasis (Green et al. 2005; O'Connor et al. 2010), neuroinflammation (Thorlin et al. 2006; Hirose et al. 2010) and oxidative stress (Hoyto et al. 2008; Poullietier de Gannes et al. 2011) in different neural cell models. However, the results from *in vitro* studies were generally inconsistent and could not support the associations between RF-EMF exposure and neurological disorders, which was primarily due to the various research models, exposure conditions and experimental protocols adopted by different groups. Therefore, the biological effects of nervous system and neurogenic cells in response to RF-EMF require further investigations.

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Astrocytes and microglia, two major types of glial cells, involved in the regulation of the immune response to pathological processes as well as in the development of brain cancer. The cerebral cortex is the largest region of the mammalian brain and plays a key role in memory, attention, perception and cognition. Cortical neurons are crucially involved in associative brain functions and have been proven to be a valuable cell model to study neuronal function and developmental neurotoxicity. Therefore, we included primary cultured astrocytes, microglia and cortical neurons in our study to evaluate the genotoxicity of 1800 MHz RF-EMF by detection of γ H2AX foci formation, an early marker of DNA double-strand breaks (DSB) (Rogakou et al. 1998). Because of the diverse functions of different neurogenic cells, we also investigated the immunological roles of astrocytes and microglia and neuronal development in cortical neurons upon RF-EMF exposure.

Materials and methods

Animal ethics

All procedures in this study were approved by the Animal Ethics Committee at Zhejiang University (Approval ID ZJU2009102020). All efforts were made to minimize the number of animals used and their suffering.

Exposure system

The sXc-1800 exposure system used in the study was designed by the Foundation for Research and Information

Technologies in Society (IT'IS Foundation, Zurich, Switzerland). A detailed description of the system and its dosimetry has been described previously (Diem et al. 2005; Xu et al. 2013). Briefly, two identical exposure chambers, one for exposure and the other for sham exposure, were placed inside a conventional incubator to ensure constant environmental conditions (37°C , 5% CO_2) (Figure 1(A)). The entire exposure set-up was controlled and monitored by a computer through specific sensors, enabling the automated control for exposure intensity and exposure time. All experiments were performed with the GSM 1800 MHz signals with intermittent exposure (5 min field on and 10 min field off) at an average Specific Absorption Rate (SAR) of 4.0 W/kg. The signal is amplitude modulated by rectangular pulses with a repetition frequency of 217 Hz and a duty cycle of 1:8 (pulse width 0.576 ms). During exposure, the temperature in the chambers was monitored and maintained at $37.0 \pm 0.1^{\circ}\text{C}$.

Cell culture

Primary newborn rat mixed glial cells were derived from postnatal day 1 (P1) rat brains (Sprague-Dawley). Briefly, meninges-free cortices from P1 rat were isolated and trypsinized. Cells were plated in T75 flask pre-coated with poly-L-lysine (PLL, Sigma, St. Louis, MO) in Minima essential medium (MEM, Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco, Carlsbad, CA) and fed every three days. After 7 d, the cultures were shaken manually for 10 min to collect microglia and remaining mixed glial

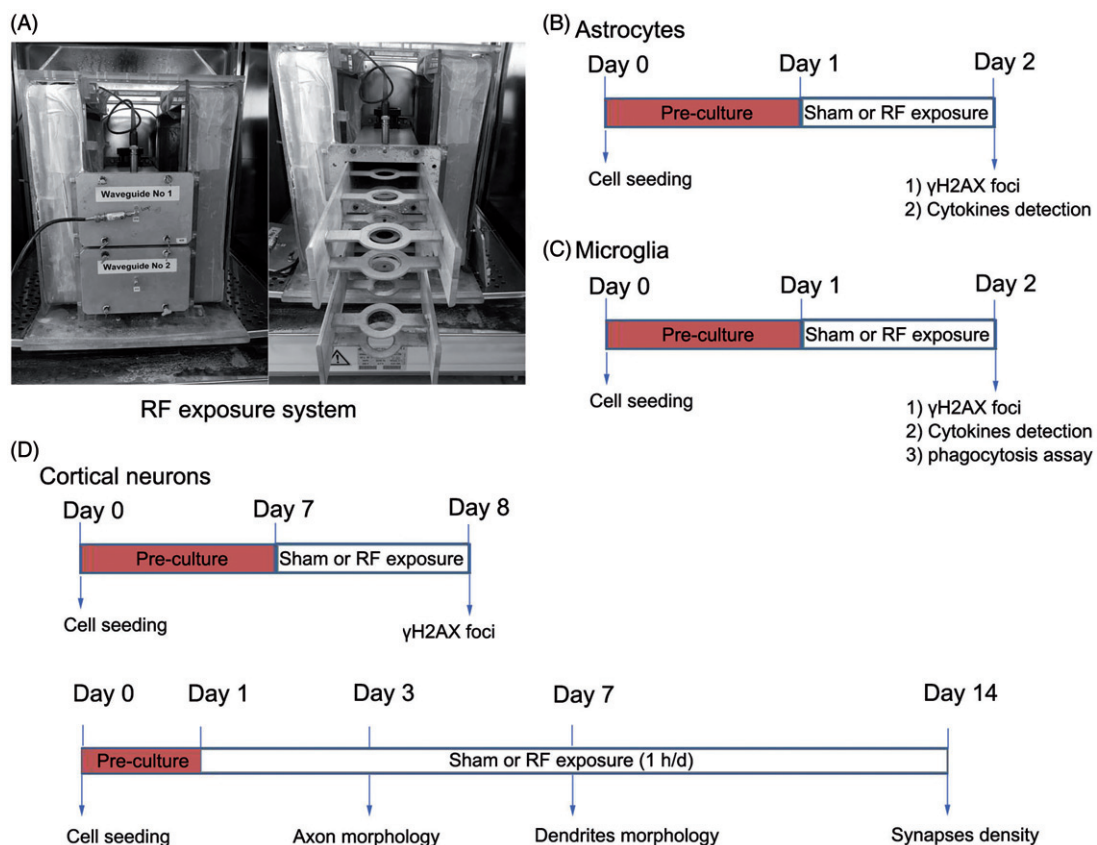


Figure 1. Schematics of experiments. (A) The RF-EMF exposure system consisted of two rectangular waveguide chambers installed in an incubator and each chamber contained six 35 mm Petri dishes; (B–D) Exposure timeline of astrocytes (B), microglia (C) and cortical neurons (D).

cells were cultured in fresh complete medium (MEM with 10% FBS). The microglia were transferred to new dishes for further culture under the complete medium. For astrocytes, the remaining mixed glial cells were cultured for additional 7 d (i.e. on 14 d) and then shaken vigorously at 240 rounds per minute for 18 h to remove microglia and oligodendrocytes for purification of astrocytes. The astrocytes were subcultured in Dulbecco's modified eagle medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% FBS. Culture purity for microglia and astrocytes was determined by staining with mouse anti-Iba1 (sc-32725; Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:400) and mouse anti-GFAP antibody (TA336707; Zhongshan Golden Bridge Biotechnology, Beijing, China; diluted 1:800), respectively. Astrocytes and microglia pre-cultured for 24 h were exposed or sham-exposed to RF-EMF to detect γ H2AX foci formation, the release of TNF- α , IL-6, and IL-1 β and phagocytic capacity of microglia (Figure 1(B,C)).

Primary cortical neurons were cultured from cortices of embryonic day 18 (E18) rats (Sprague-Dawley). Briefly, meninges-free cortices were isolated, trypsinized and plated onto PLL pre-coated 35 mm Petri dishes and maintained at 37 °C in 5% CO₂ for 4 h to let the neurons attach to the bottom. Finally, unattached cells and remaining debris were removed by replacing the serum-free Neurobasal medium (Gibco) supplemented with 2% B27 supplement (Invitrogen), 1% L-glutamine (Invitrogen), 100 units/mL penicillin (Invitrogen) and 100 μ g/mL streptomycin (Invitrogen). After 3 d, cells were treated with 2.5 μ M cytosine arabinoside (Sigma) to curb further proliferation of non-neuronal cells. The medium was half renewed every 3 d. The neurons were identified by staining with the neuronal marker microtubule-associated protein 2 (MAP2, ab11268; Abcam, Cambridge, UK; diluted 1:500) at 7 d. For γ H2AX foci formation detection, cortical neurons were exposed or sham-exposed to RF-EMF for 1, 6 or 24 h at 7 d. For analysis of neurite outgrowth, the cells were exposed or sham-exposed to RF-EMF for 1 h per day from DIV (day *in vitro*) 1 to DIV (day *in vitro*) 14 (Figure 1(D)).

Immunofluorescent detection of γ H2AX

Immunofluorescence staining was carried out as previously described (Su, Wei, et al. 2017; Su, Yimaer, et al. 2017). The number of γ H2AX foci per cell and the percentage of γ H2AX foci positive cells was used as the indicators of DNA DSB. As positive controls, 0.1 μ M of 4-Nitro-Quinoline-1-Oxide (4NQO, Sigma), a carcinogenic chemical, was used to induce DNA damage.

Enzyme-linked immunosorbent assay (ELISA) of TNF- α , IL-6 and IL-1 β

The supernatants from astrocytes and microglia were collected and stored at -80 °C until detected. TNF- α , IL-6 and IL-1 β levels were measured by commercial ELISA kits (RayBiotech, Atlanta, GA) according to the manufacturer's protocols and instructions. Briefly, the 96-well plate with standards or test samples of conditioned cell culture medium

were incubated for 2.5 h at room temperature, then washed three times followed by incubating with prepared biotin-conjugated primary antibody against rat TNF- α , IL-6 and IL-1 β for 1 h. Following incubation with prepared streptavidin conjugated secondary antibody solution for 45 min, the plates were then developed with 3,3',5,5'-tetramethylbenzidine (TMB) reagent for 30 min. The reaction was terminated by stop solution and absorbance values were measured at 450 nm using a Varioskan Flash Multimode Reader (BMG LABTECH, Cary, NC) immediately. The cells were treated with 1.0 μ g/mL of lipopolysaccharide (LPS, Sigma) for 1 h as a positive control.

Phagocytosis assay

A quantitative measurement for the phagocytic capacity of microglia was obtained by allowing the cells to engulf fluorescent microspheres. After exposure, the cells were incubated with the fluorescent microspheres (Nile Red fluorescent (535/575), 1.0 μ m diameter, Invitrogen) for 1 h at 37 °C. Dishes were then placed on ice and washed gently with the cold PBS to arrest microspheres uptake. Then, the cells were fixed in 4% formaldehyde for 15 min and more than 60 cells were counted under fluorescent microscope (Nikon, Tokyo, Japan). The phagocytic activity was determined by average number of microspheres/cell, the percentage of positive cells with microspheres and the percentage of cells with different number of microspheres. Microglia were treated with 1.0 μ g/mL of LPS for 1 h as a positive control.

Analysis of neurite outgrowth

The morphological analysis of the axon and dendrites in cortical neurons was determined by immunostaining with a monoclonal antibody to Tau (4019; Cell Signaling Technology, Boston, MA; diluted 1:800) as an axonal marker at DIV3, or with a monoclonal antibody to MAP2 (ab11268; Abcam; diluted 1:500) as a dendritic marker at DIV7. Alexa Fluor 546-conjugated goat anti-mouse IgG (Zhongshan Golden Bridge Biotechnology; diluted 1:300) was used as a second antibody. The fluorescence images were captured by Nikon confocal microscope with a 20 $\times$ objective. Axon length, axon branch length and branch number were measured and quantified by MetaMorph software (Molecular Devices, Sunnyvale, CA). The dendritic morphology analysis was focused on four different parameters: total dendritic length, number, and length of primary and higher order dendrites and Sholl analysis. These counts were performed by tracing along each neuronal projection using Imaris software (Bitplane, South Windsor, CT).

Analysis of synaptic formation

At DIV14, the cortical neurons were immunostained with a monoclonal antibody to postsynaptic density 95 (PSD95, ab2723; Abcam; diluted 1:200) and gephyrin (147011; Synaptic Systems, Gottingen, Germany; diluted 1:400) as

markers of excitatory and inhibitory synapses, respectively. The fluorescence images were captured using Nikon confocal microscope with a 60 $\times$ objective. A total of 60–70 randomly selected neurons were analyzed from three independent experiments per exposure condition. PSD95 and gephyrin cluster density in neurons were quantified using MetaMorph software. Density values were calculated as number of clusters per micrometer of dendrite length.

Statistical analysis

Values are given as mean $\pm$ standard error of the mean (SEM). Statistical significance was tested using Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS Inc., Chicago, IL). Statistical significance of γ H2AX foci formation, cytokine release, neurite outgrowth, and density of synapses between sham and RF-EMF exposure groups, or between positive control and control groups was tested by unpaired Student's *t*-test. The phagocytic ability of microglia between sham and RF-EMF exposure groups or between positive control and control groups was tested by Wilcoxon rank sum test.

Indications of statistical significance correspond to *p* values $<.05$ (*) and $p <.01$ (**).

Results

Effects of RF-EMF exposure on γ H2AX foci formation in primary cultured neurogenic cells

To evaluate genotoxicity of RF-EMF exposure, we examined γ H2AX foci formation in primary cultured neurogenic cells after RF-EMF exposure for 1, 6 or 24 h. The results showed that RF-EMF exposure did not affect the average number of γ H2AX foci per cell or the percentage of γ H2AX foci-positive cells in astrocytes (Figure 2). For microglia, exposure to RF-EMF for 6 or 24 h but not 1 h slightly increased average number of γ H2AX foci per cell and percentage of γ H2AX foci positive cells, but the differences did not reach statistical significance ($p = .21$ and $.16$) (Figure 3). For cortical neurons, the exposure did not result in changes of γ H2AX foci formation (Figure 4). However, treatment with $.04 \mu\text{M}$ of 4NQO significantly increased γ H2AX foci formation in each type of cells (Supplementary Information Figures S1–S3). Taken together,

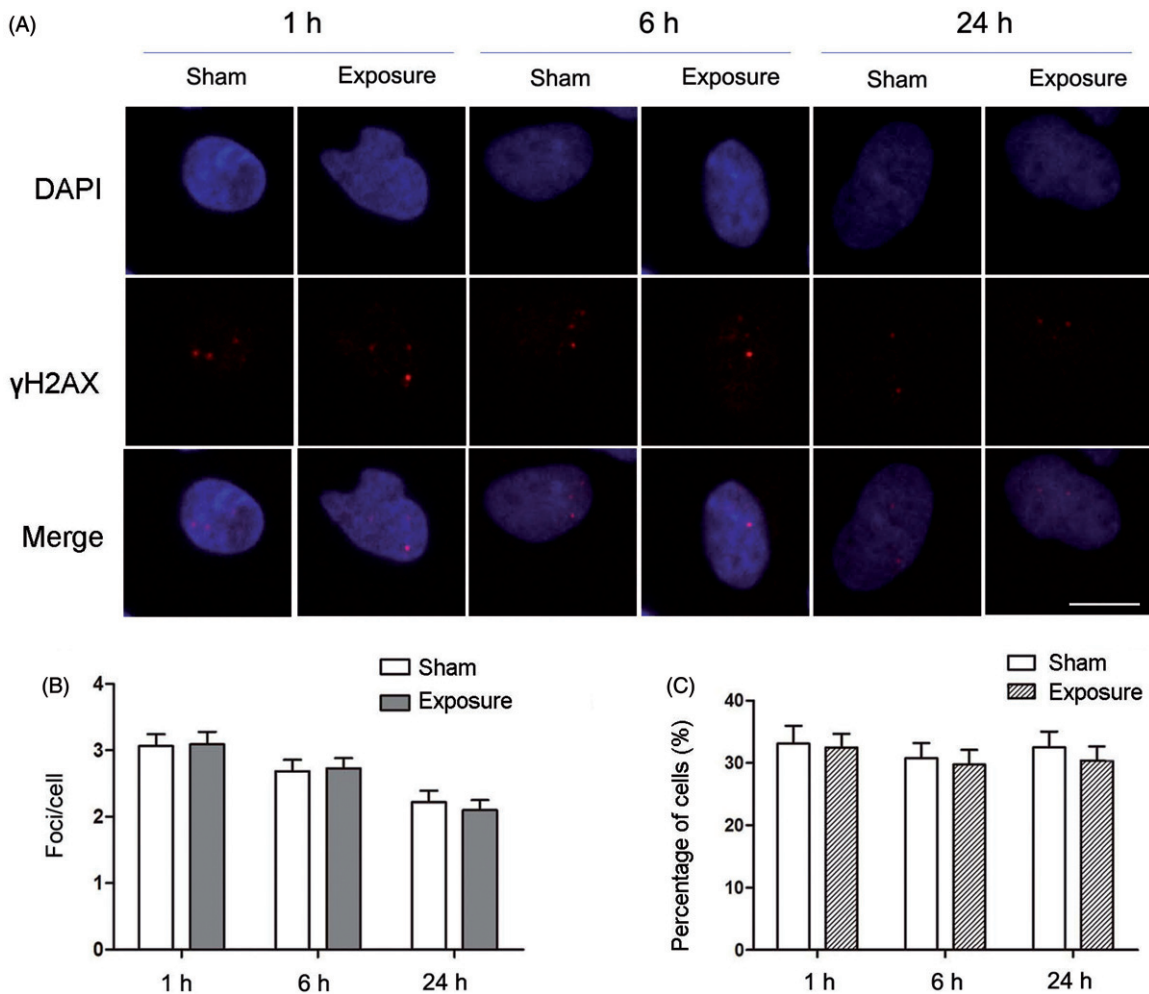


Figure 2. Effects of exposure to RF-EMF on γ H2AX foci formation in primary cultured astrocytes. (A) Representative images of γ H2AX foci formation in astrocytes after sham or RF-EMF exposure by immunofluorescent staining. Red dots showing γ H2AX foci and blue staining with DAPI showing nuclei. Histograms showing the average number of γ H2AX foci per cell (B) and the percentage of γ H2AX foci positive cells (C). Error bars indicate SEM for three independent experiments. Bar = 10 μm .

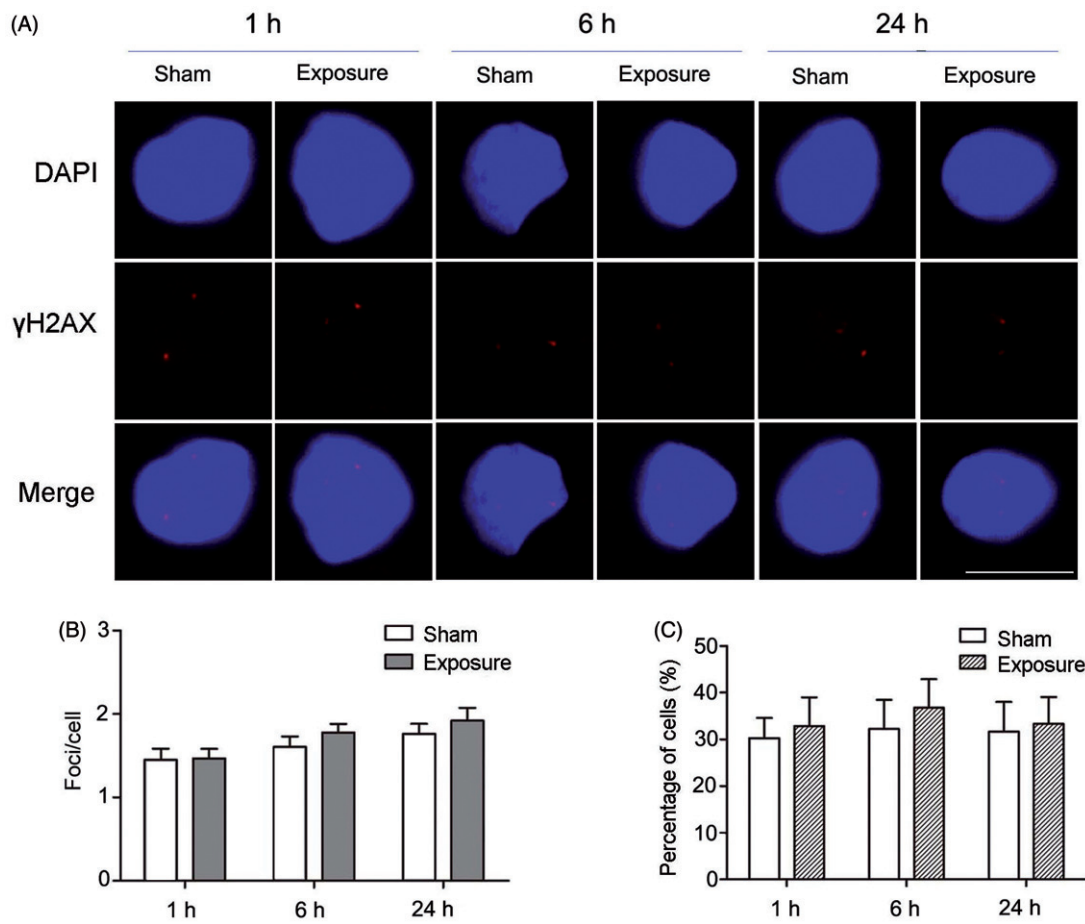


Figure 3. Effects of exposure to RF-EMF on γ H2AX foci formation in primary cultured microglia. (A) Representative images of γ H2AX foci formation in microglia cells after sham or RF-EMF exposure by immunofluorescent staining. Red dots showing γ H2AX foci and blue staining with DAPI showing nuclei. Histograms showing the average number of γ H2AX foci per cell (B) and the percentage of γ H2AX foci positive cells (C). Error bars indicate SEM for three independent experiments. Bar = 10 μ m.

our results demonstrated that RF-EMF exposure did not significantly increase γ H2AX foci formation in these primary cultured neurogenic cells.

Effects of RF-EMF exposure on cytokine release in astrocytes and microglia

To investigate whether RF-EMF exposure could influence cellular functions, the secretions of pro-inflammatory cytokines, as immunologic functions, were examined by ELISA assay in astrocytes and microglia after RF-EMF exposure. The results showed that RF-EMF exposure for 24 h did not significantly change the level of cytokines, TNF- α , IL-6 or IL-1 β , in astrocytes ($p = .59$, $.73$ and $.63$), while treatment with LPS for 1 h increased TNF- α , IL-6 and IL-1 β levels ($p < .01$, Figure 5(A)). In microglia, TNF- α , IL-6 and IL-1 β levels were not altered by RF-EMF exposure for 24 h ($p = .32$, $.47$ and $.55$). TNF- α and IL-6 but not IL-1 β level in microglia significantly increased after LPS treatment for 1 h, suggesting different effects of LPS on various cytokines in microglia (Figure 5(B)). Taken together, our data did not provide evidence that RF-EMF exposure could influence immunologic functions in astrocytes and microglia.

Alteration of phagocytic ability of microglia after RF-EMF exposure

The effect of RF-EMF exposure on the phagocytic activity of microglia was investigated using fluorescence-labeled microspheres as a prey. The results showed that RF-EMF exposure dramatically reduced the phagocytic ability of microglia, including the significant changes in the average number of microspheres per cell ($p = .03$) and the percentage of cells with none and more than six microspheres per cell ($p = .04$ and $.02$, Figure 6(A,B,D)), although no significant difference was found in the percentage of microsphere-positive cells between sham and RF-EMF exposure groups ($p = .33$, Figure 6(C)).

Effects of RF-EMF exposure on morphological development in neuron

To analyze the effects of RF-EMF exposure on neuronal morphological maturation, primary cortical neurons were exposed to RF-EMF from DIV1 to DIV14 (1 h per day). The neurite outgrowth, including axon and dendrites growth, was first evaluated at DIV3 and DIV7, respectively. We found that RF-EMF exposure significantly inhibited the axon branch

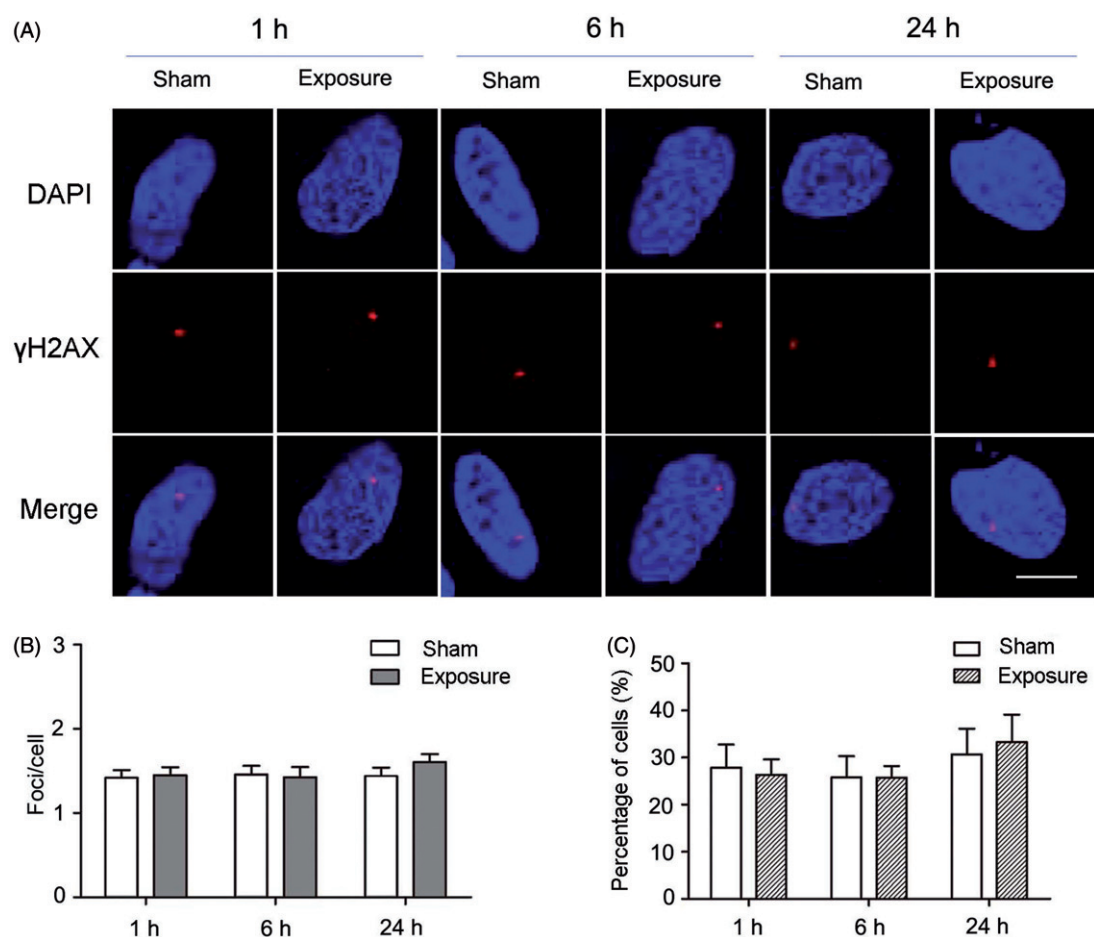


Figure 4. Effects of RF-EMF exposure on γ H2AX foci formation in primary cultured cortical neurons after sham or RF-EMF exposure by immunofluorescent staining. Red dots showing γ H2AX foci and blue staining with DAPI showing nuclei. Histograms showing the average number of γ H2AX foci per cell (B) and the percentage of γ H2AX foci positive cells (C). Error bars indicate SEM for three independent experiments. Bar = 10 μ m.

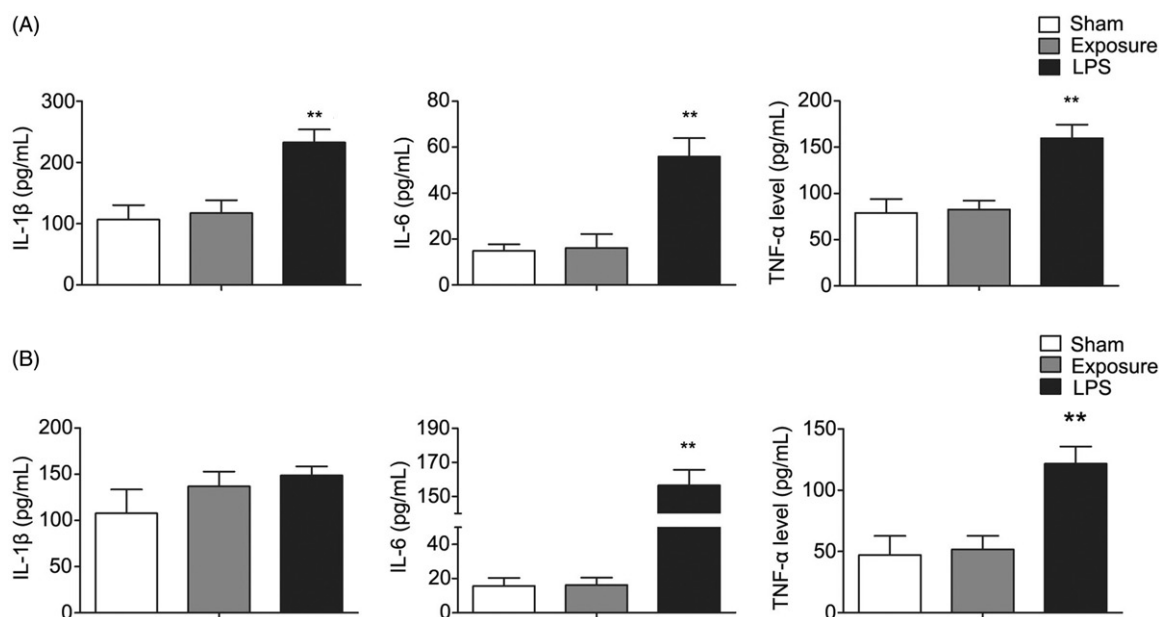


Figure 5. Effects of exposure to RF-EMF on the secretion of pro-inflammatory cytokines in astrocytes and microglia. Histograms showing the concentration of cytokine IL-1 β (left), IL-6 (middle) or TNF- α (right) in astrocytes (A) and microglia (B) after exposure to RF-EMF by ELISA assay. Values shown are mean $\pm$ SEM from three independent experiments. ** p < .01 compared to sham group.

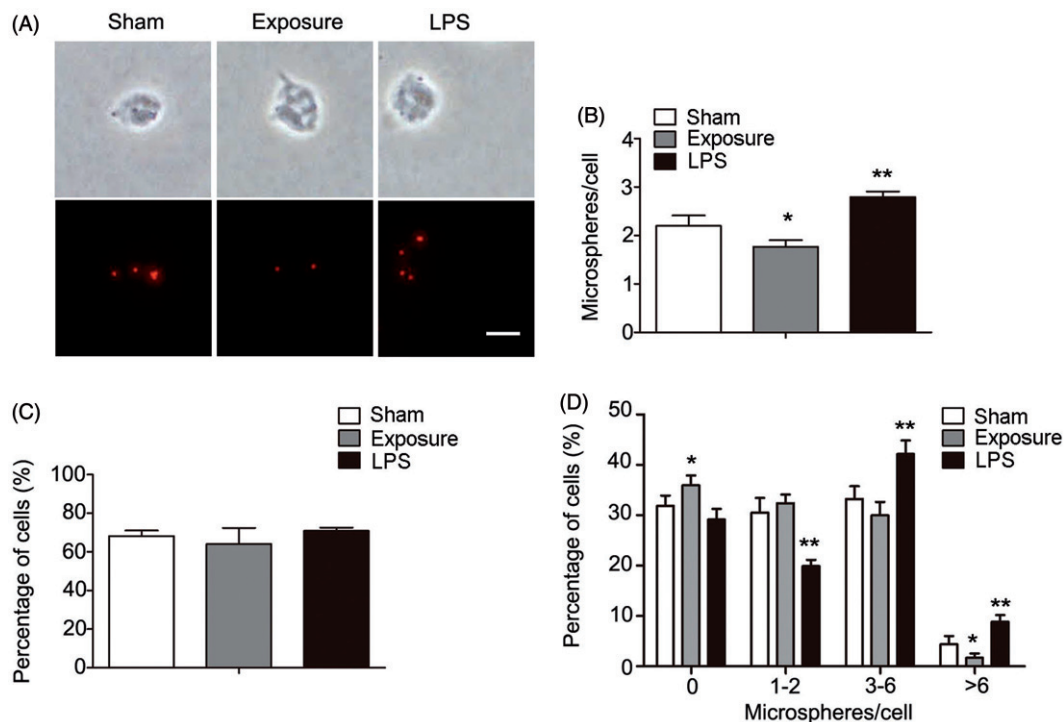


Figure 6. Effects of exposure to RF-EMF on microglial phagocytosis of microsphere. (A) Representative images of phagocytosis of microspheres by microglia after exposure to 1800 MHz RF-EMF for 24 h. Histograms showing the average number of microsphere per cell (B), the percentage of positive cells with microspheres (C) and the percentage of cells with different number of microspheres (D). Values shown are mean $\pm$ SEM from three independent experiments. * $p < .05$ and ** $p < .01$ compared to sham group. Bar = 10 μ m.

length and branch number ($p = .027$ and $.021$), but did not affect the axon length ($p = .73$, Figure 7(A)). For dendritic morphology analysis, the results showed that RF-EMF exposure did not significantly change the total dendritic length, but slightly decreased primary and higher order dendrites length while the difference did not reach significant ($p = .11$, $.23$ and $.37$). No significant change was found on the average number of branches between sham and RF-EMF exposure groups. In addition, we evaluated the number of Sholl intersections, points where dendrites cross the virtual Sholl circles and found no significant difference between sham and RF-EMF exposure groups (Figure 7(B)). As positive control, treatment with 1.0 μ g/mL of brefeldin A at DIV2 and 10 μ M of LY294002 at DIV4 significantly inhibited axonal growth and dendrite growth, respectively ($p < .01$, Figure 7(A,B)).

To further investigate the effects of RF-EMF exposure on the synaptogenesis, we detected the density of PSD95 and gephyrin clusters, as indicators of excitatory and inhibitory synapses, in the cortical neurons. The results showed no difference in the density of PSD95-stained and gephyrin-stained puncta on the dendrites between sham and RF-EMF exposure groups ($p = .24$ and $.18$, Figure 7(C)). As positive control, treatment with 10 μ M of U0126 at DIV10 significantly inhibited synaptogenesis ($p < .05$).

Discussion

In this study, we systemically evaluated the DNA damage and cellular responses to RF-EMF exposure in primary cultured neurogenic cells. We found that exposure to RF-EMF at a SAR of 4.0 W/kg did not significantly affect the γ H2AX foci

formation in the primary cultured neurogenic cells. The secretions of cytokines TNF- α , IL-6 or IL-1 β in astrocytes and microglia were not altered by RF-EMF exposure. However, RF-EMF exposure significantly reduced the phagocytic activity of microglia. For primary cortical neurons, the exposure significantly inhibited the axon branch length and branch number, but did not change the morphological indicators of dendrites or synapses.

DNA is usually damaged by endogenous and exogenous stimuli and then repaired by DNA repair enzymes (Phillips et al. 2009). Accumulation of DNA damage leads to genetic instability, which results in some diseases, e.g. cancer (Jeggo et al. 2016) and neurodegenerative diseases (Coppede and Migliore 2015). Previous studies on *in vitro* genotoxic effects of RF-EMF were mainly conducted in lymphocytes and some cell lines (Verschaeve et al. 2010), and few reports were related to neurogenic cells, especially primary cultured neurogenic cells. The results from previous study by Zuo et al. (2015) have shown that exposure to RF-EMF at a SAR of 4.0 W/kg for 24 h could not directly induce DNA damage in spiral ganglion neurons, which is consistent with our findings in cortical neurons. As to primary cultured astrocytes, our previous study (Xu et al. 2013) and this study did not show genotoxicity when the cells were exposed to RF-EMF for 24 h. Conversely, the study by Campisi et al. reported that acute exposure to 900 MHz amplitude-modulated (AM) but not continuous wave RF-EMF for 20 min produced increased DNA fragmentation in astrocytes. These inconsistencies may be due to the different RF frequency (e.g. 1800 vs. 900 MHz), modulation modes (GSM vs. AM) or exposure time (24 h vs. 20 min) used in different studies. In addition, we for the first

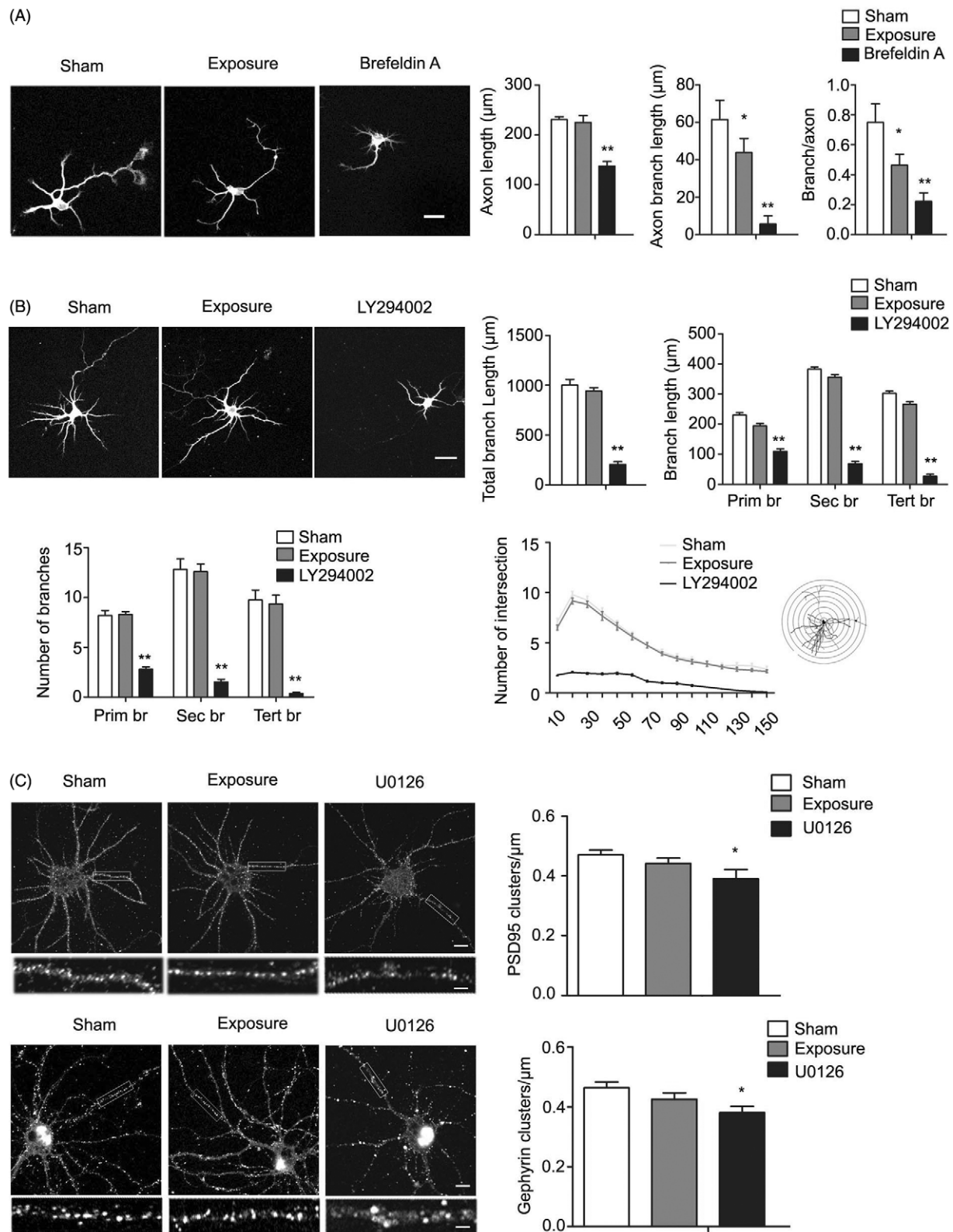


Figure 7. Effects of exposure to RF-EMF on the cortical neuronal morphology development. (A) The images showing the morphology of axons after RF-EMF exposure for 3 d. Histograms showing the average axon length per neuron, the average axon branch length per neuron and the average number of branch per axon in sham and exposure group. (B) The images showing the morphology of dendrite after RF-EMF exposure for 7 d. Histograms showing the total dendrite length per neuron, the average dendrite branch length per neuron, the average number of branches per neuron and Sholl analysis of the dendrites in sham and exposure group. Concentric circles in Sholl analysis are spaced $10\ \mu\text{m}$ apart, ranging from 10 to $150\ \mu\text{m}$ from the center of the neuronal soma. (C) The images showing the PSD95 (upper) and gephyrin (lower) clusters after exposure for 14 d. Histograms showing the density of PSD95 (upper) and gephyrin (lower) cluster in sham and exposure group. * $p < .05$ and ** $p < .01$ compared to sham group. Bar = $20\ \mu\text{m}$ (full size) and $4\ \mu\text{m}$ (insert).

time used microglia as cell model to evaluate the DNA damage of RF-EMF and found that the exposure did not induce cellular DNA damage in microglia. Generally, the currently available data did not support that RF-EMF exposure induces DNA damage in primary cultured neurogenic cells. It should be noted that DNA DSB is one type of genetic damages. In this study, we could not rule out other types of gene impair or abnormalities, such as DNA base damage and methylation change. As to neuronal cell line, the results from the study by Wang et al. have shown that exposure to RF-EMF at a SAR of 2.0 W/kg did not directly induce DNA strand breaks, but could induce oxidative DNA base damage in Neuro-2a cells (Wang et al. 2015). Thus, the other types of genotoxic effect of RF-EMF exposure on primary cultured neurogenic cells are needed further evaluation.

Astrocytes are the most numerous cell type in the CNS, which represents their potential to impact a wide range of homeostatic and pathological functions (Sofroniew and Vinters 2010). In various CNS insults including traumatic injury, ischemia, neurotoxic chemicals, tumors and infections, astrocytes are responsible for the production of cytokines and chemokines (Dong and Benveniste 2001). However, studies on the cellular responses of astrocytes to RF-EMF are very limited. Consistent with our results, previous study by Thorlin et al. showed that exposure to 900 MHz GSM RF-EMF at SAR of 3.0 W/kg did not affect the release of IL-6 or TNF- α in primary cultured astrocytes (Thorlin et al. 2006). Conversely, the study by Lu et al. used astroglial cell line C8-D1A as cellular model and found that exposure to 1800 MHz RF-EMF at SAR of 2.0 W/kg increased the release of IL-6, but did not affect the production of IL-1 β , TNF- α (Lu et al. 2014). Thus, the primary cultured astrocytes and astroglial cell lines may differentially respond to RF-EMF exposure and the underlying mechanisms remain to be elucidated.

Microglia play a critical role as resident immunocompetent and phagocytic cells in the CNS, and serve as scavenger cells in the event of infection, inflammation, trauma, ischemia and neurodegeneration in the CNS (Kim and de Vellis 2005). Interestingly, microglia were recently shown to play unexpected roles in normal brain development and adult physiology, e.g. phagocytosis of synaptic structures during postnatal development and phagocytosis of newborn neurons during adult neurogenesis (Tremblay et al. 2011). Given the important role of microglia in maintaining the homeostasis of the CNS, several studies have focused on the activation of microglia after RF-EMF exposure with different frequencies. In this study, our data showed that RF-EMF exposure did affect the release of IL-1 β , TNF- α and IL-6. Consistent with our results, previous studies have shown that exposure to 900 MHz GSM RF-EMF at SAR of 3.0 W/kg and 1950 MHz Wideband Code Division Multiple Access (W-CDMA) RF-EMF at SAR of 2.0 W/kg did not affect the release of IL-1 β , IL-6 and TNF- α in primary cultured microglia (Thorlin et al. 2006; Hirose et al. 2010). In immortalized mouse microglial N9 cells, 2450 MHz RF-EMF at SAR of 6.0 W/kg dramatically increased the production of TNF- α (Hao et al. 2010; Yang et al. 2010). Subsequently, the study by Lu et al. also found that exposure to 1800 MHz RF-EMF at SAR of 2.0 W/kg induced the release of IL-1 β , TNF- α and IL-6 in N9 cells (Lu et al. 2014).

Thus, immortalized microglial cells may be more sensitive to RF-EMF, comparing to the primary cultured microglia. On the other hand, He et al. reported that exposure to 2450 MHz RF-EMF at SAR of 6.0 W/kg depressed the phagocytic ability of N9 cells (He et al. 2014). However, it is worth noting that a high SAR of 6.0 W/kg exceeding the limit on non-thermal efficiency of RF-EMF (4.0 W/kg), with definite thermal effects, could potentially induce microglial activation. In our study, we found that RF-EMF exposure at SAR of 4.0 W/kg also reduced the phagocytic ability of primary cultured microglia. Phagocytic activity of microglia is regulated by intracellular calcium (Ca^{2+}) pathways (Heo et al. 2015; Michaelis et al. 2015). A variety of receptors for microglia functions were found on the surface of cells are involved in Ca^{2+} signaling (Brawek and Garaschuk 2013). A number of studies have evaluated the effects of RF-EMF exposure on Ca^{2+} mobilization in neuronal and non-neuronal cells, but the results are controversial (Platano et al. 2007; Rao et al. 2008; Maskey et al. 2010; O'Connor et al. 2010). Thus, whether Ca^{2+} signaling pathway is a potential mechanism underlying RF-EMF exposure inhibited the microglial phagocytosis needs to clarify in future.

RF-EMF have been suggested to affect the learning ability and memory function in human (Abramson et al. 2009; Schoeni et al. 2015) and animal (Wang and Lai 2000; Fragopoulou et al. 2010), but the cellular and molecular mechanisms remain unclear. Previously, Xu et al. found that chronic exposure (15 min per day for DIV7-DIV14) to GSM 1800 MHz RF-EMF at SAR of 2.4 W/kg decreased excitatory synaptic activity and excitatory synapse number in cultured rat hippocampal neurons (Xu et al. 2006). Subsequently, using the same exposure parameters and experimental methods, Ning et al. found that the exposure from DIV6 to DIV14 may affect dendritic development and the formation of excitatory synapses of hippocampal neurons (Ning et al. 2007). A study by Del Vecchio et al. found that GSM 900 MHz RF-EMF exposure at SAR of 1.0 W/kg caused a diminution in neurite generation from the soma without any effect on branching and neurite length in cortical neurons (Del Vecchio et al. 2009). Actually, previous studies have focused only on the effects of RF-EMF on the early or late stages of the neurons development *in vitro*. In this study, we exposed the primary cultured cortical neurons to RF-EMF from DIV1 to DIV14 at both early and late stages, and found that the exposure inhibited the axon branch length and branch number but did not significantly influence the morphological parameters of dendrites and synapse. Axons are in effect the primary transmission lines of the nervous system. Cortical neurons innervate many of their targets by collateral axon branching (Dent and Kalil 2001). It is well established that precise wiring of cortical circuits during development depends upon axon extension, guidance and branching to appropriate targets (Kalil et al. 2011). Thus, our findings might be important for better understanding of the mechanisms for cognitive performance and learning impairments after RF-EMF exposure. Overall, the currently available studies have indicated adverse effects of RF-EMF on the neuron development *in vitro*; however, this needs to be confirmed by *in vivo* studies in future.

Conclusion

In conclusion, this study systematically investigated the effects of 1800 MHz RF-EMF exposure on DNA damage and cellular functions in primary cultured neurogenic cells. Our data demonstrated that exposure to RF-EMF at SAR of 4.0 W/kg did not elicit DNA damage but inhibited the phagocytic ability of microglia and the axon branch length and branch number of cortical neurons and further studies are required to investigate the effects of RF-EMF exposure on phagocytic ability of microglia and development of cortical neuron *in vivo*.

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Disclosure statement

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