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ORIGINAL ARTICLE

Oxidative changes and apoptosis induced by 1800-MHz electromagnetic radiation in NIH/3T3 cells

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Abstract

To investigate the potential adverse effects of mobile phone radiation, we studied reactive oxygen species (ROS), DNA damage and apoptosis in mouse embryonic fibroblasts (NIH/3T3) after intermittent exposure (5 min on/10 min off, for various durations from 0.5 to 8 h) to an 1800-MHz GSM-talk mode electromagnetic radiation (EMR) at an average specific absorption rate of 2 W/kg. A 2′,7′-dichlorofluorescin diacetate fluorescence probe was used to detect intracellular ROS levels, immunofluorescence was used to detect γH2AX foci as a marker for DNA damage, and flow cytometry was used to measure apoptosis. Our results showed a significant increase in intracellular ROS levels after EMR exposure and it reached the highest level at an exposure time of 1 h ($p < 0.05$) followed by a slight decrease when the exposure continued for as long as 8 h. No significant effect on the number of γH2AX was detected after EMR exposure. The percentage of late-apoptotic cells in the EMR-exposed group was significantly higher than that in the sham-exposed groups ($p < 0.05$). These results indicate that an 1800-MHz EMR enhances ROS formation and promotes apoptosis in NIH/3T3 cells.

Keywords

1800-MHz, apoptosis, DNA damage, mobile phone radiation, ROS

History

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Introduction

Electromagnetic radiation (EMR) at 1800 MHz is used in the Global System for Mobile Communication (GSM), the GSM1X dual-mode communication system, and the 3 G mobile phone communication system. Some epidemiological studies have demonstrated that mobile phone EMR may increase the risk of developing certain types of cancer, such as uveal melanoma (Stang et al., 2001) and acoustic neuroma (Hardell et al., 2013), and induced sperm DNA damage (Aitken et al., 2005). However, some other research reported no significant effects, e.g., exposure to an 1800-MHz EMR did not cause a significant effect on mouse retinal ganglion cell responses (Ahlers and Ammermüller, 2014) apoptosis in human peripheral blood mononuclear cells (Capri et al., 2004), and reactive oxygen species (ROS) level in human lymphocytes (Lantow et al., 2006a). The point about whether exposure to EMR can lead to the development of tumors is still controversial. However, the International Agency for Research on Cancer (IARC) has classified EMR as a possible human carcinogen (2B) (IARC, 2013). Exposure limits for radiofrequency radiation are given in terms of specific absorption rate (SAR), i.e. the rate of radiofrequency energy absorption per unit mass of the body. Currently, the maximum SAR limit for a mobile phone used against the head or body in accordance with the International Commission on Non-Ionizing Radiation Protection (ICNIRP) guidelines is 2 W/kg (ICNIRP, 2009). It has been established that ROS and DNA damage are the main causes of many health problems such as carcinogenesis and aging. Environmental toxicological studies have shown that ROS can damage biomacromolecules, especially DNA, and lead to apoptosis. Oxidative stress has also been implicated in the initiation and promotion of carcinogenesis. However, whether 1800-MHz EMR can induce DNA damage remains to be determined. In this study, we investigated intracellular ROS production, DNA damage and apoptosis in mouse embryonic fibroblasts NIH/3T3 cells exposed to the 1800-MHz EMR.

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Materials and methods

Cell culture

Mouse embryonic fibroblasts NIH/3T3 cells (China Union Medical University Center for Basic Medical Cell, Beijing, China) were used in the research. NIH/3T3 cells are sensitive to environmental factors and being used frequently to screen potential carcinogens. Cells were maintained in Dulbecco's Modified EAGLE's Medium (DMEM; Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) in an incubator at 37 °C with 5% CO_2 (Thermo Scientific, New York, MA), and subcultured every 3–4 days.

Figure 1. The sXc-1800-MHz exposure system. (A) Dish holder, (B) waveguids in an incubator, (C) signal generator.

Exposure system

The sXc (System for Exposure of Cells; IT'IS, Zurich, Switzerland) 1800-MHz exposure system was used to expose cells to EMR. The system consists of a signal generator, an arbitrary function generator, a narrow band amplifier and two rectangular waveguide chambers (Figure 1). The two waveguides, one for EMR exposure and the other for sham exposure, with accessory probes and fans were placed inside a conventional incubator. The probes and fans were connected to a computer controlling the exposure parameters including SAR, exposure time and exposure pattern automatically (Schuderer et al., 2004). A culture dish holder placed inside the waveguide ensured that dishes were placed in the H-field maximum of the standing wave and exposed simultaneously to E-polarization inside the chamber. The temperature was kept at $37 \pm 0.1\,^{\circ}C$ for the entire duration of exposure and the temperature difference between the exposed and the sham-exposed cultures never exceeded 0.1 °C. Therefore, the data were obtained under relatively constant temperature conditions. A HP-8592C spectrum analyzer and a Narda 7620 type microwave radiation tester were used to periodically monitor exposure parameters.

Exposures were performed by using a pulse-modulated 1800-MHz GSM signal at a repetitive rate of 217 Hz. The NIH/3T3 cells were intermittently (field on for 5 min, field off for 10 min) exposed to the 1800-MHz EMR at an average SAR of 2 W/kg, (as calculated by IT'IS based on the dielectric properties and the thickness of the medium in the culture disks) which is now defined as the safety limit for mobile phone radiation by the ICNIRP. Cells were exposed for different periods of time (0.5, 1, 1.5, 2, 4, 6, or 8 h).

ROS level measurement

Intracellular ROS levels were measured by flow cytometry using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Applygen Technologies Inc., Beijing, China). DCFH-DA diffuses into cells and is deacetylated by cellular esterases to non-fluorescent 2′,7′-dichlorodihydrofluorescin (DCFH), which is rapidly oxidized to highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF) by ROS. The fluorescence intensity is proportional to the ROS levels within the cell cytosol. A cell density of 10^5/ml was placed in each 35-mm culture plate (Nunc, Denmark). After exposure, cells were washed twice with phosphate-buffered saline (PBS; Sigma, Santa Clara, CA) and incubated in buffer with 1 μM DCFH-DA at 37 °C for 30 min in a dark room. After washing three times, a fraction of the cells was photographed under a microscope, while the remaining cells were detached using 0.25% trypsin (Hyclone, Logan, UT). Following digestion, fluorescence intensities of resuspended cells were measured by flow cytometry. As a positive control, we treated cells with 30 μM H_2O_2 (Sigma, Santa Clara, CA) for 30 min. For each sample, 10,000 cells were measured. Each exposure condition was repeated three times with two experimental replicates (dishes) each.

γH2AX foci detection

Approximately 10^5 cells were cultured in each 35-mm plate. After 24 h, cells were exposed to the 1800-MHz radiation for different time periods. The positive control cells were treated with 30 μg/ml 2-acetamidofluorene (Sigma, Santa Clara, CA) for 2 h to chemically induce DNA damage. After exposure, cells were washed with PBS to remove residual medium, followed by fixation with 4% paraformaldehyde (Sigma, Santa Clara, CA) for 15 min at 4 °C. Cells were then permeabilized in 0.2% TritonX-100 (Sigma, Santa Clara, CA) for 15 min at 4 °C and the plates were blocked with a normal goat serum working solution (Beijing Sequoia Biological Technology, Beijing, China) for 2 h. After washing, cells were then incubated with a rabbit monoclonal anti-γH2AX antibody (Cell Signaling Technology, Boston, MA) for 2 h at a concentration of 1:500 and with fluorescein isothiocyanate (FITC) conjugated goat-anti-rabbit secondary antibody (Beijing CoWin Bioscience Company, Beijing, China) for 1 h at a concentration of 1:200 in a dark room. The samples were also incubated with propidium iodide (PI) (Sigma, Santa Clara, CA) and stained for 15 min. Finally, cells were washed with PBS three times at each step and mounted with 90% glycol (Sigma, Santa Clara, CA). The cells were then photographed under a fluorescent microscope (Olympus AX70, Hamburg, Germany). Cellular γH2AX foci were monitored in approximately 100 cells per sample. Each experiment was repeated three times.

Apoptosis detection

Approximately 10^5 cells were placed in each 35-mm culture plate. After 24 h, cells were exposed to the 1800-MHz EMR for different periods of time. They were then detached by

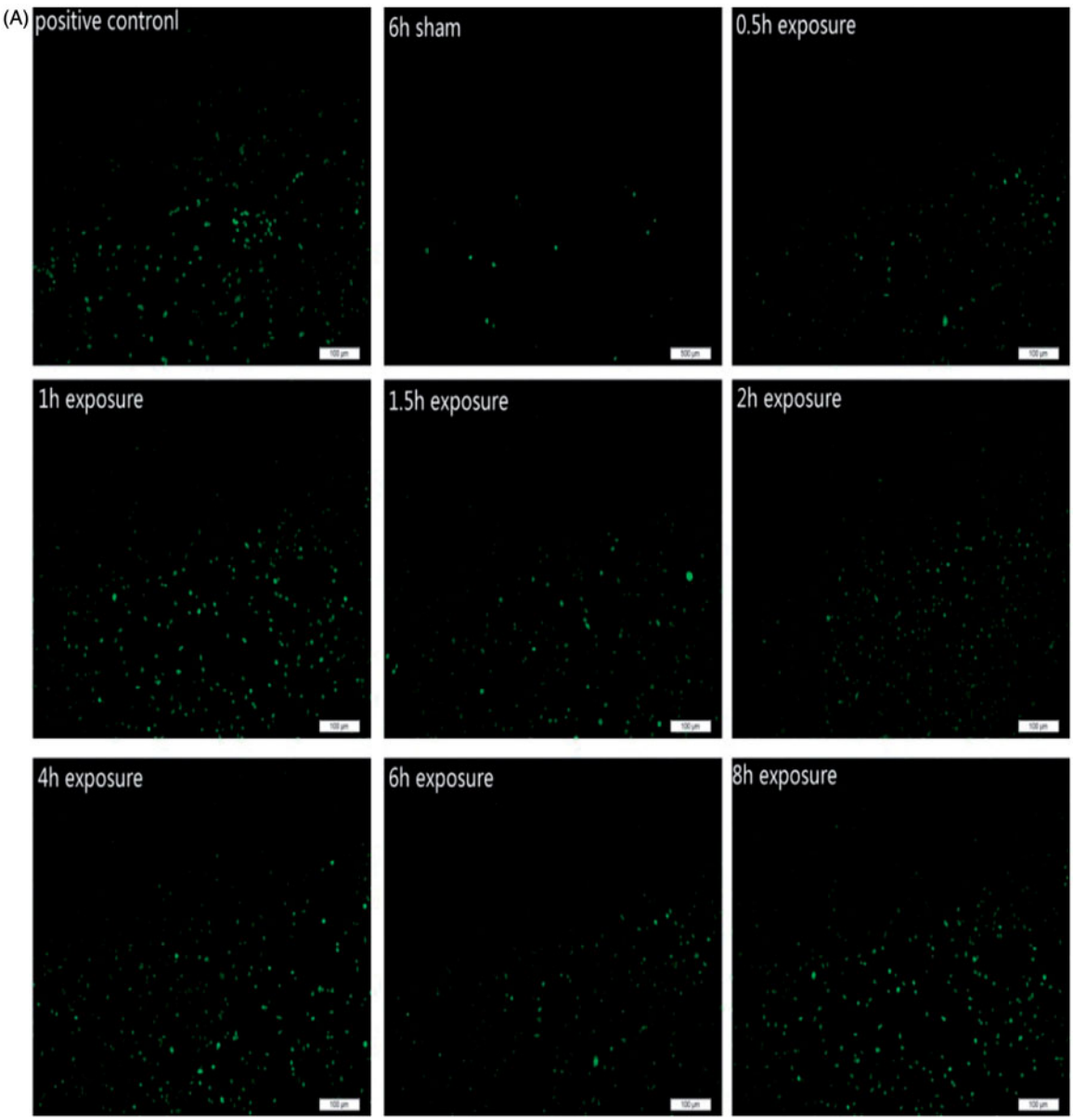

Figure 2. Intracellular ROS in NIH/3T3 cells after exposure to an 1800-MHz EMR. (A) Fluorescence microscopy showing changes in intracellular ROS (magnification ×100). (B) Flow cytometry data showing the percentage of ROS positive cells of each group. Since intracellular ROS levels among each sham-exposed group were essentially the same, only the 6 h sham group is shown. (C) Bar graphs comparing percentages of ROS positive cells after different exposure durations; $*p < 0.05$.

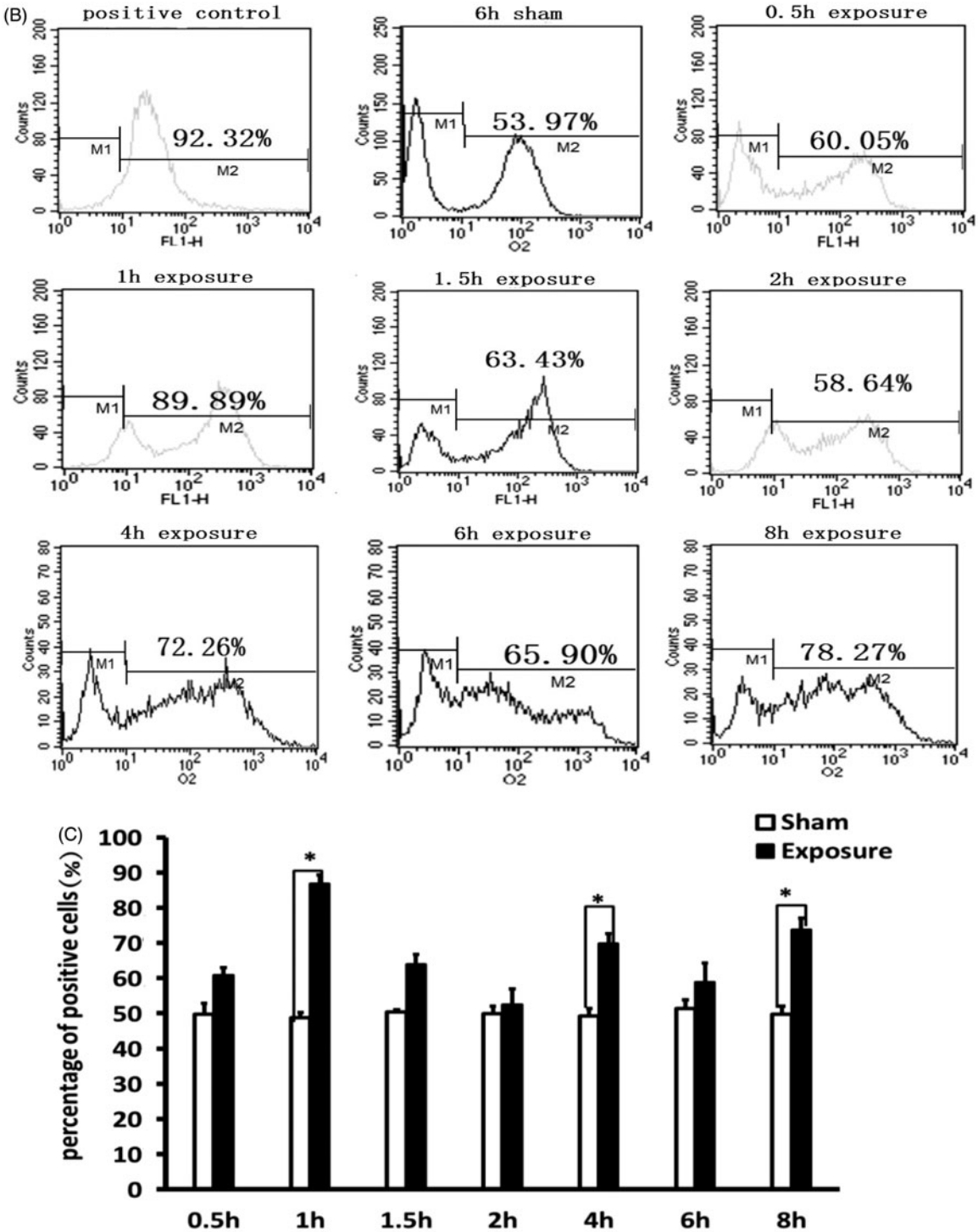

Figure 2. Continued.

0.25% trypsin digestion. Cells were washed with cold PBS and 5 µl FTTC-annexin V (Becton Dickinson and Company, Franclin Lakes, NJ) and 10 µl PI (Becton Dickinson and Company, Franclin Lakes, NJ) were added to 100 µl of the cell suspension for a final cell concentration of 10^6 cells/ml. The mixture was incubated for 15 min in a dark room at room temperature. The cells were then washed twice with binding buffer and adjusted to a final cell concentration of 10^6 cells/ml. Cell apoptosis was analyzed using a flow cytometer (Becton Dickinson and Company, Franclin Lakes, NJ) within 1 h. Each exposure condition was repeated three times with two replicate dishes each.

Statistical analysis

Statistical analyses were performed using the SPSS 17.0 software. Each experiment was repeated three times. Data are presented as the mean ± standard deviation (SD) of each group. Statistical analyses were carried out using analysis of variance (ANOVA) and difference between two treatment

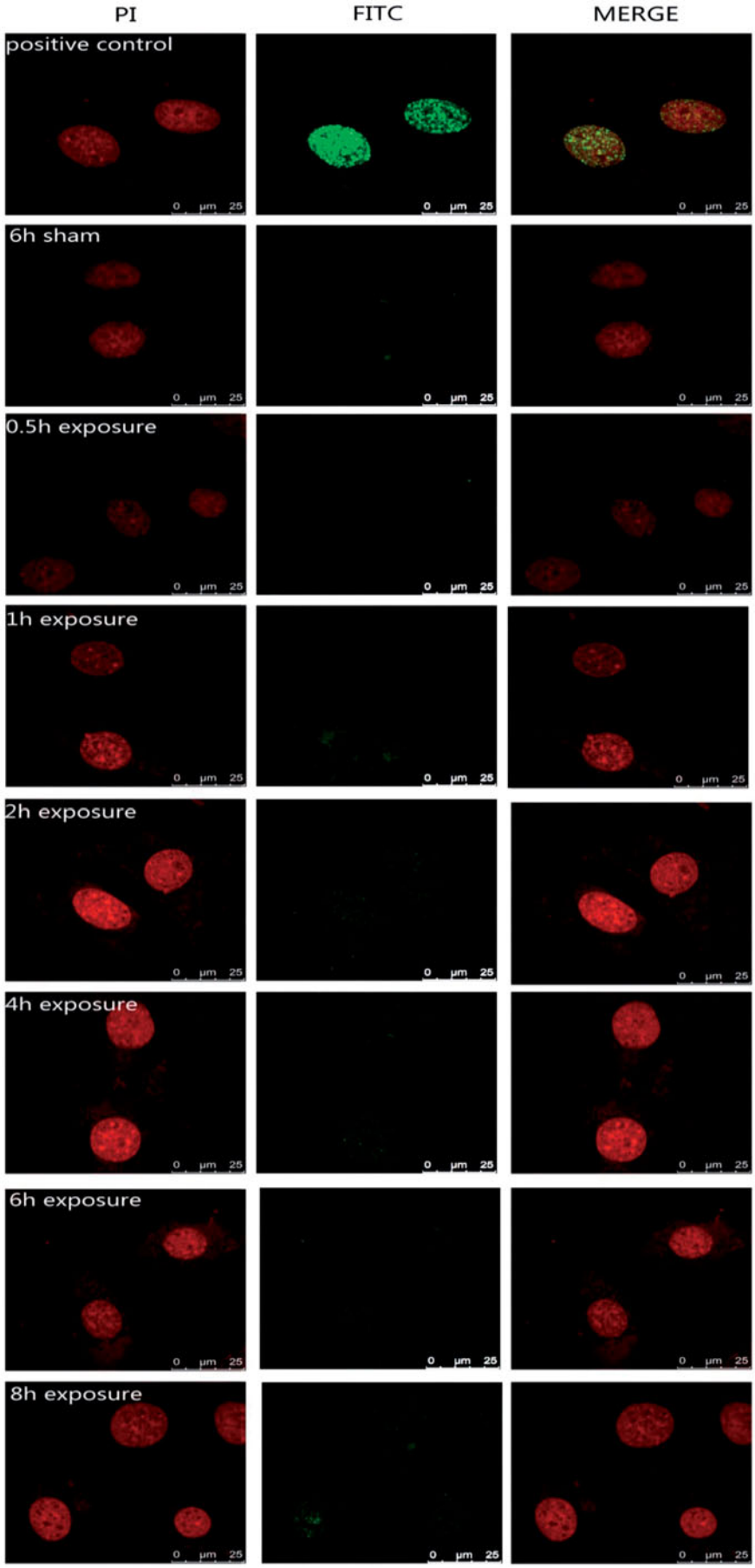

Figure 3. Effects of an 1800-MHz EMR on the number of cells showing γH2AX foci. (A) Fluorescence microscopy showing changes in intracellular γH2AX foci in cells (magnification ×200) after EMR exposure. (B) Bar graphs comparing percentages of cells with intracellular γH2AX foci.

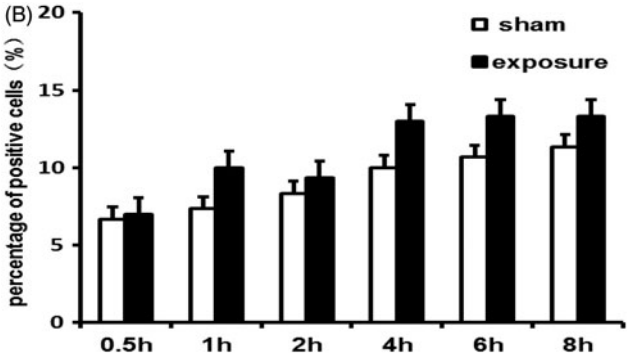

Figure 3. Continued.

groups was compared using the Tukey Test. A difference at $p < 0.05$ was considered statistically significant.

Results

Intracellular ROS after exposure

Results of effects of exposure to an 1800-MHz EMR on intracellular ROS level are presented in Figure 2. Representative micrographs (Figure 2A), flow cytometry data (Figure 2B) and percentage of positive cells (Figure 2C) are presented. Intracellular ROS levels in the EMR-exposure groups at 1, 4 and 8 h were significantly increased ($p < 0.05$, compared to respective sham-exposed controls). And 1 h-exposure group showed the highest ROS level ($0.001 < p \leq 0.023$) confirmed by a two-way ANOVA considering two factors: exposure (sham/EMR) and exposure duration. No significant difference was found in the groups exposed to the EMR for 0.5, 1.5, 2 and 6 h.

DNA damage

Data of DNA double-strand break (γH2AX foci) are presented in Figure 3. Because the number of γH2AX foci is relatively low in sham-exposed groups as reported previously (d'Adda di Fagagna et al., 2003), a cell with one or more γH2AX foci was defined as γH2AX-foci positive. Approximately 100 cells were analyzed in each sample. After exposure to the 1800-MHz EMR for 2, 4, 6 or 8 h, the number of γH2AX foci was slightly but not statistically significantly increased in the EMR-exposed groups (as compared with their respective sham-exposed controls). Because the number of γH2AX foci was not significantly different between each sham-exposure groups, only the foci image of the sham-exposure group at 6 h is shown.

NIH/3T3 cell apoptosis

Approximately 10,000 NIH/3T3 cells were analyzed by flow cytometry in each sample. Data are presented in Figure 4. After exposed for 1, 4 or 8 h, the number of late-apoptotic cells increased significantly when compared with their respective sham-exposed groups.

Discussion

ROS (i.e. O^{-2}, ·OH, H_2O_2, HOCl, NO, O^3) are by-products of physiological oxygen metabolism and harmful to most cells. Elevated ROS can damage DNA directly and hamper functions of proteins, reducing some molecular flows and signal communications, leading to subsequent apoptosis. Agarwal et al. (2009) found that cell phone EMR increased the ROS level in sperms, and decreased their vitality and mobility. Wu et al. (2008) found that intermittent exposure to an 1800-MHz EMR for 24 h at an average SAR of 4 W/kg could increase the levels of ROS in lens epithelial cells. Our results showed that ROS levels in NIH/3T3 cells were significantly increased ($p < 0.05$) after 1, 4 and 8 h of intermittent exposure to an 1800-MHz EMR at an average SAR of 2 W/kg, and 1 h-exposure group showed the highest ROS levels. This is possibly due to the fact that cells have self-protective mechanisms, such as releasing carotenoid in response to oxidative stress, that prevent further increase in ROS level (Lu et al., 2012).

γH2AX foci are one of the earliest markers of DNA double-strand breaks in eukaryotes. Following a DNA double-strand break, serine residue 139 (ser-139) in the highly conserved carboxy-terminal SQE domain of H2AX is rapidly phosphorylated, generating histone γH2AX (Andrievski and Wilkins, 2009). Many reports have shown that the number of γH2AX foci were proportional to the number of DNA double-stand breaks. The higher the number of γH2AX foci, the more extensive is DNA damages. Therefore, the γH2AX assay has become an established method for detecting DNA damage. Zhao et al. (2007) found that there was no formation of γH2AX foci after exposure to an 1800 MHz radiation at an SAR of 3 and 4 W/kg for 2 h in lens epithelial cells. Yokus et al. (2008) found that ROS levels, mitochondrion proliferation and DNA strand breaks significantly increased after exposure to an 1800-MHz EMR (SAR values gradually increased from 0.4 to 27.5 W/kg) in human sperm cells. Speit et al. (2007) found that there was no obvious DNA damage in human diploid fibroblasts that were exposed to an 1800-MHz EMR at an SAR of 2 W/kg for 1, 4 or 24 h. In our study, the γH2AX foci number did not show a significant increase in the EMR-exposed cells. Although the γH2AX focus is a sensitive and early intracellular DNA damage marker by far, it can only detect double-strand breaks in DNA. Other types of DNA damage, e.g. mononucleotide damage, may be possible. Other types of DNA damage and oxidative damages of other macromolecules may explain the increase in apoptosis in the EMR-exposed NIH/3T3 cells.

Apoptosis is a well-conserved process of autonomic programmed cell death, which often occurs after damage to key molecules such as DNA, lipid or proteins. Capri et al.

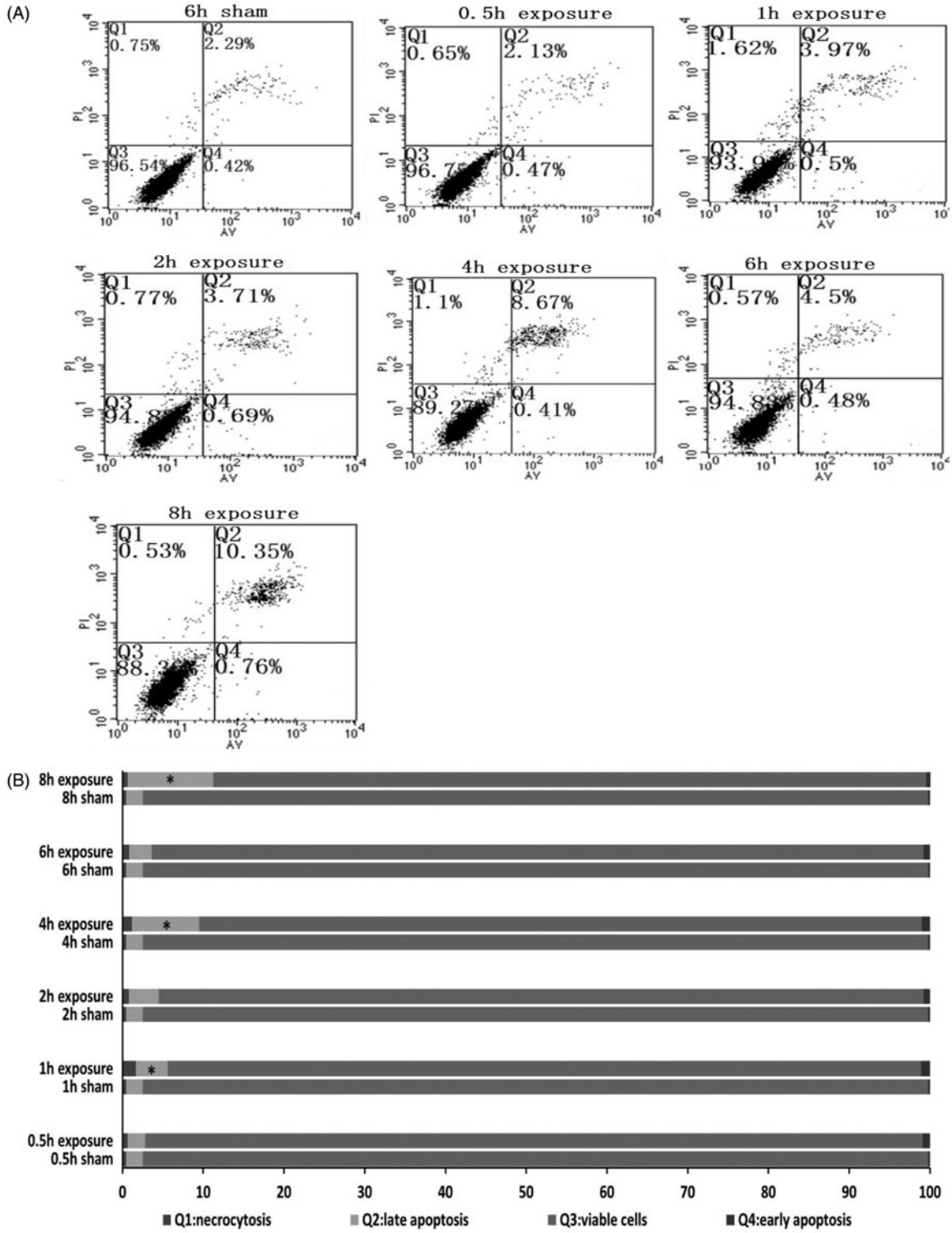

Figure 4. Effects of an 1800-MHz EMR on apoptosis in NIH/3T3 cells. (A) Flow cytometry results. Since there were no significant differences in the apoptosis cell number among the sham-exposed groups at each time point, only the 6 h sham group is shown. (B) Comparison of the percentage of apoptosis cells. $*p < 0.05$ when compared with sham.

(2004) found that exposure to an 1800-MHz EMR did not promote apoptosis in peripheral blood mononuclear cells. Lantow et al. (2006b) found that an 1800-MHz EMR at an average SAR of 2 W/kg promoted apoptosis in human immune cells (MAC6 and K562 cells). Dasdag et al. (2008) found that rats exposed to 900 MHz radiation 2 h/day (7 days/week) for 10 months showed no apoptosis activation effect in testicle cells. Our results showed that the number of late apoptotic cells increased after exposure of NIH/3T3 cells to an 1800-MHz EMR at an average SAR of 2 W/kg. The reasons why the data are inconsistent may attribute to factors such as differences in cell type, level of differentiation and cell cycle, that may affect a cell's sensitivity to EMR.

Conclusion

Results of this study showed a significant increase in intracellular ROS level in NH/3T3 cells after intermittently exposed to an 1800-MHz EMR at an SAR of 2 W/kg, and it reached the highest level at the exposure time of 1 h followed by a slight decrease even if the exposure lasted as long as 8 h. The exposure did not cause a significant increase in DNA double-strand breaks as measured by γH2AX focus formation. However, the exposure promoted apoptosis in NIH/3T3 cells.

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Declaration of interest

The authors confirm that there are no known conflicts of interest associated with the paper ''Oxidative Changes and Apoptosis Induced by 1800-MHz EMR in NIH/3T3 Cells'' and the financial support could not influence the research results. They confirm that the manuscript has been read and approved by all named authors and there are no other persons who satisfied the criteria for authorship but not listed. They understand that the Minglian Wang and Qingxia Hou are the contacts for the editorial process. They are responsible for communicating with the other authors.

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