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Research Report

Exposure to 1800 MHz radiofrequency radiation induces oxidative damage to mitochondrial DNA in primary cultured neurons

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ABSTRACT

Increasing evidence indicates that oxidative stress may be involved in the adverse effects of radiofrequency (RF) radiation on the brain. Because mitochondrial DNA (mtDNA) defects are closely associated with various nervous system diseases and mtDNA is particularly susceptible to oxidative stress, the purpose of this study was to determine whether radiofrequency radiation can cause oxidative damage to mtDNA. In this study, we exposed primary cultured cortical neurons to pulsed RF electromagnetic fields at a frequency of 1800 MHz modulated by 217 Hz at an average special absorption rate (SAR) of 2 W/kg. At 24 h after exposure, we found that RF radiation induced a significant increase in the levels of 8-hydroxyguanine (8-OHdG), a common biomarker of DNA oxidative damage, in the mitochondria of neurons. Concomitant with this finding, the copy number of mtDNA and the levels of mitochondrial RNA (mtRNA) transcripts showed an obvious reduction after RF exposure. Each of these mtDNA disturbances could be reversed by pretreatment with melatonin, which is known to be an efficient antioxidant in the brain. Together, these results suggested that 1800 MHz RF radiation could cause oxidative damage to mtDNA in primary cultured neurons. Oxidative damage to mtDNA may account for the neurotoxicity of RF radiation in the brain.

1. Introduction

The world-wide and rapidly growing use of mobile phones (MP) has raised considerable concerns about their potentially hazardous effects on human health. Compared to the other organs, the brain is exposed to relatively high specific absorption rates (SAR) due to the close proximity of the mobile telephone device to the head. Exposure to radiofrequency (RF) radiation emitting from MP has been postulated to result in a variety of neurological effects, including headaches, changes in sleep patterns, modifications in neuronal electrical activity, increases in the permeability of the blood–brain barrier (BBB) and disturbances in neurotransmitter release (Hossmann and Hermann, 2003; Repacholi, 1998; Valentini et al., 2007). There is

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Abbreviations: MP, mobile phone; RF, radiofrequency; SAR, average special absorption; 8-OHdG, 8-hydroxyguanine; BBB, blood–brain barrier; mtDNA, mitochondrial DNA; mtRNA, mitochondrial RNA; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; DCFH-DA, 2′,7′-dichlorofluorescein diacetate

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growing evidence to suggest that RF radiation induces oxidative stress in the brain and in other tissues (Ilhan et al., 2004; Irmak et al., 2002; Koylu et al., 2006; Moustafa et al., 2001; Oktem et al., 2005; Ozguner et al., 2005a,b; Sokolovic et al., 2008; Yurekli et al., 2006). Oxidative stress is known to be essential for pathophysiological processes in the brain, and it plays critical roles in cell apoptosis, general and specific gene expression, DNA damage, cell proliferation, inflammation process and mitochondrial dysfunction (Hayashi et al., 2008; Lin and Beal, 2006). The involvement of oxidative stress in manifold processes is compatible with the diverse nature of the observed effects induced by RF radiation (Balik et al., 2005; Cleary et al., 1996; Diem et al., 2005; Koylu et al., 2006; Schwarz et al., 2008; Zhao et al., 2007a,b). Thus, oxidative stress may be involved in the adverse effects of RF radiation on the nervous system.

Mitochondria are the major sites of production of reactive oxygen species (ROS). ROS are toxic by-products of respiration, and excess ROS are known to cause oxidation of unsaturated fatty acid, proteins, and DNA. Mitochondrial DNA (mtDNA) is particularly susceptible to oxidative stress for a variety of reasons, including its lack of protective histone-like proteins, its insubstantial capacity for repair following damage, and its proximity to the respiratory chain in the mitochondrial inner membrane (Evans et al., 2004). MtDNA damage is reflected by the presence of mtDNA mutation, by a decline in mtDNA copy number, and by a reduced number of mitochondrial RNA (mtRNA) transcripts (Ide et al., 2001). Each of these types of mtDNA damage can amplify oxidative stress by encoding the deficient critical proteins for the respiratory chain, which exacerbates further production of ROS and aggravates the oxidative damage to mitochondrial function (Evans et al., 2004; Wallace, 1999). Indeed, oxidative damage to mtDNA contributes to mitochondrial dysfunction in various mitochondrial-related diseases including aging, neurodegenerative diseases and ischemia. The presence of mtDNA mutations is closely related to a variety of neurological symptoms, such as migraine, dementia, and ataxia (Lin and Beal, 2006; Taylor and Turnbull, 2005; Xu et al., 2009).

Therefore, there is the possibility that RF radiation could cause oxidative damage to mtDNA in nerve cells, and this may account for the adverse effects of RF radiation on the nervous system. In order to address this, we exposed primary cultured cortical neurons to 1800 MHz radiofrequency radiation at the SAR value of 2 W/kg. We observed a striking degree of oxidative damage to mtDNA in these neurons. In addition, we discovered that these oxidative injuries to mtDNA could be successfully prevented by melatonin, a pineal neurohormone with known antioxidant capacity especially in the brain (Lee et al., 2001; Sokolovic et al., 2008).

2. Results

In the RF radiation group, exposure to RF radiation for a period of 24 h significantly increased the production of ROS compared with that of the sham-control group ($P<0.01$; Fig. 1). A similar result was obtained with the hydrogen peroxide (H_2O_2) group. H_2O_2 was applied as a positive control for measuring the effects of oxidative stress (Fig. 1). The increased amount of ROS in the radiation group could be reduced by pretreatment with melatonin ($P<0.05$; Fig. 1). These results indicated that RF radiation induced oxidative stress in neurons, and that this effect could be reversed by melatonin pretreatment.

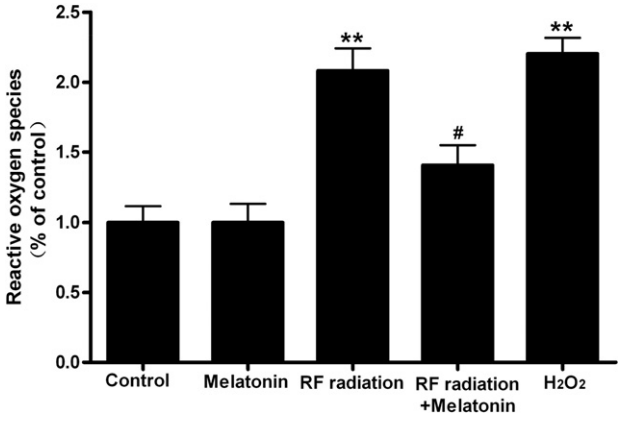

Fig. 1 – ROS production in primary cultured cortical neurons exposed to RF radiation. After exposure to RF radiation, neurons were incubated with DCFH-DA at 37 °C for 20 min. Fluorescence images were acquired with a Leica fluorescence microscope at 485 nm for excitation and 530 nm for emission. Fluorescence intensity for each dish was measured within 25×25 pixel regions of interest and analyzed for pixel intensity by NIH Image. Cellular fluorescence intensity was expressed as the multiple of the level in control groups. $^{**}p<0.01$ versus sham-exposed control group and melatonin group, $^{\#}p<0.05$ versus RF radiation group. Values are means±SE, $n=6$.

To detect the oxidative damage of mtDNA that was induced by RF radiation, we performed immunostaining detection and HPLC analysis to determine the levels of 8-OHdG in mitochondria. 8-OHdG is the most prominent DNA damage, and it is often used as a biomarker for the oxidative damage of DNA. As shown in Fig. 2A, few 8-OHdG-immunopositive cells were observed in either the control or the melatonin group. However, after exposure to RF radiation, the number of 8-OHdG-positive cells significantly increased. We also observed that most of the 8-OHdG-positive cells were colocalized with MitoTracker Red CMXRos in the cytoplasm of the neurons (Fig. 2A). This result suggested that the RF radiation damaged DNA in the mitochondria of neurons. Consistent with the immunostaining detection results, HPLC analysis revealed that there was an approximately two-fold greater increase in the levels of 8-OHdG in mitochondria of the RF radiation group compared to those of the control and melatonin groups ($P<0.01$), and melatonin pretreatment almost completely attenuated this type of mtDNA oxidative damage (Fig. 2B).

It has been reported that mtDNA damage is not only reflected by an increase in mtDNA mutations, but also by a decline in mtDNA copy numbers and a reduction in the numbers of mtRNA (Ide et al., 2001). Our study showed that RF radiation reduced the mtDNA copy numbers by half (Fig. 3). In addition, the mtDNA transcript levels of ND1, COX I and ND6

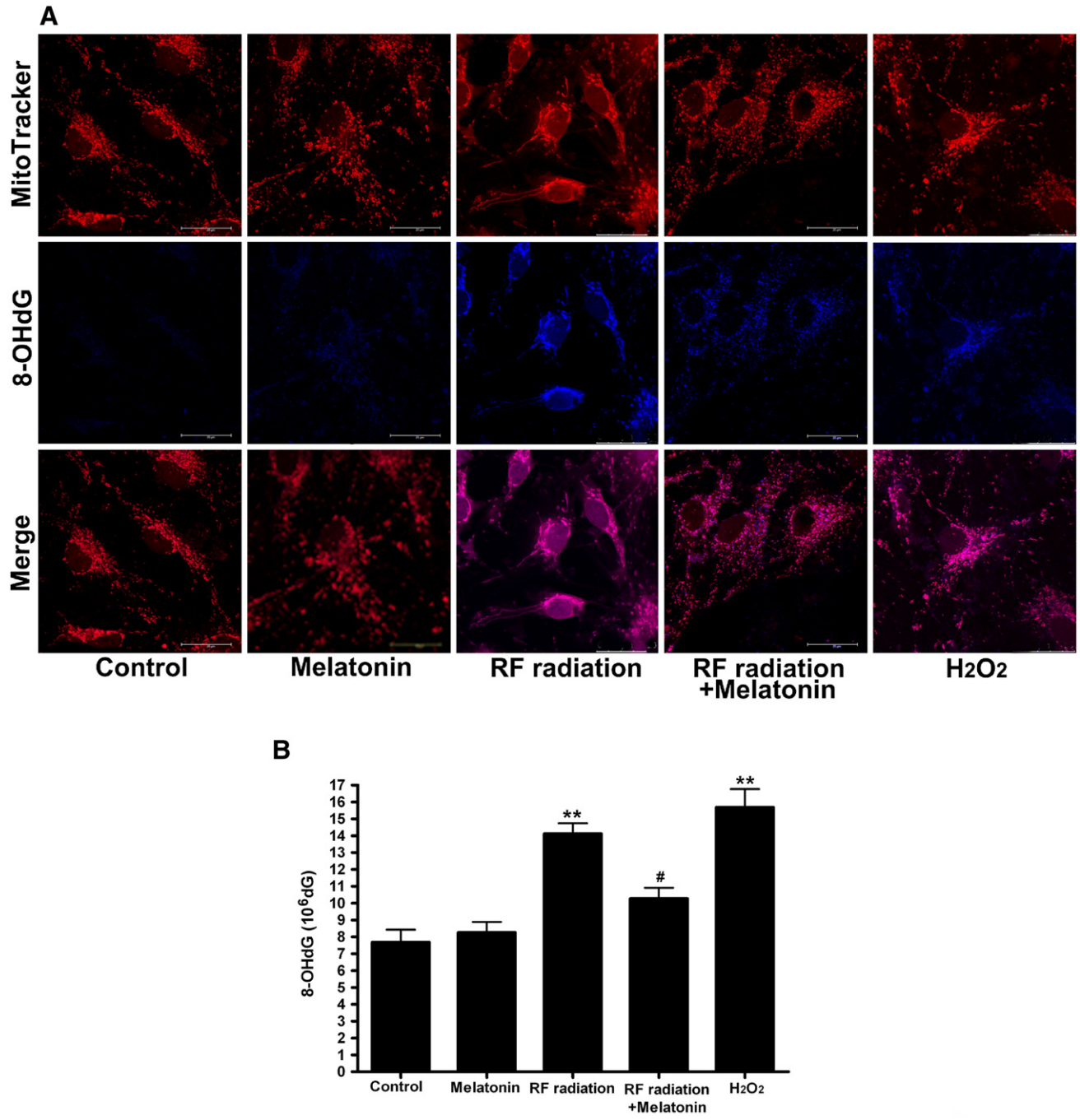

Fig. 2 – Increase of 8-OHdG levels in mitochondria after RF radiation. (A) After exposure to RF radiation, neurons were incubated with MitoTracker Red CMXRos probe and then with 8-OHdG monoclonal antibody. Confocal microscopic scanning photographs indicated the fluorescence signals of MitoTracker Red CMXRos (red) and 8-OHdG immunostaining (blue). The merged images of red and blue fluorescence appeared as a bluish red color. The presented image is the representative of three independent experiments. (B) For HPLC analysis, mtDNA in exposed neurons was extracted and digested to deoxynucleotides using nuclease P1 and alkaline phosphatase. The 8-OHdG content of mitochondria for each experimental group was measured by HPLC using an electrochemical detection system in experimental groups. 8-OHdG amount was expressed as the number of 8-OHdG molecules per 10^6 dG, determined simultaneously with a UV monitor coupled to the HPLC system. $^{**}p<0.01$ versus sham-exposed control group and melatonin group, $^{\#}p<0.05$ versus RF radiation group. Values are means ± SE, $n=3$.

in RF-irradiated cells were 49%, 28% and 64% lower than those of the controls, respectively (Fig. 4). All of these reductions in mtDNA copy number and mtDNA transcripts were significantly prevented by melatonin pretreatment. These results further proved that RF radiation damaged mtDNA in neurons (Figs. 3 and 4).

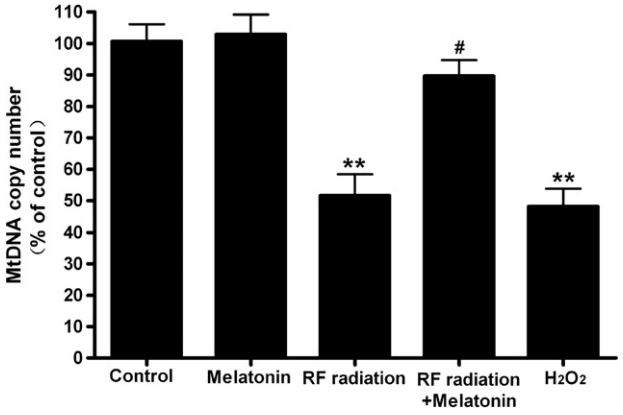

Fig. 3 – Decrease of mtDNA copy number after RF radiation. Quantitative real-time PCR was used to measure mtDNA copy number. A specific fragment of Complex IV was designed for mtDNA copy number quantification. The amounts of mtDNA were normalized to the internal control, β-actin. $^{**}p<0.01$ versus sham-exposed control group and melatonin group, $^{\#}p<0.05$ versus RF radiation group. Values are means±SE, $n=6$.

3. Discussion

Oxidative stress makes a substantial contribution to various pathological processes in the nervous system (Lin and Beal, 2006). As a result of its high metabolic rate, its inefficient oxidant defense mechanism and its diminished cellular turnover, the nervous system is particularly vulnerable to oxidative stress. Increasing evidence indicates that the radio-frequency fields of MP may affect brain biological activity by inducing oxidative stress (Ilhan et al., 2004; Koylu et al., 2006; Sokolovic et al., 2008; Zhao et al., 2007a). Consistent with this, we demonstrated that 1800 MHz RF radiation significantly damaged mtDNA maintenace by increasing the production of ROS in primary cultured cortical neurons. To our knowledge, this study is the first to demonstrate a relationship between RF radiation exposure and mtDNA damage in neurons.

Oxidative stress refers to an imbalance between the intracellular production of free radicals and cellular defense mechanisms (Tsutsui et al., 2006). In the present study, we showed that RF radiation obviously elevated the ROS production in cortical neurons. Consistent with previous studies, this indicated that RF radiation induced oxidative stress in neurons (Ilhan et al., 2004; Koylu et al., 2006; Sokolovic et al., 2008; Zhao et al., 2007a). An explanation for these findings could be that RF radiation produced an excess of ROS via inhibition of the mitochondrial respiratory chain, prolongation of the life-span of free radicals and impairment of the antioxidant defense system (Koylu et al., 2006; Moustafa et al., 2001). However, several studies have reported that radiation emitted from MP has no effect of inducing oxidative stress (Ferreira et al., 2006; Hook et al., 2004; Oktem et al., 2006; Simko et al., 2006; Zeni et al., 2007). For example, Hook et al. did not detect the induction of oxidative stress in J774.16 mouse macrophage cells that were exposed for 20–22 h at 0.8 W/kg to either frequency-modulated continuous-wave field at 835.62 MHz or code division multiple access field at 847.74 MHz. The discrepancies between our results and those of previous studies may be due to differences in the RF signals that were used, such as frequency, orientation, modulation, power density and duration of exposure (Ilhan et al., 2004; Simko, 2007; Zeni et al., 2007). Furthermore, even when the same experimental design is used, distinct cell types may react differently to the same stimulus (Schwarz et al., 2008; Simko, 2007).

MtDNA encodes 13 key subunits of the respiratory chain, and it is highly susceptible to oxidative damage. In our study, there was a significant increase in 8-OHdG in mitochondria after RF field exposure. Concomitant with this alteration, the mtDNA copy number and the mtRNA transcript levels also decreased after RF radiation. These reductions might result from a ROS-induced mutation at the origins of replication and transcription (Coskun et al., 2004; Ide et al., 2001). In addition, the expression of mitochondrial-encoded genes is largely

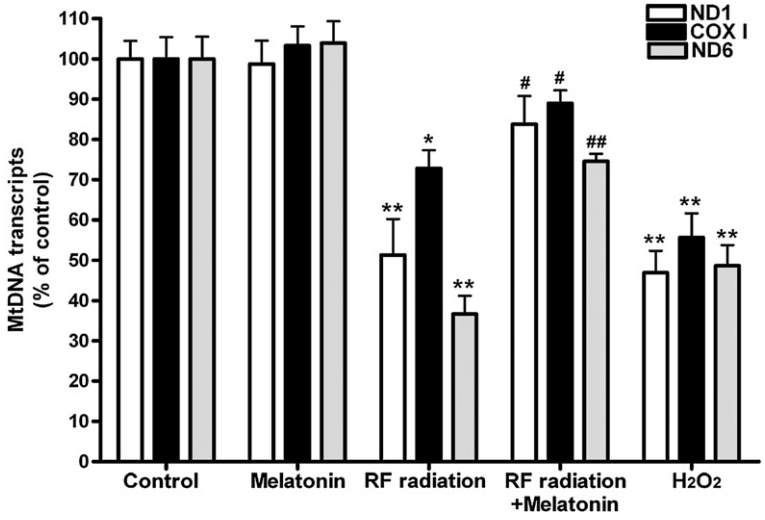

Fig. 4 – Decrease of mtRNA transcripts levels after RF radiation. Quantitative real-time PCR was used to measure the levels of mtRNA transcripts. The amounts of mtRNA were normalized to the internal control, β-actin. $^{**}p<0.01$, $^{*}p<0.05$ versus sham-exposed control group and melatonin group, $^{\#\#}p<0.01$, $^{\#}p<0.05$ versus RF radiation group. Values are means±SE, $n=6$.

regulated by the copy number of mtDNA (Kanki et al., 2004). Therefore, a decrease in the mtDNA copy number may result in a corresponding decrease in mtRNA transcripts, which could ultimately lead to mitochondrial dysfunction. Furthermore, oxidative stress could directly degrade mtDNA and mtRNAs by damaging the mtDNA nucleoid structure and the stability of mtRNAs (Kang et al., 2007; Rodriguez-Gabriel et al., 2003). Importantly, oxidative stress could be amplified by the damaged mtDNA through encoding of the deficient critical proteins for the respiratory chain (Evans et al., 2004). It is conceivable that RF radiation-induced oxidative stress caused mitochondrial dysfunction through damage to mtDNA. In turn, reduced mitochondrial function might further exacerbate the production of ROS, which could induce further oxidative damage to mtDNA. This vicious cycle might proceed until mitochondrial failure and apoptosis occurred. Indeed, recent data showed that exposure to radiofrequency radiation decreased cerebral cytochrome c oxidase activity and activated apoptosis signaling pathways in primary cultured neurons (Ammari et al., 2008; Zhao et al., 2007b).

Melatonin is a neurohormone that is synthesized in and released from the pineal gland during the dark period. It is capable of rapidly crossing the blood–brain barrier and accumulating in high concentrations in the cortical and hippocampal cells (Cabrera et al., 2000). Melatonin is considered to be a potent antioxidant that works to detoxify a variety of ROS and stimulate anti-oxidative enzymes in many pathophysiological states, particularly in the brain (Gupta et al., 2003). In our study, melatonin administration could effectively attenuate the oxidative damage of mtDNA. It further indicated that oxidative stress was the critical neurotoxic effect of RF radiation. This was consistent with the previous facts that melatonin improved oxidative phosphorylation and protected mtDNA against oxidative damage at the mitochondrial level by decreasing ROS production (Acuna-Castroviejo et al., 2001; Chen et al., 2005; Gurkok et al., 2009; Jou et al., 2007; Pappolla et al., 1999).

In conclusion, our study indicates that 1800 MHz RF radiation induced oxidative damage to mtDNA, but that melatonin prevented this damage. Based on the facts that mtDNA defects are the key factors in various mitochondria-associated behavioral symptoms and diseases of the nervous system (Dong et al., 2007; Lin and Beal, 2006; Loeb et al., 2005; Warner et al., 2004), oxidative damage to mtDNA may be an important mechanism underlying the adverse effects of RF radiation that is emitted from MP on brain activity. However, it may be difficult to directly extrapolate our results (which were obtained using rodent cells) to humans for different exposure conditions and SAR values. In future studies, to investigate the neurotoxicity of MP radiation in mtDNA defects, greater care will need to be taken to replicate the actual RF exposure conditions for humans.

4. Experimental procedures

4.1. Cell culture and RF radiation

Cortical neurons were dissected from newborn SD rats that were purchased from the animal center of Third Military Medical University. All procedures were carried out in strict accordance with the guidelines of the Animal Care Committee. Minced neocortexes were digested with trypsin-EDTA at 37 °C for 20 min. Cells were plated onto 35 mm dishes that were pre-coated with poly-L-lysine-coated (Sigma, St. Louis, MO), at a density of 5×10^5 cells/2 ml in DMEM/F-12 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 10% horse serum (HyClone, Logan, UT) and 50 mg/ml penicillin/streptomycin. After 24 h, cells were cultured in Neurobasal™ medium (Gibco BRL, Paisley, UK) containing $1\times B_{27}$ supplement (Invitrogen, Carlsbad, CA) and 0.5 mM L-glutamine.

On day 8, neurons were exposed to a carrier frequency of 1800 MHz intermittently (5 min fields on/10 min fields off) or sham-exposed for 24 h at an average SAR of 2 W/kg as previously described (Diem et al., 2005; Zhao et al., 2007a). The RF-exposure system used in these experiments is the sXc-1800 system (Zurich, Switzerland). This system is designed for the testing of RF electromagnetic field exposures from mobile communication devices in the 1800 MHz region such as the GSM digital communication system. The fields were amplitude-modulated by rectangular pulses with a repetition frequency of 217 Hz and a duty cycle of 1:8. The exposure system was composed of a RF generator, an arbitrary function generator, a narrow band amplifier and two rectangular waveguides. Exposed and sham-exposed cell dishes were simultaneously placed inside a commercial incubator in which the environmental conditions were constant (37 °C, 5% CO_2, 95% humidity). During the exposure, the value of SAR was monitored and the temperature response of the medium was assessed as described previously (Sun et al., 2006). The temperature difference between sham- and RF-exposed cultures never exceeded 0.05 °C. To enable blind exposures, the entire setup was controlled with a computer, which automatically controlled the exposure parameters, including exposure strength (SAR) and exposure time.

The cultured cells were assigned to one of five groups: (1) sham-exposed control, (2) Melatonin, (3) RF radiation, (4) RF radiation+Melatonin (5) hydrogen peroxide (H_2O_2) exposure. In groups (2) and (4), melatonin (Sigma, St. Louis, MO) was dissolved in ethanol and then freshly diluted with cell medium before application. Melatonin was used 4 h prior to RF radiation at a concentration of 200 nM. In group (5), cells treated with 100 μM H_2O_2 for 24 h were used as a positive control of oxidative stress. In groups (2) and (5), cells were put into the incubator for sham-exposure. Before the experiment, we also designed an incubator control and found there were no differences between the cells in the normal incubator and those in the sham-exposed waveguide at an average SAR of 2 W/kg for 24 h.

4.2. Assay of intracellular reactive oxygen species (ROS)

The determination of intracellular oxidant production was based on the oxidation of DCFH-DA (Beyotime Company, China). Briefly, exposed cells were incubated with DCFH-DA at 37 °C for 20 min. DCFH-DA passively diffused into the cells and was deacetylated by esterases to form nonfluorescent 2′,7′-dichlorofluorescein (DCFH). In the presence of ROS,

DCFH reacted with ROS to form the fluorescent product DCF, which was trapped inside the cells. The amount of emitted fluorescence was correlated with the quantity of ROS in the cell. Fluorescence images were acquired using a Leica fluorescence microscope at 485 nm for excitation and 530 nm for emission. The fluorescence intensity for each dish was measured within 25×25 pixel regions of interest and analyzed for pixel intensity by NIH Image. The experiment was repeated six times and cellular fluorescence intensity was expressed as the multiple of the level in control groups.

4.3. *Immunostaining of 8-OHdG in mitochondria*

To measure the levels of 8-OHdG located in mitochondria, exposed cells were reacted with 200 nM MitoTracker Red CMXRos probe for 30 min at 37 °C according to the manufacturer's instructions. After being washed twice in cold PBS, cells were fixed with 2% (w/v) paraformaldehyde in PBS at 4 °C for 20 min. Then the cells were permeabilized with 0.5% Triton X-100 in PBS for 15 min at room temperature. The cells were washed twice with PBS, before being blocked with normal goat serum at 37 °C for 30 min. Subsequently, the cells were incubated with a mouse monoclonal antibody against 8-OHdG at 1:100 dilution overnight at 4 °C, and then with Cy5-labeled goat anti-mouse IgG (H+L) (Beyotime Company, China) at 1:200 dilution for 1 h at 37 °C. The cells were mounted in mounting medium and visualized under a Leica confocal laser scanning microscope (TCS SP2, Germany). For two-color fluorescence, the red and blue signals were collected simultaneously and then superimposed. Colocalization appeared as a bluish red color due to mixing of the red and blue signals.

4.4. *HPLC analysis of 8-OHdG*

To analyze the 8-OHdG contents in mitochondria, mtDNA was isolated from the neurons using a Qiagen Plasmid Midi kit, as previously described (Cahill et al., 1997). After mtDNA was digested with nuclease P1 and Escherichia coli alkaline phosphatase, the contents of 8-OHdG were assessed using high-performance liquid chromatography (HPLC) with an electrochemical detection system as reported. The 8-OHdG amount was expressed as the number of 8-OHdG molecules per 10^6 dG, determined simultaneously with a UV monitor coupled to the HPLC system (Cahill et al., 1999).

4.5. *Detection of mtDNA copy number and mtDNA transcripts levels*

We applied real-time PCR to detect mtDNA copy number and mtRNA transcripts levels as previously described (Xu et al., 2009). The real-time PCR was studied with the iQ™5 Real-Time PCR Detection System (Bio-Rid, USA) and using the SYBR Green I detection method. Each real-time PCR reaction (25 μl total volume) contained 1 μl of template DNA (10 ng) or cDNA, 12.5 μl of 2×SYBR® Green Realtime PCR Master (Toyobo, Osaka, Japan), 1 μl of each of the forward and reverse primers and 9.5 μl ultrapure water. Samples were denatured by heating at 95 °C for 4 min, followed by 40 cycles of amplification and quantification (95 °C for 15 s and 58 °C for 15 s) and by a final extension cycle (72 °C, 90 s). Each measurement was repeated six times and was normalized against the control.

For mtDNA copy number analysis, total cellular DNA from cells was extracted using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. As previously described, we compared relative amounts of mtDNA and nuclear DNA copy numbers. The mtDNA amplicons were generated from Complex IV segment. The nuclear amplicon was generated through amplification of a β-actin segment. The mtDNA primers (Accession No. NC_001665) were 5-AGCAGGGATACCTCGTCGTTAC-3 and 5-GTTTTGATGCGAAGGCTTCTCAAA-3 (fragment length, 140 bp), and there were designed to minimize amplification of mtDNA pseudogenes embedded in the nuclear DNA. The primers for β-actin (Accession No. NC_005111), which were chosen as the internal standards, were 5-TCCCAGCACACTTA ACTTAGC-3 and 5-AGCCACAAGAAACACTCAGG-3 (fragment length, 98 bp). The threshold cycle number (Ct) values of β-actin and the mtDNA were determined for each individual quantitative PCR run. The –ddCt (mtDNA to β-actin) represented the mtDNA copy number in a cell.

For mtRNA transcripts quantification, total RNA was extracted with TriPure (Roche, Indianapolis, IN) reagent. Reverse transcription was performed with ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). The primers for NADH dehydrogenase subunits 1 (Accession No. X14848.1 (2729-3685)) were 5-CGAATACGCCGCAGGAC C-3 and 5-TGCTAGGAAAATTGGCAG GGA-3 (fragment length, 298 bp). The primers for cytochrome c oxidase subunits I (Accession No. X14848.1 (5309-6853)) were 5-AGCAGGGATACCTCGTCGTTAC-3 and 5-GTTTTGATGCGAAGGCTTCTCAAA-3 (fragment length, 140 bp). The primers for NADH dehydrogenase subunits 6 (Accession No. X14848.1 (13531-14049)) were 5-GATTGATTGTTAGTGGATGTATTG-3 and 5-CTCAGTAGCCATAGCAGTTG-3 (fragment length, 137 bp). As mentioned above, β-actin was selected as the internal standard. The threshold cycle number (Ct) values of β-actin and mtDNA transcripts were determined. The –ddCt (mtDNA transcripts to β-actin) represented the mtDNA transcripts in a cell.

4.6. *Statistical analysis*

All the experimental data were expressed as the means ± SE. Each experiment was carried out at least three times. Statistical significances of differences between two groups were determined using Student's *t*-test. All *P* values were two-sided, and $P<0.05$ was considered statistically significant.

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