Letter to the Editor

Real-time Assessment of Cytosolic, Mitochondrial, and Nuclear Calcium Levels Change in Rat Pheochromocytoma Cells during Pulsed Microwave Exposure Using a Genetically Encoded Calcium Indicator*

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Little information is available about the effects of exposure to pulsed microwaves on neuronal Ca^{2+} signaling under non-thermal conditions. In this study, rat pheochromocytoma (PC12) cells were exposed to pulsed microwaves for 6 min at a specific absorption rate (SAR) of 4 W/kg to assess possible real-time effects. During microwave exposure, free calcium dynamics in the cytosol, mitochondria, and nucleus of cells were monitored by time-lapse microfluorimetry using a genetically encoded calcium indicator (ratiometric-pericam, ratiometric-pericam-mt, and ratiometric-pericam-nu). We established a waveguide-based real-time microwave exposure system under accurately controlled environmental and dosimetric conditions and found no significant changes in the cytosolic, mitochondrial, or nuclear calcium levels in PC12 cells. These findings suggest that no dynamic changes occurred in $[Ca^{2+}]_c$, $[Ca^{2+}]_m$, or $[Ca^{2+}]_n$ of PC12 cells at the non-thermal level.

Over the past few decades, we have been increasingly exposed to pulsed microwaves emitted by telecommunications and medical devices, but whether and how the microwaves interact with living cells remains controversial. Exposure to microwaves at frequencies > 100 kHz can lead to significant energy absorption and temperature increases, which are often considered thermal effects. The restrictions in these guidelines when establishing exposure limits were based primarily on thermal effects[1]. Although many non-thermal effects have been reported, the published results are extremely difficult to replicate and were not widely accepted by the scientific community. The International Commission on Non-Ionizing Radiation Protection (ICNIRP) set guidelines about occupational and general public exposure limitations mainly based on the specific absorption rate (SAR) rather than the peak power of pulsed fields. Little information regarding pulsed microwave bio-effects were considered in the exposure guidelines[1]. With the wide application of high power microwaves in radar and other emitters, hazards from occupational and accidental microwave exposure should be taken seriously. Therefore, we chose high-peak-power microwave pulses (200 W/cm^2 peak power), with relatively short pulse widths. The effects under such microwave pulses have often been ignored due to the low average power of microwaves despite high-peak-power. Single pulses with short pulse duration may produce higher energy densities than the threshold for potential hazards.

The spatial and temporal properties of Ca^{2+} signals are particularly important features for the functional diversity of cells, such as energy metabolism, neurotransmitter release, enzyme activation, intracellular signal transduction, and gene expression[2]. In addition, calcium-binding proteins, acting as calcium buffers, determine the dynamics of free calcium inside neurons[3]. Changes in Ca^{2+} might be the reason for microwave-induced neural injury. A number of studies have evaluated the effects of microwave on Ca^{2+} signaling in neuronal and non-neuronal cells, but the results have been controversial[4]. PC12 cells represent a well characterized, transformed cell line that exhibits numerous features of neurons and has been frequently used as a model neuronal cell line[5].

Moreover, physiological effects were previously assessed after electromagnetic treatment[6]. Many

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transient and recoverable effects may not be observed. Studies on the early interactions between microwaves and cells lagged behind due to limitations in real-time microwave exposure equipment. Therefore, a real-time pulsed microwave exposure system is essential for a real-time bioelectromagnetic study.

In the present study, we designed a real-time pulsed microwave exposure system connected with a laser scanning confocal microscope (LSCM), which contained a signal generator, a pulse amplifier, a circulator, a directional coupler, a waveguide cell, a power meter and the LSCM, for real-time recording (Figure 1A). The exposure chamber had two windows (Φ 60 mm and 20 mm) at the center of the top and bottom wall to visualize the cell under the microscope and to place cell samples and the optical fiber temperature probe. The glass-bottom (Φ 20 mm) dish (NEST, Shanghai, China) containing the biological sample was positioned on the center window of the lower wall of the chamber to ensure uniformity of the electromagnetic field distribution. To reduce leakage, center window (Φ 20 mm) annular metal accessories were used to cover the top of the window after placing the cell samples. The main transmission mode in the waveguide was TE10 mode, and the electric field was perpendicular to the medium surface when the magnetic field was parallel

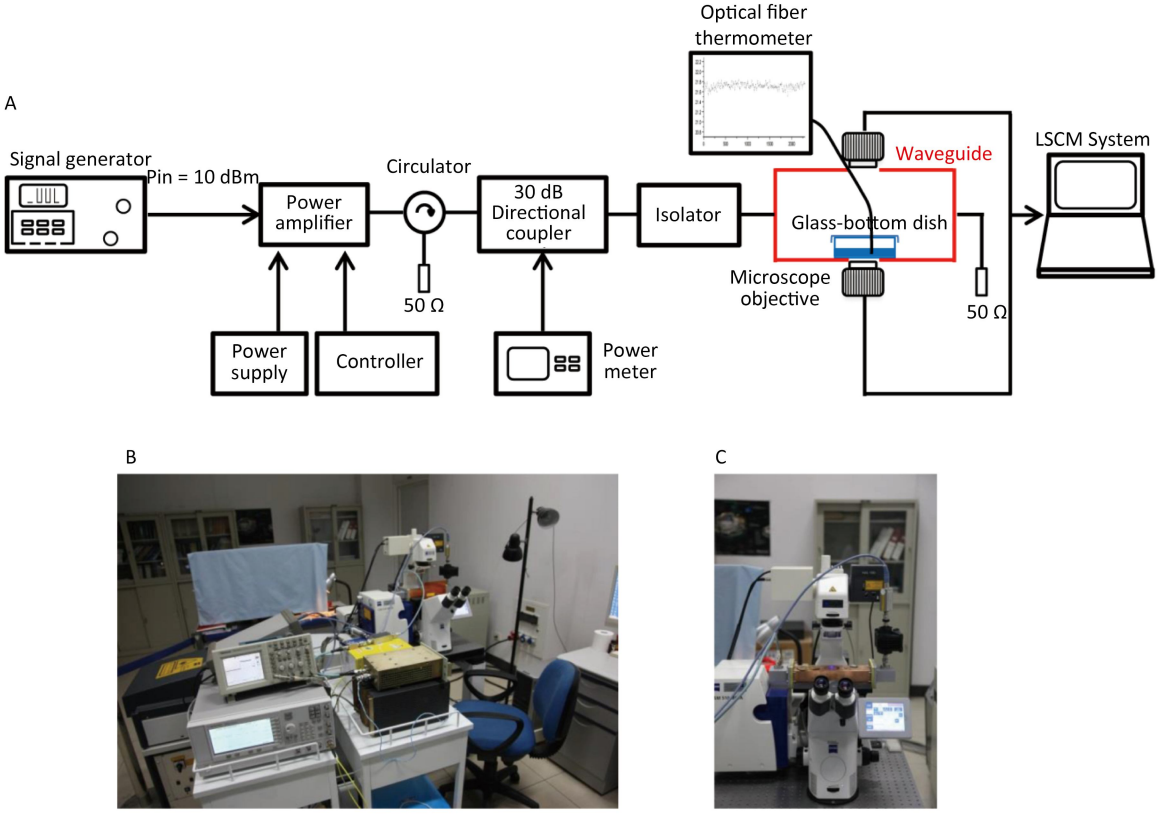

Figure 1. Schematic and photographs of the cell exposure system. (A) Schematic diagram of the experimental microwave exposure setup. The microwave exposure system is composed of a signal generator (Agilent E8257D ESG-D Series generator; Agilent Technologies, Santa Clara, CA, USA), an amplifier (IMP2729; Microwave Amplifiers, Shijiazhuang, China), a circulator, a bidirectional coupler (11667A power splitter; Hewlett-Packard, Palo Alto, CA, USA), and a customized waveguide (WR260, 72.14 mm width × 34.04 mm height, 270 mm length). The fluorescence measurement system is mainly composed of the customized waveguide and a laser scanning confocal microscope (LSCM). The glass-bottom dish containing cells is placed above the opening window on the bottom side of the waveguide. The temperature probe is inserted into the culture medium vertically and is close to the bottom of the dish to obtain the average temperature of the field; (B) Photograph of the exposure system; (C) photograph of the customized waveguide on the LSCM microscope stage.

to the medium surface. The Agilent E4417A EPM-P Series Power Meter (Agilent Technologies, Santa Clara, CA, USA) and signal generator were connected, allowing continuous monitoring of the power level that was adjusted according to the required SAR. Empire 5.0 software (IMST, Kamp-Lintfort, Germany), based on the finite difference time domain method, was employed for the dosimetry simulation to evaluate SAR[7].

PC12 cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% horse serum (Invitrogen), 5% fetal bovine serum (Invitrogen), 10^5 U/L of penicillin, and 10^5 U/L of streptomycin. The culture medium was changed every 2 days, and the cells were split 1:4 every week. The PC12 cells were seeded at a density of 2×10^4 cells/cm^2 in poly-L-lysine-coated glass-bottom dishes for differentiation. Six hours later, the cells were induced with 5 ng/mL nerve growth factor-2.5S (Sigma-Aldrich, St. Louis, MO, USA) in DMEM plus 1% horse serum for 5-7 days.

Ratiometric-pericam (RP), ratiometric-pericam-mt (RP-mt), and ratiometric-pericam-nu (RP-nu) are calcium indicators based on fusion of circularly permuted yellow fluorescent protein and calmodulin constructed by Atsushi Miyawaki Laboratory[8]. An advantage of these Ca^{2+}-sensing fluorescent proteins is that they were engineered to selectively combine with specific cellular organelles or subcellular regions. The RP, RP-mt, and RP-nu were selectively expressed in the cytosol, mitochondria, and nucleus of cells, respectively, which made it easy to dynamically monitor the cytosolic calcium concentration ($[Ca^{2+}]_c$), mitochondrial calcium concentration ($[Ca^{2+}]_m$), and nuclear calcium concentration ($[Ca^{2+}]_n$).

The RP, RP-mt, and RP-nu plasmids were generous gifts of Dr. Miyawaki (RIKEN, Saitama, Japan). A total of 1 μg DNA and 2 μL Lipofectamine2000 (Invitrogen) was premixed according to the manufacturer's instructions and added to cells growing on glass-bottom dishes in 2 mL medium. After 6 h, the medium was replaced with 2 mL of fresh supplemented medium. At 48 h after transfection, the culture media were removed and replaced with 2 mL Krebs/HEPES buffer. The central cell sample was observed for 2 min before microwave exposure, and then exposed to an average SAR of 4 W/kg (frequency: 2,856 MHz, pulsed width: 2 μs, one pulse every 265 μs) for 6 min. After the microwaves were powered off, the sample was observed for an additional 2 min. Sham-exposed cells were treated under the same conditions but without microwave exposure (sham exposure group).

Ratiometric imaging was obtained every 2 s for 10 min using a 40× objective lens on an inverted fluorescence microscope (Eclipse Ti-E; Nikon, Tokyo, Japan) with the Ultra View spinning-disk confocal scanner unit (Perkin Elmer, Waltham, MA, USA). Areas of interest containing one or more moderately bright cells expressing RP were suitable for acquiring images sequentially by alternatively exciting at 488 and 405 nm. Once the experiment was completed, the imaging sequences were viewed and analyzed with Volocity software. Fluorescence intensity was measured in regions of interest. Moreover, the ratio of 488 nm/405 nm fluorescence was presented as a trace representing the change in calcium levels.

Data are expressed as mean ± standard error. Repeated-measures analysis of variance was applied, and a P-value < 0.05 was considered significant.

To detect the thermal or non-thermal effects, we used the optical fiber thermometer (m3300, Luxtron Corp, Santa Clara, CA, USA) placed in culture medium and an exposure chamber to monitor the temperature of the culture medium and exposure chamber dynamically. The sampling frequency was 1 s, and temperature resolution was 0.1 °C. Microwave exposure (SAR = 4 W/kg) did not induce significant changes in temperature (Figure 2), indicating no thermal effects in the exposed samples.

Representative recordings of sham and exposed PC12 cells are shown in Figure 3. Each pericam (RP, RP-mt, RP-nu) was transfected into PC12 cells, and the fluorescence (green and blue) was uniformly distributed throughout the cytosolic, mitochondrial, and nuclear compartments, respectively (Figure 3A, C, E). The cytosolic, mitochondrial, and nuclear $[Ca^{2+}]$

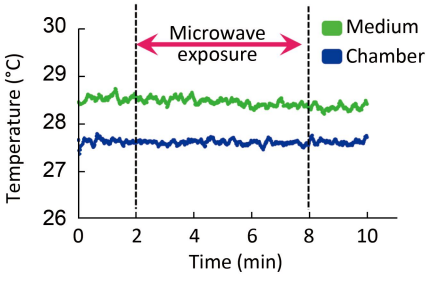

Figure 2. Temperature monitoring using an optical fiber thermometer. No significant change in temperature was observed with time.

of the PC12 cells did not show any significant changes during the entire pulsed microwave exposure (Figure 3B, D, F). Analysis of the mean values obtained from each experiment during the baseline, exposure, and post-exposure periods demonstrated no significant effects of pulsed microwave exposure on any of the calcium levels measured (Table 1). These results indicate that exposure to 4 W/kg pulsed microwaves did not have any effects on calcium signaling in PC12 cells.

Using this exposure system, the high-peak-power pulsed microwave at SAR of 4 W/Kg for 6 min did not induce thermal effects and showed no evidence of any consistent or biologically relevant effect on intracellular Ca^{2+} homeostasis in PC12 cells. The average SAR of 4 W/Kg was chosen mainly based

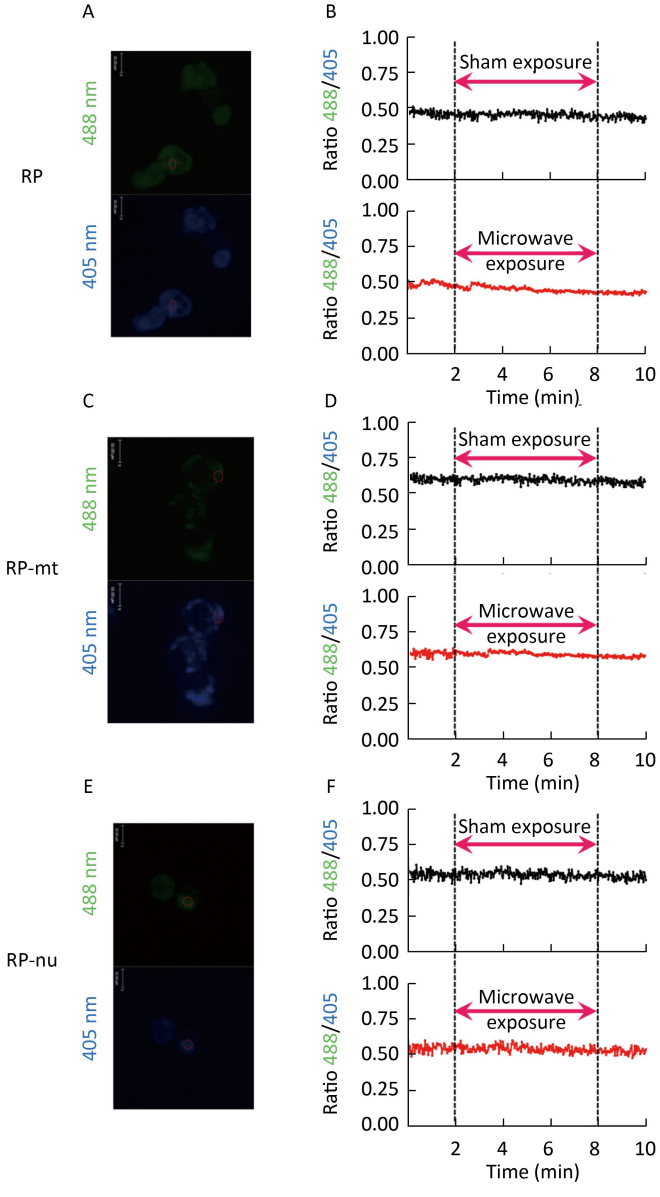

Figure 3. Real-time effects of pulsed microwave exposure on cytosolic, mitochondrial, and nuclear [Ca^{2+}] in PC12 cells. (A), (C), (E) Subcellular localization of RP, RP-mt, and RP-nu. This image shows PC12 cells transfected with RP, RP-mt, and RP-nu. (Scale bar = 20 μm); (B), (D), (F) Calcium dynamics in the cytoplasm, mitochondria, and nuclei of PC12 cells during pulsed microwave exposure. Black and red traces represent the kinetic changes in the 488/405 nm ratio over the region of interest of a single representative cell in the sham and microwave exposure groups, respectively.

Table 1. Real-time Effect of 4 W/kg Pulsed Microwaves on Calcium Levels in PC12 Cells

Timing (min)	Average Relative $[Ca^{2+}]_c$		Average Relative $[Ca^{2+}]_m$		Average Relative $[Ca^{2+}]_n$	
	Sham (n = 21)	Microwave (n = 25)	Sham (n = 25)	Microwave (n = 30)	Sham (n = 24)	Microwave (n = 29)
0-2	0.453 ± 0.021	0.459 ± 0.021	0.607 ± 0.031	0.601 ± 0.033	0.543 ± 0.033	0.541 ± 0.023
2-8	0.455 ± 0.026	0.451 ± 0.022	0.599 ± 0.028	0.580 ± 0.032	0.548 ± 0.032	0.550 ± 0.022
8-10	0.452 ± 0.019	0.452 ± 0.024	0.603 ± 0.029	0.598 ± 0.035	0.543 ± 0.030	0.543 ± 0.026

Note. Average relative $[Ca^{2+}]_c$, $[Ca^{2+}]_m$, and $[Ca^{2+}]_n$ are represented by ratio 488/380 of RP, RP-mt, RP-nu respectively. Results are presented as mean ± SEM from all sham and exposed condition experiments.

on the safety criteria established by the ICNIRP, which is 10 times greater than the occupational exposure restriction in the ICNIRP guidelines[1]. A review by D'Andrea summarized studies about disrupted animal behavior in response to threshold microwaves (4 W/kg) as a potential hazard of microwave radiation absorption[9].

In our experiments, the cell response to pulsed microwaves was observed in real-time with the calcium indicators. A microfluorimetry technique was successfully used in this study to investigate whether $[Ca^{2+}]$ changed in PC12 cells during application of microwaves. The electromagnetic field of the microwaves can interfere with the signal recorded by the electrodes, so dynamic observations by LSCM may be more suitable than the patch-clamp technique. This real-time pulsed microwave exposure system combined with different fluorescent dyes can be employed to detect other real-time bioelectromagnetic effects *in vitro*, such as reactive oxygen species, cell membrane potential, mitochondrial membrane potential and cycling of synaptic vesicles. This exposure system provides a novel platform and a new research approach to study the interactions between electromagnetic fields and cells.

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