● RESEARCH ARTICLE

Quinacrine pretreatment reduces microwave-induced neuronal damage by stabilizing the cell membrane
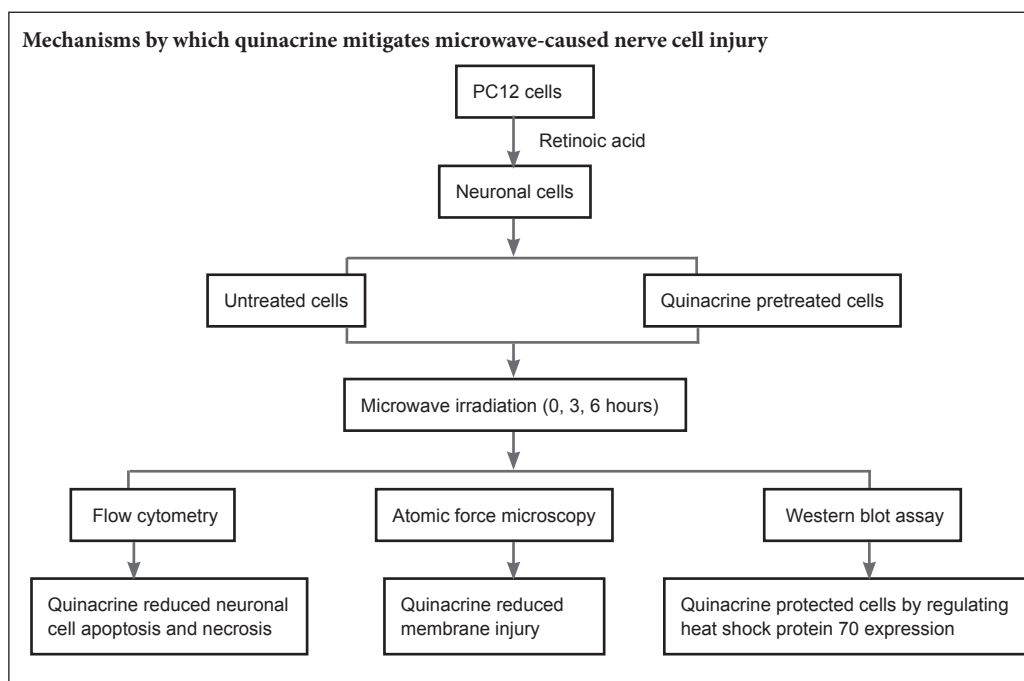
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Graphical Abstract

Mechanisms by which quinacrine mitigates microwave-caused nerve cell injury

PC12 cells
→ Retinoic acid
Neuronal cells
→
Untreated cells | Quinacrine pretreated cells
→
Microwave irradiation (0, 3, 6 hours)
→

Flow cytometry → Quinacrine reduced neuronal cell apoptosis and necrosis

Atomic force microscopy → Quinacrine reduced membrane injury

Western blot assay → Quinacrine protected cells by regulating heat shock protein 70 expression

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Abstract

Quinacrine, widely used to treat parasitic diseases, binds to cell membranes. We previously found that quinacrine pretreatment reduced microwave radiation damage in rat hippocampal neurons, but the molecular mechanism remains poorly understood. Considering the thermal effects of microwave radiation and the protective effects of quinacrine on heat damage in cells, we hypothesized that quinacrine would prevent microwave radiation damage to cells in a mechanism associated with cell membrane stability. To test this, we used retinoic acid to induce PC12 cells to differentiate into neuron-like cells. We then pretreated the neurons with quinacrine (20 and 40 mM) and irradiated them with 50 mW/cm^2 microwaves for 3 or 6 hours. Flow cytometry, atomic force microscopy and western blot assays revealed that irradiated cells pretreated with quinacrine showed markedly less apoptosis, necrosis, and membrane damage, and greater expression of heat shock protein 70, than cells exposed to microwave irradiation alone. These results suggest that quinacrine stabilizes the neuronal membrane structure by upregulating the expression of heat shock protein 70, thus reducing neuronal injury caused by microwave radiation.

Key Words: nerve regeneration; quinacrine; microwave; irradiation; heat shock; cell apoptosis; cell necrosis; thermal effect; cell membrane; heat shock protein 70; nerve cells; neural regeneration

Introduction

Microwaves are widely used in daily life. Their effects, both thermal and non-thermal, have been studied extensively (Mausset-Bonnefont et al., 2004; Brillaud et al., 2007; Zuo, 2014; Deshmukh et al., 2016; Cantres-Rosario et al., 2017; Tan et al., 2017). One of the most important characteristics of microwaves is their thermal effect, which we harness for heating and cooking food, and which greatly facilitates our life (Hermann et al., 1997; Sinha et al., 2008; de Tommaso et al., 2009; Zhao et al., 2012; Xiong et al., 2013; Deshmukh, 2015; Masuda et al., 2015; Xiong et al., 2015; Hinrikus et al., 2017). However, under some conditions, this thermal effect may damage human organs and the central nervous system (Ono et al., 2004; Campisi et al., 2010; Wang et al.,

2015; Zhao et al., 2017; Zhi et al., 2017). Therefore, it is important to find effective drugs against microwave-induced heat injury. Quinacrine (6-chloro-9-[(4-diethylamino)-1-methyl-butyl]-amino-2-ethoxyacridine) was once widely used for treating protozoan infections, Giardia intestinalis, Taenia saginata, and malaria (Lalle, 2010; Meyer zu Hörste et al., 2010; Ehsanian et al., 2011; Growe et al., 2013; Haseman et al., 2015; Kodera et al., 2017). During World War II, quinacrine was used as a first-line antimalarial drug, saving many lives. Today, although quinacrine is no longer used as an antimalarial drug in higher-income countries, as more effective substituted compounds have been found, research on this drug is ongoing (Chauhan and Srivastava, 2001; Kalia and Dutz, 2007; Manna et al., 2016). Recently, quinacrine was found to stabilize the cell nucleus by binding to DNA and other nuclear proteins (Reyes et al., 2001; Hossain et al., 2008; McNamee et al., 2016). It is also an inhibitor of phospholipase-A2 (PLA2), which is responsible for hydrolyzing membrane phospholipids to release free fatty acids and lysophospholipids. So quinacrine may also be involved in stabilizing cell membranes (Markaverich et al., 2007; Ortiz et al., 2014; Sharma A et al., 2017). Furthermore, mounting evidence suggests quinacrine has a protective effect against heat injury, including that caused by microwaves (Zhao et al., 2004; Gao et al., 2009). Indeed, our previously published study showed that quinacrine protected animals against microwave-induced heat injury (Gao et al., 2009). However, the molecular mechanism of this process remains to be elucidated.

In the present study, we pretreated neurons derived from PC12 cells with quinacrine, then exposed them to microwave irradiation, to explore the role of quinacrine in microwave-induced heat damage to neurons.

Materials and Methods
PC12 cell culture and neuronal differentiation
PC12 cells (1.5×10^4) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and maintained at 37°C with 5% CO_2 in a tissue culture incubator for up to 8 days (Fujino et al., 2013; Tarjányi et al., 2013). The cultured PC12 cells were transferred into poly-D-lysine-coated dishes (35 mm) and cultured in DMEM containing 1% fetal bovine serum and 1% horse serum (Gibco Life Technologies, Grand Island, NY, USA), before being induced with retinoic acid to differentiate into neuronal cells. About 7–8 days after induction, the induced cells formed a network of neurites. Next, the differentiated cells were characterized with antibodies against neuron-specific enolase and neurofilament (Sigma, St. Louis, MO, USA) as neuronal markers (Scheibe et al., 1991; Sakimura et al., 1995).

PC12 cell pretreatment with quinacrine before microwave irradiation
Quinacrine (low concentration, 20 mM; high concentration, 40 mM) was applied to differentiated PC12 cells and the dishes were immediately sealed with Parafilm to prevent bacterial contamination. The sealed dishes were put into a homemade microwave generator as described

previously (Wang et al., 2015; Zhao et al., 2017; Zhi et al., 2017). In brief, the 2.9 GHz generation system consisted of a pulse microwave generator (BZJ1500M-300W, Glory MV Electronics, China) and a 45 dB gain power amplifier (VE1079A, Beijing Vacuum Electronics Research Institute, China). Microwave energy was transmitted by a rectangular waveguide and a 12 dB standard-gain horn antenna to an anechoic chamber ($12.5 \times 9.7 \times 7.5$ m^3). The diagonal of the antenna was 17.2 cm. The interior walls of the anechoic chamber were covered with 500 mm and 300 mm pyramidal microwave absorbers to minimize reflections (> 45 dB). The emitted power was measured using a power sensor (N1921A, Keysight, Santa Rosa, CA, USA) connected to a directional coupler at one port of a circulator. The peak and average power densities were measured using a calibrated waveguide antenna, a power meter (N1912A, Keysight) and a power sensor (N1921, Keysight). Microwave pulses were delivered at 50 pps, 100 pps and 300 pps, respectively, with a pulse width of 500 ns. The peak power densities for the three exposure groups were 200 W/cm^2. The average power densities were 5, 10 and 30 mW/cm^2. The output power of the radiation source for the three exposure groups was 3 MW. The cells were irradiated with 50 mW/cm^2 microwaves for 3 or 6 hours. Untreated differentiated cells were used as the negative control. Microwave conditions were as follows: temperature, 15–25°C; humidity, 30–40%; frequency, 2.856 GHz; pulse length, 500 ns; dishes 2 meters from radiation source. The cover of the dish did not affect microwave transmission (Marchiarullo et al., 2013).

Flow cytometry
After irradiation, cell apoptosis and necrosis were examined using an annexin-V flow cytometry kit (Thermo Fisher Scientific, New York, NY, USA) as described previously (Qiao et al., 2014; Dai et al., 2015).

Membrane surface ultrastructure assay using atomic force microscopy
Atomic force microscopy was performed as previously described (Zhang et al., 2017; Marsh et al., 2018). In brief, the pretreated PC12 cells were fixed with 1.25% glutaraldehyde solution for 30 minutes at 4°C and washed three times with 0.9% NaCl. The treated cells were scanned using an atomic force microscope (SPM-9500J3, Shimadzu, Kyoto, Japan) at 25°C and images were acquired using the contact mode and 125 mm × 125 mm scanner at a rate of 2 Hz. Images were processed with specific atomic force microscopy software (SPM online and offline software; Shimadzu) (Yingge et al., 2003; Plodinec et al., 2015). Membrane integrity was analyzed using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA). The darker the membrane in the image, the more severe the damage.

Western blot assay
After microwave exposure, cells were immediately harvested and lysed using radioimmune precipitation assay lysis buffer (Sigma). Protein samples were subjected to sodium dodecyl

sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking with 5% skim milk in phosphate-buffered saline (PBS), the membrane was incubated with polyclonal antibody against HSP70 (1:1,000; Abcam, Cambridge, UK) and polyclonal β-actin (1:1,000; Abnova, Taipei, China) for 2 hours at room temperature. Peroxidase-coupled detection was performed using enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK) (Huang et al., 2013). The relative expression level of HSP70 was calculated using Quantity One software (Bio-Rad, Hercules, CA, USA) as the gray value ratio of the HSP70 and actin bands.

Immunohistochemistry
Seven days after induction with retinoic acid, the cells were fixed with 4% paraformaldehyde/PBS at 4°C overnight. To block non-specific binding, the cells were incubated with 5% goat serum in PBS (pH 7.4) for 1 hour at room temperature, and then with rabbit polyclonal anti-neuron-specific enolase (NSE) and anti-neurofilament (NF) antibodies (1:500; Sigma) in PBS containing 5% goat serum and 0.1% Triton X-100 at 4°C overnight. After three washes in PBS, the cells were incubated with goat anti-rabbit secondary antibody (Abcam) conjugated with horseradish peroxidase for 2 hours at room temperature. Immunoreactivity was examined using diaminobenzidine as a chromogen. NSE- and NF-immunoreactive cells were counted in five randomly placed visual fields in each group, the ratio of the number of NSE- or NF-positive cells and the number of total cells was calculated as a percentage of neuronal cells. The mean percentage for each group was used in the final analysis.

Statistical analysis
Statistical analyses were performed using Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Comparisons between three or more groups were made using one-way analysis of variance and the Tukey's *post-hoc* test, as described previously (Fan et al., 2015; Long et al., 2015). Data are presented as the mean ± SEM. $P \leq 0.05$ was considered statistically significant.

Results
PC12 cells were successfully induced to differentiate into neurons
PC12 cells differentiated into neuron-like cells after induction. Long neurites were observed in most induced cells (**Figure 1**). Immunohistochemistry showed that more than 90% of differentiated cells were positive for NSE and more than 70% were positive for NF, confirming that the induced cells could be used for the subsequent experiment.

Quinacrine pretreatment reduced the apoptosis and necrosis rates of microwave-exposed cells
To understand the effect of quinacrine on microwave-induced neuronal apoptosis and necrosis, induced PC12 cells were exposed to microwave radiation, with or without pretreatment with quinacrine. The cells were then examined using flow cytometry. Compared with the control group, cells irradiated for either 3 or 6 hours showed significantly more neuronal apoptosis and necrosis (**Figure 2**). However, compared with the microwave-only group, cells pretreated with low- or high-concentration quinacrine showed significantly less apoptosis and necrosis after 3- and 6-hour irradiation. In cells exposed to 3-hour irradiation (**Figure 2A–C**), both concentrations of quinacrine significantly reduced microwave-induced apoptosis, with no significant difference between the concentrations; however, the rate of necrosis was significantly lower after high-concentration quinacrine pretreatment than after low-dose quinacrine ($P < 0.01$). This might be due to the different processes involved in necrosis and apoptosis. In addition, apoptosis and necrosis caused by 6-hour microwave irradiation (**Figure 2D**) were lower in cells pretreated with low- or high-concentration quinacrine, with no significant difference in effect between the two concentrations.

Quinacrine pretreatment reduced membrane damage caused by microwave irradiation
To understand the mechanism underlying the protective effect of quinacrine on microwave-exposed neuronal cells, we examined membrane integrity using atomic force microscopy. For both irradiation durations, cells pretreated with high-concentration quinacrine showed markedly less membrane injury than non-pretreated cells (**Figure 3**), suggesting that quinacrine protects neurons by stabilizing the cell membrane.

Quinacrine reduced microwave-induced neuronal membrane damage by increasing HSP70 expression
To further investigate the molecular mechanism underlying the protective effect of quinacrine, we examined the expression of HSP70, a commonly used heat injury marker, using western blots. HSP70 expression did not differ significantly between non-irradiated cells, cells irradiated for 3 hours, and cells pretreated with low-concentration quinacrine and irradiated for 3 hours; however, high-concentration quinacrine resulted in significantly higher HSP70 expression than in control cells (**Figure 4**). In contrast, 6-hour microwave irradiation resulted in significantly lower HSP70 expression ($P < 0.05$); this may be caused by the greatly enhanced neuronal apoptosis and necrosis. Both low- and high-concentration quinacrine rescued this decrease in HSP70 expression ($P < 0.05$), with no significant difference found between concentrations (**Figure 4**). These results indicate that quinacrine may promote neuronal cell survival by regulating HSP70 expression, though it may be not the only mediator in the signaling pathway.

Discussion
Due to the extensive use of microwaves in our daily life and work, there is increasing concern about the interactions of electromagnetic radiation with human health, in particular brain health (Zhao et al., 2012; Deshmukh et al., 2015; Xiong et al., 2015). The thermal effect is one of the most import-
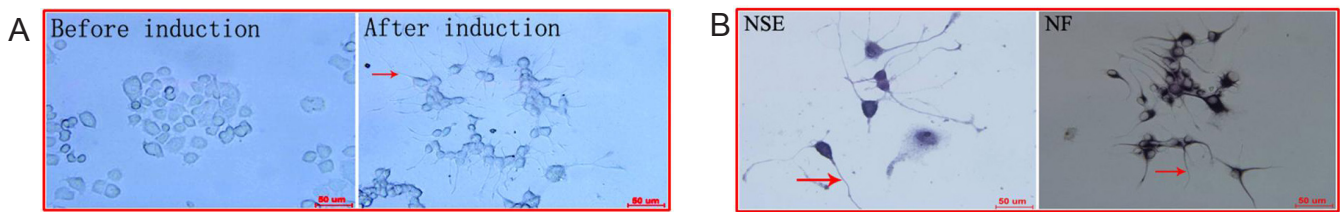
Figure 1 PC12 cells were successfully induced into neuronal cells.
Before induction with retinoic acid, PC12 cells were cultured in Dulbecco's modified Eagle's medium containing 1% fetal bovine serum and 1% horse serum. After induction, the differentiated cells were characterized using immunohistochemistry with NSE (neuron) and NF (neuron) polyclonal antibody. (A) Morphology of PC12 cells before and after induction. Neurites (arrow) in the induced PC12 cells are observed. (B) Compared with the control, large numbers of neurites (arrows) were observed in NSE- and NF-immunoreactive cells. Scale bars: 50 μm. NSE: Neuron-specific enolase; NF: neurofilament.
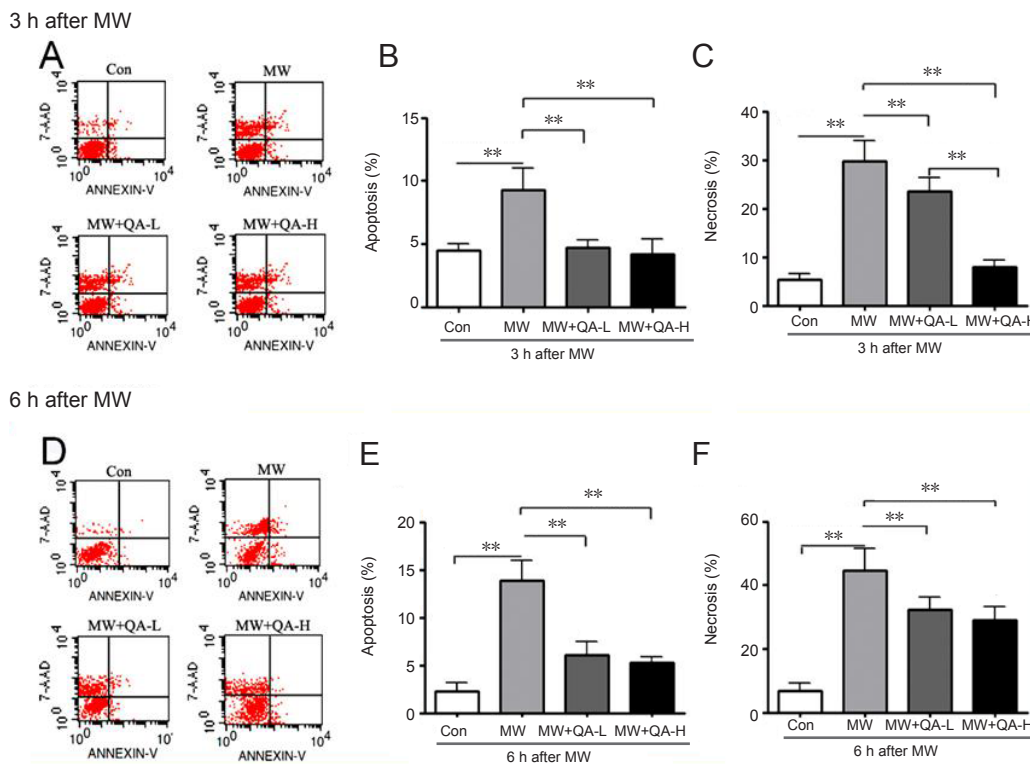
Figure 2 Quinacrine reduced microwave-induced neuronal cell apoptosis and necrosis.
The induced PC12 cells were exposed to microwaves (MW) with or without quinacrine pretreatment at low (20 mM; QA-L) or high (40 mM; QA-H) concentration for 3 and 6 hours separately. The treated cells were examined using flow cytometry. (A) Apoptosis and necrosis rates of control (3 h), MW group (3 h), MW + QA-L (3 h), and MW + QA-H (3 h). (B, C) Statistical analysis of apoptosis (B) and necrosis (C) in the three groups (3 h). (D) Apoptosis and necrosis rates of control (6 h), MW (6 h), MW + QA-L (6 h), and MW + QA-H (6 h) groups. (E, F) Statistical analysis of apoptosis and necrosis in the three groups (6 h). $**P < 0.01$ (mean ± SEM, $n = 3$, one-way analysis of variance analysis followed by Tukey's *post-hoc* tests). Experiments were performed in triplicate. Con, Normal control; MW (3, 6 h), MW exposure for 3 and 6 h, respectively; MW + QA-H (3, 6 h): 40 mM quinacrine pretreatment followed by MW exposure for 3 or 6 h, respectively; MW + QA-L (3, 6 h), 20 mM quinacrine pretreatment followed by MW exposure for 3 or 6 h, respectively. MW: microwave; QA: quinacrine; QA-L: low-dose quinacrine; QA-H: high-dose quinacrine; h: hours.

ant effects of microwaves. It is this effect that is harnessed to make our lives easier, but excessive microwave radiation may cause neuronal heat injury and affect human brain function. Our previous study showed that quinacrine administration reduced microwave-induced heat injury to the hippocampus (Gao et al., 2009). Here, to characterize the molecular mechanism, we explored the effects of quinacrine on microwave-induced heat injury in PC12 cells differentiated into neurons after induction with retinoic acid. PC12 cells differentiated into NSE- and NF-immunoreactive cells, and outgrowth of neurites indicated successful establish-

ment of the neuronal cell model. Subsequent investigation showed that microwave radiation under our conditions caused neuronal apoptosis and necrosis, consistent with previously published studies (Martin et al., 2009; Motawi et al., 2014). However, in the presence of quinacrine, this damage was markedly decreased, further supporting our previous study in an animal model. Subsequent analysis showed that there was no significant difference in apoptosis reduction between low- and high-concentration quinacrine at either 3- or 6-hour microwave irradiation, indicating that low-dose quinacrine is enough to prevent neuronal cell apoptosis in
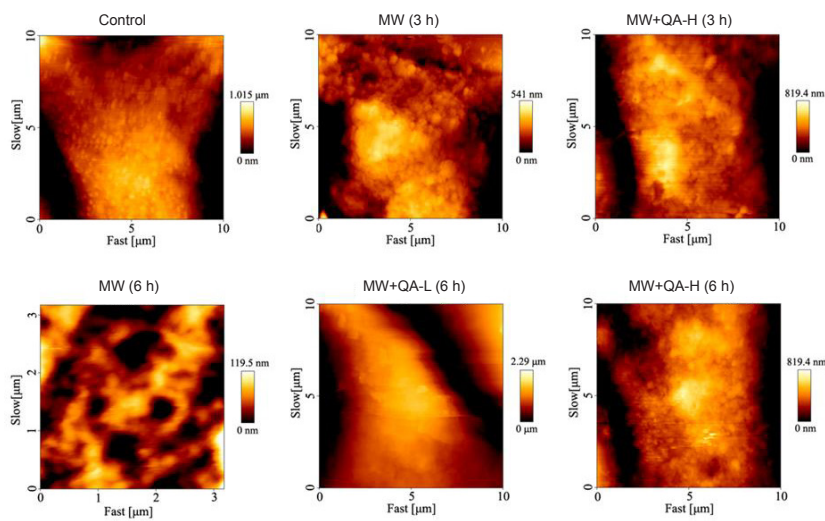
Figure 3 Quinacrine pretreatment reduced membrane injury caused by microwave exposure.

Atomic force microscopy images showing membrane surface ultrastructure of microwave-exposed PC12 cells. Darker areas represent more severe membrane damage. At 3 and 6 hours after microwave exposure, cells pretreated with high-concentration quinacrine showed markedly less membrane injury than non-pretreated cells. Relative injury intensity was analyzed using Image-Pro Plus software. The scale represents the height of the outermost surface: the lighter the color, the greater the height of the surface. MW (3, 6 h): microwave exposure for 3 or 6 hours, respectively; QA-H: 40 mM quinacrine pretreatment; QA-L: 20 mM quinacrine pretreatment.
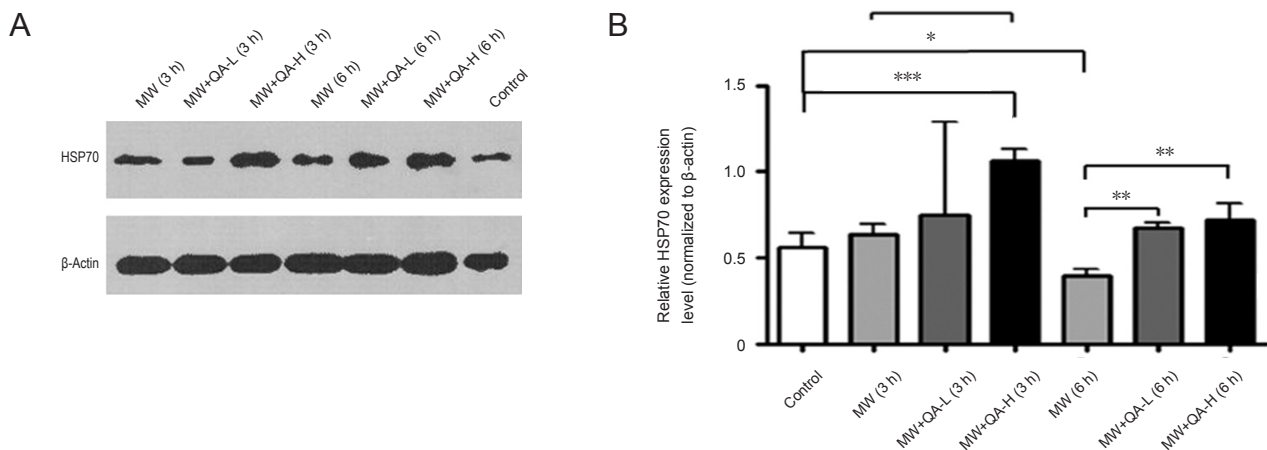
Figure 4 Quinacrine pretreatment reduced microwave-induced neuronal membrane injury by increasing HSP70 expression.
(A) Western blot assay of HSP70 expression level among different groups. (B) Statistical analysis of HSP70 expression level using Quantity One software. Cells pretreated with 40 mM quinacrine before 3-hour microwave exposure (QA-H+MW (3 h)) showed greater HSP70 expression than those not pretreated (MW (3 h)) or pretreated with 20 mM quinacrine (QA-L+MW (3 h)). In cells exposed to 6 hours of microwave irradiation, both 20 and 40 mM quinacrine pretreatment groups (QA-L+MW (6 h) and QA-H+MW (6 h)) showed greater HSP70 expression than those not pretreated (MW (6 h)). $*P < 0.05$, $**P < 0.01$ (mean ± SEM, $n = 3$, one-way analysis of variance analysis followed by Tukey's *post-hoc* tests). Experiments were performed in triplicate. HSP70: Heat shock protein 70; h: hours.

this cell model. For 3-hour irradiation, the high concentration of quinacrine reduced the rate of necrosis more than the low concentration, and quinacrine even reduced the rate of necrosis to a similar level as control (non-exposed) cells. In the 6-hour exposure groups, no significant difference in cell necrosis rate was detected between low- and high-concentration quinacrine, although the necrosis rate in the quinacrine treatment groups was significantly higher than that in the control group, indicating that the necrosis caused by 6-hour microwave irradiation might be not reversible. Because PLA2 hydrolyzes membrane phospholipids to release free fatty acids and lysophospholipids, whereas quinacrine is an inhibitor of PLA2, quinacrine may reduce cell necrosis by inhibiting PLA2 (Markaverich et al., 2007; Ortiz et al., 2014). Further investigations on cell membrane integrity using atomic force microscopy showed that the membrane structure in cells exposed to 6-hour microwave irradiation was

worse than that in 3-hour groups. In addition, high-concentration quinacrine had a better effect on stabilizing membrane structure than low-concentration quinacrine in the 3-hour groups. In the 6-hour groups, low-concentration quinacrine had better protective effects on membrane structure. HSP70 is a protector in heat stroke injury (Horowitz and Robinson, 2007; Kim et al., 2012; Liu et al., 2016; Shi et al., 2017). To find the related molecular mechanism involved in this process, we examined HSP70 expression levels using western blots. In the 3-hour groups, quinacrine at high, but not low, concentration resulted in a significant upregulation of HSP70 expression. In the 6-hour irradiation groups, both low-dose and high-dose quinacrine unregulated HSP70 expression compared with irradiation alone. However, 6-hour, but not 3-hour, irradiation caused significant downregulation of HSP70 compared with the control group, which also supports the flow cytometry results. Therefore, HSP70 may be in-

volved in regulating the protective effect of quinacrine on microwave-induced neuronal injury. However, this effect may be limited to certain cell lines, as data from other labs have demonstrated that quinacrine can induce apoptosis in SGC-7901 cells, metastatic breast cancer stem cells, and human leukemia K562 cells (Wu et al., 2012; Changchien et al., 2015; Siddharth et al., 2016). Therefore, the roles of quinacrine in different cell types or cell lines should be explored in future.

In summary, we induced differentiation of PC12 cells into neurons and exposed the neurons to microwave irradiation. We found that quinacrine protected the cells against thermal damage from microwaves by upregulating HSP70 expression and stabilizing the cell membrane structure *via* PLA2 inhibition. These results will inform discussions on new uses of quinacrine in thermal brain damage.

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