Methanol extract of Kang-fu-ling attenuates high power microwave-induced oxidative stress in PC12 cells via the Nrf2/ARE pathway

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Abstract: Kang-fu-ling (KFL) is a polybotanical traditional Chinese medicine which exhibits activity against oxidative stress in protecting the high power microwave (HPM) injured rats. In this study, KFL was found significantly increased the protein level of Nrf2 in the nucleus of rat hippocampal pyramidal neurons. KFL consists of a six-ingredient comprehensive formulation, so we look for the essential component for its function. We selected the aqueous (KFL-A) and methanol (KFL-M) extract as the candidate for antioxidant defense evaluation, and both of them showed strong free radical scavenging activity and non-toxicity. However, only the KFL-M treatment significantly suppressed the production of malondialdehyde and intracellular ROS even under the HPM exposure. The molecular mechanism investigation showed that the pre-incubation of cells with KFL-M before HPM exposure increased the protein level of nuclear factor erythroid 2-related factor 2 (Nrf2) in nucleus and the mRNA expression of target genes, such as HO-1, GST-mu3 and NQO1. The protective effect of KFL-M could provide a promising approach for the treatment of HPM injury.

Keywords: KFL-M, high power microwave, oxidative stress, Nrf2/ARE pathway

Introduction

In recent years, with the significant advances in the development of microwave technology, there is more public concern about the possibility that exposure to electromagnetic fields might cause health problem, especially in central nervous system. Studies have demonstrated that the high density microwave exposure could cause detrimental effects on hippocampal morphology and cognitive behavior [1-4]. Recently, more and more powerful microwave emitter (communications equipment and radar) are being developed, leading to an increased potential for accidental exposure. Yet, currently available therapeutic agents are still lacking. Thus, there is a high unmet need for the development of highly effective and orally available treatment options.

Our previous work has clearly provided the information that excessive accumulation of reactive oxygen species induced by microwave was involved in the neurological injury, although its underlying mechanism has not yet been fully elucidated [5]. Thus, preventing oxidative stress may be a potential therapeutic strategy for the microwave-induced disorders. Oxidative stress activates an adaptive mechanism aimed to protect cells against ROS-induced injury. This response is regulated by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) [6-8]. Under physiological conditions, Kelch-like ECH-associated protein-1 (Keap1) promotes Nrf2 ubiquitination degradation. Under electrophilic and oxidative stress, reactive cysteine residues of Keap1 become modified, and result in the release of Nrf2 and its translocation to the nucleus, where it binds to antioxidant response elements (ARE) in genes encoding antioxidant enzymes [9].

We have developed traditional Chinese medicine Kang-fu-ling (KFL) with neuroprotective effects against high power microwave (HPM), which ameliorates oxidative stress via the Nrf2/
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ARE pathway [5, 10]. In this study, we further delineate Nrf2 expressing cell-types in the hippocampus. We found KFL treatment in HPM-exposed rats leads to an increase in free Nrf2 in pyramidal neurons rather than glial cells in hippocampus CA1 area. So the PC12 cells were chosen to evaluate the possible essential component of KFL as it is well-established model system for studying neuronal injury and neuroprotective effect [11-13]. The PC12 cell line is initially derived from a rat pheochromocytoma, and the completely differentiated PC12 cells possess the phenotypic properties of sympathetic neurons [14].

Based on our previous study, we speculated some electrophilic compounds contained in KFL which could cross blood-brain barrier exert neuroprotective effects dependent on Nrf2 mediated anti-oxidative pathways. Due to the complexity of the chemical composition of KFL, it is difficult to determine which specific compounds are effective. In order to investigate which fraction play the most important role in the stimulation of Nrf2, solvents of different polarity were used to divide KFL into several fractions. We confirmed that the methanol extract of KFL was a new inducer of Nrf2 for the treatment of HPM injury like t-BHQ. In summary, Nrf2/ARE pathway is involved in the neuroprotective effects against HPM and the supplement KFL-M is a promising natural complex, which ameliorates oxidative stress.

Materials and methods

Immunohistochemistry

The primary antibodies against Nrf2 used in immunohistochemistry, IF, and immunoblotting was purchased from Santa Cruz. All animal experiments protocols were performed in accordance with the protocol outlined in the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health and approved by Institutional Ethical Committee for Care and Use of Laboratory Animals of the Academy of Military Medical Sciences. Immunohistochemistry staining for Nrf2 was carried out on the paraffin-embedded rat brain tissue, followed by secondary antibody and 3,3′-diaminobenzadine disclosure (ZS-Bio), and microscopic imaging and analysis. Nuclei were counterstained with haematotoxyn. Images were captured using a Leica DM6000 microscope (Leica, Germany). The widely accepted German semi-quantitative scoring system in considering the staining intensity and area extent was used [15]. Each specimen was assigned a score according to the intensity of the nucleic, cytoplasmic, and membrane staining (no staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3) and the extent of stained cells (0-5% = 0, 5-25% = 1, 26-50% = 2, 51-75% = 3, 76-100% = 4). The final immunoreactive score was determined by multiplying the intensity score with the extent of score of stained cells, ranging from 0 (the minimum score) to 12 (the maximum score).

Isolating KFL by different dissolvent

KFL was prepared as described before [5]. In order to purify and isolate the different fractions of KFL, 10 g of powdered KFL was successively extracted in Soxhlet extractor and partitioned with petroleum ethre, trichloromethane, ethyl acetate, methanol, and last the distilled water (1:30, w/v). The extracts were dried using a rotary vacuum evaporator and stored in a desiccator. After each fraction had been dried, they were dissolved in DMSO at a concentration of 50 mg/ml and stored at 4°C for further experiments. The stock solution was filtered by DMSO-Safe Acrodisc® Syringe Filter before use.

Measurement of hydroxyl radical scavenging activity by electron spin resonance (ESR) spectroscopy

The hydroxyl radical scavenging ability was evaluated in a cell-free, in vitro system using Bruker ESP A300 EPR spectrometer (Bruker BioSpin, Germany) as described previously [5, 16]. For detection of hydroxyl radical (OH·), the signal intensity was evaluated by measuring the second peak of DMPO/OH spin adduct in reaction buffer containing 5 μl FeSO₄ (2 mM), 5 μl DTPAC (1 mM), 5 μl DMPO (0.8 mM), 5 μl H₂O₂ (3 mM), and 30 μl sample.

PC12 cells culture and treatments

PC12 cells were cultured in DMEM (Corning) supplemented with 10% horse serum (Hyclone), 5% fetal bovine serum (Hyclone), 10° U/L of penicillin, and 10° U/L of streptomycin at 37°C with a 5% CO₂ atmosphere. The cells were cultured at 37°C for 48 h. Then they were incu-
bated with corresponding drugs (50, 100, and 200 μg/ml of the KFL-A or KFL-M, or 10 t-BHQ, or the vehicle alone) for another 48 h, then exposed to 30 mW/cm² HPM exposure for 6 min.

**Microwave exposure system and dosimetry**

The microwave exposure system, which consists of radio frequency generator, klystron amplifier, rectangular waveguide, 16-dB standard-gain horn antenna, electromagnetic shield chamber, and oscilloscope, has been described previously [5]. The cells were exposed to pulsed microwave at an average power density of 30 mW/cm² for 6 min. Sham-exposed cells were treated under the same conditions without microwave. The SAR calculation was based on the finite difference time domain (FDTD) method as described [17-19]. In our study, the average SAR were calculated to be 19 W/kg.

**Intracellular ROS analysis**

Intracellular ROS level was determined by dichlorofluorescein diacetate (DCFH-DA), a cell permeable nonfluorescent molecular probe oxidized by ROS to fluorescent compound dichlorofluorescein (DCF). After the indicated treatments as described above, cells were incubated with 10 μM DCFH-DA for 30 min at 37°C in darkness and then were washed twice with PBS. The fluorescence intensity of DCF was detected by FACS Aria flow cytometer (BD, USA) or fluorospectrophotometer (PerKinElmer, USA) at an emission wavelength of 535 nm and an excitation wavelength of 485 nm.

**Immunofluorescence staining and cell imaging**

For subcellular localization analyses, cells were grown on coverslips and fixed with 4% paraformaldehyde, permeabilized in 0.2% Triton X-100/PBS and blocked with 5% BSA. Proteins were stained using the indicated antibodies and detected with a TRITC-conjugated or FITC-conjugated secondary antibody. The nuclei were stained with DAPI (Molecular Probe), and images were visualized with a Zeiss LSM 510 Meta inverted confocal microscope (Zeiss, Germany).

**Preparation of cell lysates and immunoblotting**

Cytosolic and nuclear extracts from cells were prepared according to the manufacturer's instructions (BestBio). Proteins were separated by SDS-PAGE, transferred onto PVDF membrane and examined by immunoblotting with the indicated primary antibodies and appropriate secondary antibody, followed by detection with Super Signal chemiluminescence kit (Pierce). The intensity of the protein bands after immunoblotting were quantitated by using Quantity One 4.6.3 Image software (Bio-Rad, USA) and normalized against proper loading controls.

**Quantitative real-time PCR (qPCR)**

Total RNA of cells was isolated using the TRizol reagent (Invitrogen) following the manufacturer’s instructions. 1 μg of total RNA was converted to cDNA using the GoScriptTM Reverse Transcription System (Promega, USA). qPCR for rat β-actin, HO-1, GSTmu3 and NQO1 was completed using the GoTaqR qPCR Master Mix (Promega) using the following primers: β-actin (5’-AGA TCA AGA TCA TTG CTC CTC CT-3’; 5’-ACG CAG CTC AGT AAC AGT CC-3’), HO-1 (5’-GAG TTT CCG CTC AGT AAC AGT CC-3’), GSTmu3 (5’-TGG ACA TTC CCA ATT TGC CCT-3’; 5’-CAA AGT CAG GAC TGC AGC AA-3’) and NQO1 (5’-CAG AAA CGA CAT CAC AGG GGA-3’; 5’-AGC ACT CTC TCA AAC CAG CC-3’).

**Estimation of lipid peroxidation**

Malondialdehyde (MDA) reacts with thiobarbituric acid (TBA) to produce a red adduct. Levels of MDA were measured in PC12 cell lysates with a spectrophotometer at a wavelength of 535 nm. The method described in the MDA detection kit was employed from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. The protein content was measured using the Bradford method with bovine serum albumin as the standard.

**Statistical analysis**

Statistical calculations were carried out using SPSS 19.0. Data are expressed as the mean ± SEM. The results of semi-quantitative pathological evaluation were compared with Mann-Whitney Rank Sum Test. One-way ANOVA followed by Dennett’s post hoc test was used for multiple-group comparisons. Differences were considered to be statistically significant at P<0.05.
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Results

Nrf2 was activated by the KFL in hippocampal neurons

In our previous study, KFL could reduce the ROS accumulation and ameliorate the spatial memory impairment caused by HPM in rat, and KFL might activate the Nrf2/ARE signaling pathway in hippocampus [5]. To investigate whether the observed up-regulated Nrf2/ARE pathway effects of KFL were only on neurons/glial cells or rather both. We further delineate Nrf2 expressing profiles on the protein level in the hippocampus using immunohistochemistry. After KFL treatment, there was a clear increase of Nrf2 immunoreactivity in the hippocampus, especially in the pyramidal cell layer of CA1 area. At high magnification (400×), we found that Nrf2 protein mainly expressed in the nucleus of pyramidal neurons (Figure 1A-E). The expression of Nrf2 protein was confirmed by semi-quantitative scoring system (Figure 1F). In summary, KFL treatment in HPM-exposed rats leads to an increase in free Nrf2 in neurons rather than glial cells in hippocampus CA1 area.

Figure 1. KFL increased the protein level of Nrf2 in nucleus of rat hippocampal neurons. Rats were exposed to 30 mw/cm² microwave for 15 min and then orally administered different dose of KFL once daily for 14 days. Animals were anaesthetized, decapitated, and removed brain immediately. Then brain tissue was sectioned, fixed, dehydrated, and embedded in paraffin. Immunohistochemistry staining for Nrf2 was carried out on the paraffin-embedded rat brain tissue. A: Nrf2 expression was detected by immunohistochemistry in rat of shame exposure. B: Nrf2 expression detected by immunohistochemistry after microwave exposure. C-E: Nrf2 expression was detected by immunohistochemistry after KFL treatment in microwave-exposed rats. Scale bar, 50 μm. F: Box plot of Nrf2 expression in rat hippocampus (n = 15).

Both KFL-A and KFL-M possessed free radical scavenging activity

KFL was separated by solvents with different polarity, and the products were named as KFL-PE (0.27 g, 3%, w/w), KFL-T (0.62 g, 6%, w/w), KFL-EA (0.41 g, 4%, w/w), KFL-M (5.38 g, 54%, w/w) and KFL-A (3.27 g, 33%, w/w), respectively (Figure 2A). The percentage of methanol (KFL-M) and aqueous extract (KFL-A) was much higher than others, and we also found that most the possible antioxidant elements in KFL were dissolved in these two extracts.

So we then tested the effect of KFL-A and KFL-M on hydroxyl radical scavenging activity in a Fenton reaction system with DMPO, a spin trapping agent. In vitro studies showed that both KFL-A and KFL-M possessed free radical scavenging abilities. The typical ESR signals of DMPO-OH adducts were concentration-dependently attenuated when addition of KFL-M or KFL-A 0.6, 3, 6, 30 or 60 mg/ml (Figures 2B,
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In vitro, we first analyzed the toxicity of KFL extracts on PC12 cells. PC12 cells were treated with KFL-M and KFL-A for 48 h, and the highest exposure concentration of different extracts induced no injury is 400 μg/ml (Figure 3A). The concentration which is low than 400 μg/ml was chosen to test the protective effect of different extracts. The pre-incubation with KFL-M but not the KFL-A could inhibit the production of ROS induced by HPM (Figure 3B, 3C). We also tested the level of MDA, another marker of oxidative stress which is formed by degradation of polyunsaturated lipids by ROS. HPM increased MDA levels compared with the control group, while pre-incubation with KFL-M but not KFL-A caused a decrease in the levels of MDA (Figure 3D). Notably, although KFL-M and KFL-A both showed the scavenged hydroxyl ability in vitro, only KFL-M decreased HPM-induced oxidative stress in PC12 cells.

KFL-M activated Nrf2/ARE pathway in PC12 cells

To further study whether Nrf2/ARE signaling was required for the KFL-M neuroprotective effects, we found that KFL-M could increase Nrf2 expression in the nucleus of PC12 cells the same as t-BHQ (Figure 4A). Next, nuclear and cytoplasmic proteins were extracted, and equal amounts were separated by SDS-PAGE and immunoblotted with specific Nrf2 antibody. The data showed that KFL-M could induce the accumulation of Nrf2 proteins in the nucleus (Figure 4B, 4C). Next, we verified whether KFL-M could active Nrf2/ARE pathway under microwave exposure. The translocation of Nrf2 was measured by western blot. HPM exposure slightly increased the amount of nuclear Nrf2. After treatment with KFL-M, the nuclear protein expression of Nrf2 significantly increased (Figure 4D, 4E). The qPCR results showed that pre-treatment with KFL-M resulted in a dramatic increase in mRNA expression of three Nrf2-regulated antioxidant genes (HO-1, GSTmu3, and NQO1), which also confirmed the activation of Nrf2/ARE signaling (Figure 4F). These data suggested that KFL-M attenuated the microwave-induced oxidative stress in PC12 cells by activating the Nrf2/ARE pathway.

Discussion

The signaling mechanism of neurological injury induced by microwave is probably a complex process and has not been well studied [2]. The
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Oxidative stress leads to the damage and even cell death, thus prevention and control of oxidative stress in neuronal injury is a promising therapeutic target [20, 21]. Our previous work has confirmed that oxidative stress is involved in the injury induced by HPM and we also suggested that KFL enhanced the oxidative stress response in rat. Several studies have demonstrated that Nrf2-mediated neuroprotection may not only be directly mediated, but critically involves effects via astrocytes [22-24]. The astrocytes can improve antioxidant defenses in co-cultured neurons by releasing GSH [25]. However, our animal experiment results supplied the protection role of Nrf2/ARE pathway in neurons cells. The specificity of Nrf2 expressing cell-types was proven by immunohistochemistry. After KFL treatment, there was a clear increase of Nrf2 immunoreactivity in pyramidal neurons rather than glial cells in hippocampus CA1 area.

Here we also provided more data on the effective extraction of KFL for neuroprotective function. After separation, we found that the antioxidant effectors from the original materials were almost included in the methanol extract. Although both of KFL-M and KFL-A showed

Figure 3. Function detection of the different extracts from KFL. A: The extracts from KFL were dissolved in DMSO to different concentration. PC12 cells were exposed to 400 μg/ml KFL-M or KFL-A for 48 h. The cell viability was detected by MTT (n = 10). B: PC12 cells were pre-treated with 50, 100, 200 μg/ml KFL-M or KFL-A for 48 h before HPM exposure. Cells were loaded by DCFH-DA and detected by fluorospectrophotometer (n = 6). C: The fluorescence intensity of DCF was detected by flow cytometer. D: MDA level in cells were tested by MDA detection kit (n = 6).
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Figure 4. Effect of KFL-M on Nrf2 pathway in PC12 cells. A: PC12 cells were grown on coverslips, treated with 50, 100, 200 μg/ml KFL-M or 10 μM t-BHQ for 48 h, then fixed, permeabilized and probed with anti-Nrf2 antibody followed by TRITC-tagged secondary antibody. Cells were also stained with DAPI to visualize the nuclei (blue). Scale bar, 10 μm. B: Nrf2 protein level was detected by western blot in nuclear and cytosolic extracts of PC12 cells after KFL-M or t-BHQ 48 h pre-treatment. C: The Nrf2 band intensities were quantified and plotted. The results represent the means ± SEM of 3 separate experiments. D: PC12 cells were grown on plates, treated with or without KFL-M for 48 h, then exposed or sham exposed to HPM for 6 min. Nrf2 protein level was detected by western blotting. The results represent the means ± SEM of 3 separate experiments. E: The Nrf2 band intensities were quantified and plotted. F: HO-1, GSTmu3, and NQO1 mRNA level in PC12 cells was detected by qPCR (n = 3).
scavenging hydroxyl radical activity in a cell-free system, only the KFL-M functioned as a stimulator of Nrf2/ARE pathway. Further studies are needed to confirm the monomeric chemical substances as well the precise mechanism of KFL-M. A wide variety of plant-derived and synthetic compounds have been proven to be Nrf2 activators and have potent antioxidant properties [24, 26, 27]. For use in CNS, the capacity to cross blood-brain barrier and access to CNS tissue is believed to be a major issue [28]. Therefore, the polarity of candidate should be considered.

In this study, we provided some insights into the cellular mechanisms of HPM defense by using neuron-like PC12 cells. Moreover, we also revealed the scientific basis for KFL-M usefulness as traditional Chinese medicine. These results suggest that supplementation with KFL-M may be an effective strategy for preventing the brain oxidative mitochondrial damage and cognitive dysfunction associated with microwaves.

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Disclosure of conflict of interest

None.

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