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Neuroprotective effects of lotus seedpod procyanidins on extremely low frequency electromagnetic field-induced neurotoxicity in primary cultured hippocampal neurons

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ABSTRACT

The present study investigated the protective effects of lotus seedpod procyanidins (LSPCs) on extremely low frequency electromagnetic field (ELF-EMF)-induced neurotoxicity in primary cultured rat hippocampal neurons and the underlying molecular mechanism. The results of MTT, morphological observation, superoxide dismutase (SOD) and malondialdehyde (MDA) assays showed that compared with control, incubating neurons under ELF-EMF exposure significantly decreased cell viability and increased the number of apoptotic cells, whereas LSPCs evidently protected the hippocampal neurons against ELF-EMF-induced cell damage. Moreover, a certain concentration of LSPCs inhibited the elevation of intracellular reactive oxygen species (ROS) and Ca^{2+} level, as well as prevented the disruption of mitochondrial membrane potential induced by ELF-EMF exposure. In addition, supplementation with LSPCs could alleviate DNA damage, block cell cycle arrest at S phase, and inhibit apoptosis and necrosis of hippocampal neurons under ELF-EMF exposure. Further study demonstrated that LSPCs up-regulated the activations of Bcl-2, Bcl-xl proteins and suppressed the expressions of Bad, Bax proteins caused by ELF-EMF exposure. In conclusion, these findings revealed that LSPCs protected against ELF-EMF-induced neurotoxicity through inhibiting oxidative stress and mitochondrial apoptotic pathway.

1. Introduction

Electromagnetic fields are now a ubiquitous part of modern life, people increasingly have a focus on the impact of electromagnetic fields. An extremely low frequency electromagnetic fields (ELF-EMF) is an electromagnetic wave of frequency 0–300 Hz that emits a non-thermal effect of non-ionizing radiation, and is mainly caused by the electric appliances and power lines. It has raised concern about their potential adverse health effects because people are increasingly exposed to ELF-EMF. Several studies have shown the influences of ELF-EMF on behavior [1], cognition [2] and neurophysiology [3].

Some epidemiological studies have shown that acute exposure to ELF-EMF increases the risks for the central nervous system diseases (CNS), Alzheimer's disease, and Amyotrophic lateral sclerosis (ALS) [4]. Oxidative stress appears to be a possible mechanism. Oxidative stress is due to imbalance between oxidants derivatives production and antioxidants defenses [5]. The generation of ROS in the brain when exposure to ELF-EMF, however, obviously exceeds the affordability of the antioxidant system. Excessive ROS impair membrane phospholipids, destroy mitochondrial function, and damage a wide variety of components of the cell, including proteins, sugars, lipids, RNA and DNA. Finally, these changes induce neurodegeneration in the brain, so ELF-EMF is regarded as a useful tool to model neuropathology. Some studies have suggested that induced by ELF-EMF exposure at 8 mT for a short time can impair consolidation of spatial memory [6]. Furthermore, studies also found that exposure to 2 mT magnetic fields for 60 min significantly decreased cholinergic activities of hippocampus [7]. The hippocampus is functionally related to intellectual activities and vital behaviors, such as learning and memory [8,9]. Thus, ELF-EMF exposure severely injures the neuronal functions in the hippocampus.

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Procyanidins, widely distributed in fruits, vegetables, seeds and bark, represent a ubiquitous group of plant polyphenols [10]. As natural antioxidants, procyanidins are known to possess a broad spectrum of biological, pharmacological and chemoprotective properties against free radicals and oxidative stress [11]. Lotus seedpod procyanidins (LSPCs) are procyanidin compounds extracted from lotus seedpod, in which the oligomeric procyanidins are considered to be the main active constituents [12]. LSPCs have the functions of antioxidantion activities and antimyocardial ischemia. Indeed, it has reported that brain tissue is prone to oxidation and deterioration in learning and memory abilities with age, however, which can be modified by LSPCs, most likely through the ability of LSPCs to scavenge oxygen free radicals and to stimulate antioxidant enzyme activity [13]. Our previous research demonstrated that dietary LSPCs protected the hippocampus of ICR mouse against oxidative damage after ELF-EMF exposure *in vivo* experiments [14]. However, there are few articles mentioning about the effects of LSPCs on ELF-EMF induced cell damage *in vitro* experiments. Thus, in this study, we investigated the effects of LSPCs on ELF-EMF induced neurotoxicity in primary cultured rat hippocampal neurons.

2. Materials and methods

2.1. Primary culture of hippocampal neurons

Newborn Sprague-Dawley (SD) rats of either gender (less than 24 h old) were provided by the Experimental Animal Center of Jiangsu University. All experimental procedures were performed in accordance to the guidelines of the Animal Care Committee. Primary hippocampal neuronal cultures were prepared as described previously [15] with some modifications. Hippocampi were dissected from the brain on ice and minced in sterile ice-cold phosphate-buffered saline (PBS) with the blood vessels and meninges carefully removed. The tissues were digested with 0.25% trypsin for 25 min at 37 °C and then the digestion procedure was stopped by adding DMEM medium with 10% fetal bovine serum (FBS), 2% B27 supplement, 10,000 units/ml Penicillin and 10000 μg/ml Streptomycin. Cell suspension was centrifuged at 1000 rpm for 5 min and then resuspended in DMEM medium. The cells were counted using a hemacytometer and seeded into cell culture plates coated with poly-L-lysine. Then the cells were incubated at 37 °C in a humidified 5% CO_2 incubator. After 24 h seeding, the medium was full replaced by serum-free DMEM medium. On the 4th day, cytarabine (4 μg/ml) was injected to inhibit the growth of glial cells. Then the next day, the medium was full replaced by serum-free DMEM medium again. Half of the medium was changed every 2 days. Cultured neurons were used for *in vitro* studies on the 7th day.

2.2. ELF-EMF exposure system

The electromagnetic field was generated with a pair of Helmholtz coils (1.0 mm wire diameter, 9 cm internal diameter, 13 cm external diameter) in 400 turns. A pair of parallel Helmholtz coils, parallel to the ground, was connected to a 220 V AC power supply via the adjustable transformer. The AC current (50 Hz) was passed through a pair of coils, producing an electromagnetic field of 0–15 mT at the center of the coils. The intensity of the electromagnetic field was measured in 6 different points by using a digital teslameter (Lz-630H, Lianzhong Technology, China) to ensure the homogeneity of the magnetic field during the experiment. According to our previous studies, cell culture plates were exposed to ELF-EMF (50 Hz, 8 mT) for 90 min in the experiments.

2.3. Preparation of LSPCs

Lotus seedpod was collected from Honghu Lantian Lake (Hubei, China). This variety of *N. nucifera Gaertn*. was named Number 2 Wuhan plant and authenticated by the Department of Botany, Wuhan Plant Institute of the Chinese Academy of Science.

Fresh plant material was lyophilized first. In order to obtain maximum amounts of procyanidin polymers in intact plant tissues, the preparations avoided exposuring to heat, light and air. LSPCs were extracted with Me_2CO/H_2O three times and purified by Sephadex LH-20 column chromatography, with a purity of >98%. As a part of our ongoing research, we have already demonstrated the main molecular weight distribution of LSPCs (range 291.1–1155.3) by electrospray ionisation-mass (ESI–MS) analysis. LSPCs polymerization was $\leq$4 and contained monomers, dimers and tetramers in which the amounts of dimers were the greatest and catechin and epicatechin were the base components. For all experiments, final concentrations (2.5, 5, 10 μg/ml) of the LSPCs were prepared by diluting the stock with serum-free DMEM medium.

2.4. Cell viability assays

The cell viability of cultured hippocampal neurons was determined by the reduction 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan, the amount of formazan was proportional to the number of living cells. In brief, hippocampal neurons were seeded into 96-well plates at a density of 1×10^6 cells per well for 7 days. After pretreatment with LSPCs (2.5, 5, 10 μg/ml) for 4 h, each group was exposed to ELF-EMF at 8 mT for 90 min with the exception of control. After incubation for 24 h, 100 ul of MTT (1 mg/ml) was added to each well and then incubated for 4 h at 37 °C. Finally, the insoluble formazan was dissolved in DMSO. The absorbance was detected at 570 nm using a microplate reader (Multiskan MK3, USA).

2.5. Morphological observation

Hippocampal neurons were cultured in 6-well plates at a density of 6×10^5 cells per well for 7 days. After treatments as mentioned above, morphological changes were observed with inverted phase contrast fluorescence microscope (Leica DMI 4000B, Germany). Fluorescence microscopy was the most common method for morphological observation of cells. Apoptotic cells could be discriminated from normal cells by shape of the cell nucleus [16]. The cells were dyed with Hoechst 33342 before being examined.

2.6. Determination of SOD activity and MDA level

SOD and MDA levels were measured by assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). SOD is an important antioxidant enzymes, which can catalyze the dismutation of superoxide anion to generate H_2O_2 and O_2, and the vitality level of SOD indirectly reflects the body's ability to scavenging oxygen free radicals. In brief, hippocampal neurons (1×10^6 cells/well) were plated in 6-well plates for 7 days. Neuronal cells were exposed to ELF-EMF (50 Hz, 8 mT) for 90 min and then incubated for 24 h, cells were lysed by adding 150 μl of lysis buffer (10 mM Tris, 20 nm EDTA, 0.25% Triton X-100, pH 8.0) for 30 min on ice and centrifuged at 12000*g* at 4 °C for 5 min. Then the supernatants were saved.

SOD activity was determined by inhibiting the oxidation of hydroxylamine by the xanthine–xanthine oxidase system. After reaction, the absorbance at 450 nm was monitored using a microplate reader (Multiskan MK3, USA). MDA is a natural product