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5-Hydroxymethylfurfural from wine-processed *Fructus corni* inhibits hippocampal neuron apoptosis

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Research Highlights

(1) This study, for the first time, used cytobiological and molecular biology methods to investigate the molecular mechanisms underlying the neuroprotective effects of 5-hydroxymethylfurfural, a compound extracted from wine-processed *Fructus corni*, on rat hippocampal neurons injured by oxidative stress.
(2) Results from this study confirmed that 5-hydroxymethylfurfural, can protect against H_2O_2-induced rat hippocampal neuron apoptosis and that the underlying mechanism may be related to regulation of expression levels of the apoptosis-related genes p53, bcl-2, bax and caspases.
(3) This finding provides theoretical evidence for the treatment of neurodegenerative disease with 5-hydroxymethylfurfural extracted from wine-processed *Fructus corni* and lays the foundations for clinical prevention and treatment of oxidative injury-related diseases in the brain.

Abstract

Previous studies have shown that 5-hydroxymethylfurfural, a compound extracted from wine-processed *Fructus corni*, has a protective effect on hippocampal neurons. The present study was designed to explore the related mechanisms. Our study revealed that high and medium doses (10, 1 μmol/L) of 5-hydroxymethylfurfural could improve the morphology of H_2O_2-treated rat hippocampal neurons as revealed by inverted phase-contrast microscopy and transmission electron microscopy. MTT results showed that incubation with high and medium doses of 5-hydroxymethylfurfural caused a significant increase in the viability of neuronal cells injured by H_2O_2. Flow cytometry assays firmed that H_2O_2 could induce cell apoptosis, while high and medium doses of 5-hydroxymethylfurfural had a visible protective effect on apoptotic rat hippocampal neurons. Real-time PCR and western blot analysis showed that high and medium doses of 5-hydroxymethylfurfural prevented H_2O_2-induced up-regulation of p53, Bax and caspase-3 and antagonized the down-regulation of Bcl-2 induced by H_2O_2 treatment. These results suggested that 5-hydroxymethylfurfural could inhibit apoptosis of cultured rat hippocampal neurons injured by H_2O_2 *via* increase in Bcl-2 levels and decrease in p53, Bax and caspase-3 protein expression levels.

Key Words

neural regeneration; traditional Chinese medicine; neurodegenerative disease; 5-hydroxymethylfurfural; *Fructus corni*; hippocampus; neuron; oxidative stress; apoptosis; grants-supported paper; neuroregeneration

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Hai Gu and Zequn Jiang contributed equally to this work.

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Author contributions: Gu H was responsible for the majority of the study. Jiang ZQ drafted the manuscript. Wang MY revised the manuscript. Jiang HY designed PCR primers. Zhao FM coordinated data and performed statistics. Ding X identified the structure of 5-hydroxymethylfurfural. Cai BC wine-processed the *Fructus corni*. Zhan Z was in charge of funding. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: This study received full ethical approval from the Animal Ethics Committee of Nanjing University of Chinese Medicine, China.

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INTRODUCTION

Fructus corni is one of the most important traditional Chinese medicines used clinically[1-3]. In the past few decades, Fructus corni has been pharmacologically investigated. Studies have shown that Fructus corni has a protective effect on hepatocytes, and has anticancer, antioxidative, anti-inflammatory and antiseptic properties[4-6]. Recently, many biologically active components of Fructus corni have been studied. For example, gallic acid has an antitumor properties[4] and oleanolic acid has antiulcer property[6]. With high performance liquid chromatography, we found that the content of 5-hydroxymethylfurfural in wine-steamed Fructus corni increased markedly compared with that in the crude drug[7]. We separated 5-hydroxymethylfurfural from wine-streamed Fructus corni, identified its structure by infrared spectroscopy, ultraviolet-visible spectroscopy, and gas chromatography-mass spectrometry and compared it with the standard sample[8].

It has been reported that 5-hydroxymethylfurfural has antioxidant and anti-ischemic properties, and can enhance erythrocyte deformation and alter blood hemorheology in rats[9]. 5-Hydroxymethylfurfural was also found to improve acute liver injury in mice[8] and could inhibit the oxidative damage to hepatocytes caused by H_2O_2 in human LO_2 hepatocytes[10]. All these suggest the anti- apoptosis mechanism of 5-hydroxymethylfur- fural. Our previous results suggested that 5-hydroxymethylfurfural had a protective effect on neurons in rats, and the effect may be related to antioxidant properties. It is clearly important to understand the molecular basis of apoptosis in neurons and how these events can be manipulated.

Oxidative stress has long been considered as a primary cause of neuronal damage, which is related to several human neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease and acute brain disorders such as stroke[11-15]. It has been well established that balanced antioxidants help to control the cognitive and motor functions of the cerebral cortex and the hippocampus[16-17]. Oxidative stress has been considered as one of the major risk factors that exacerbate neuronal damage in neurodegenerative disorders via different molecular pathways[18-19]. H_2O_2 is a harmful factor for neurons, and can be used to establish a cell model of oxidative stress, which leads to the accumulation of reactive oxygen species and an imbalance between the cell oxidation system and the antioxidant system[20]. In fact, H_2O_2 can cause cell damage in many aspects, but the basic mechanism of injury is through the Haber-Weiss or Fentons reaction, which generate·OH. Neurons have reduced levels of glutathione compared with other kinds of cells, so neurons are more sensitive to oxidative stress[21]. Therefore, the search for neuroprotective drugs of natural origin against H_2O_2-induced neuronal death has attracted increasing interest. The experimental report showed that H_2O_2 at concentrations higher than 10 μmol/L could decrease cell survival rate and superoxide dismutase activity[22], but increase malondialdehyde production in a dose-dependent manner. In our experiments, the concentration of 300 μmol/L H_2O_2 was used in reference to the general literature commonly used.

At least, to some extent, oxidative stress injury can induce marked neuronal death, which is mainly linked to an apoptotic process. It has been accepted that apoptosis is a gene-directed process and a number of genes, including p53, bcl-2, bax and caspases, have been reported to be involved in this process[23-25].

Thus, this paper evaluated the neuroprotective effects of 5-hydroxymethylfurfural on H_2O_2-induced neurotoxicity and the roles of p53, bcl-2, bax and caspases in neuronal apoptosis induced by H_2O_2 in hippocampal neurons. This study may provide further insights on the mechanism of action of 5-hydroxymethylfurfural on neurodegenerative disorders in a comprehen-

sive way.

RESULTS

5-Hydroxymethylfurfural improved the morphology of H_2O_2-treated rat hippocampal neurons

Rat hippocampal neurons were pre-incubated with 5-hydroxymethylfurfural (0.1, 1, 10 μmol/L) for 24 hours, and then apoptosis was induced with 300 μmol/L H_2O_2 for another 24 hours. The treated cells were observed and photographed under an inverted phase-contrast microscope. In the control group, a typical pyramidal morphology of layer V neurons was observed: a single apical dendrite extending toward the pia with secondary branches, basal dendrites and a single axon running straight down (Figure 1A). However, the cells in the H_2O_2 group appeared aggregated in large clusters, which occurs possibly because of degenerating dendrites and abnormal axonal trajectories (Figure 1B). After treatment with 5-hydroxymethylfurfural, the rat hippocampal neurons exhibited considerable improvement in shape, including clear soma, dendrites and axons (Figure 1C–E).

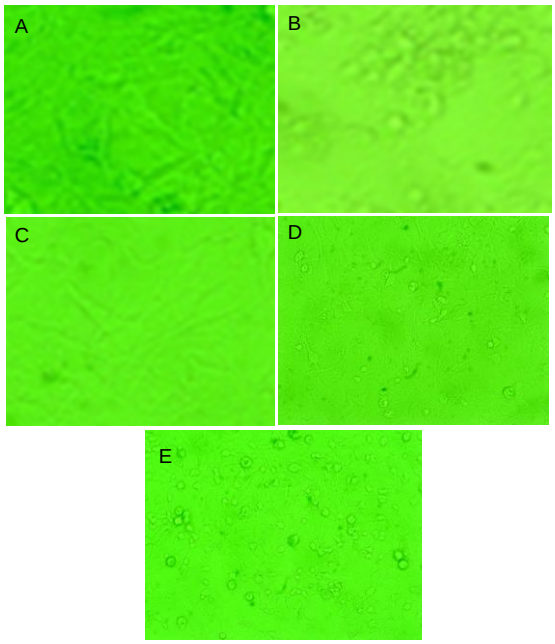

Figure 1 Effect of 5-hydroxymethylfurfural (5-HMF) on H_2O_2-injured rat hippocampal neurons (inverted phase-contrast microscope, × 400).

(A) Cells in the control group appeared flat and spindly; (B) cells in the H_2O_2-treated cultures were shrunken, separated, and irregular; (C, D) cells treated with H_2O_2 and 5-HMF (10, 1 μmol/L) exhibited obvious improvement in appearance, *i.e.*, axons and dendrites were maintained, while cells treated with H_2O_2 and 5-HMF (0.1 μmol/L) exhibited no obvious improvement.

5-Hydroxymethylfurfural reduced ultrastructural damage of rat hippocampal neurons injured by H_2O_2

To conduct further research, the ultrastructural changes of the cells were observed under the transmission electron microscope. As shown in Figure 2, cells in the control group exhibited normal morphology and regular nuclei. However, after H_2O_2 treatment, cells displayed apoptotic morphology, characterized by the condensation of chromatin and nuclear shrinkage.

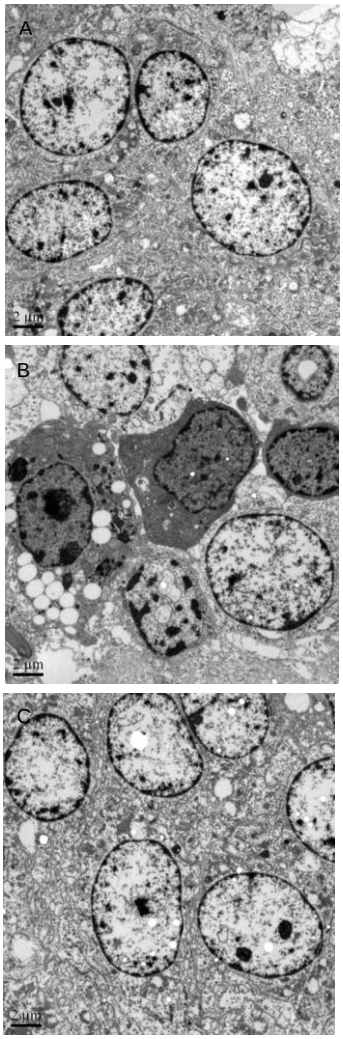

Figure 2 Effects of 5-hydroxymethylfurfural on H_2O_2-injured ultrastructure of rat hippocampal neurons (transmission electron microscope, × 4 000).

(A) Control group: cells displayed normal nuclear morphology, intact nuclear membrane and uniformly distributed chromatin. Most mitochondria were normal.

(B) H_2O_2 group: mitochondria were obviously swollen and fragmentation of mitochondrial crista was obvious.

(C) 5-Hydroxymethylfurfural high dose group: some mitochondria were swollen, but morphology was mostly normal.

After treatment with H_2O_2 and a high dose of 5-hydroxymethylfurfural, only a small number of vacuoles were observed, and the nuclear membrane remained intact. These results suggest that cells were protected by 5-hydroxymethylfurfural against H_2O_2.

5-Hydroxymethylfurfural caused significant increases in the viability of neuronal cells injured by H_2O_2

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure cell viability. Compared with the control group, the viability of rat hippocampal neurons in the H_2O_2 group was decreased ($P < 0.05$); compared with the H_2O_2 group, the cell viability in the 5-hydroxymethylfurfural high and medium dose groups significantly increased ($P < 0.05$); there was no statistical significance in cell viability between 5-hydroxymethylfurfural low dose group and the H_2O_2 group ($P > 0.05$; Figure 3).

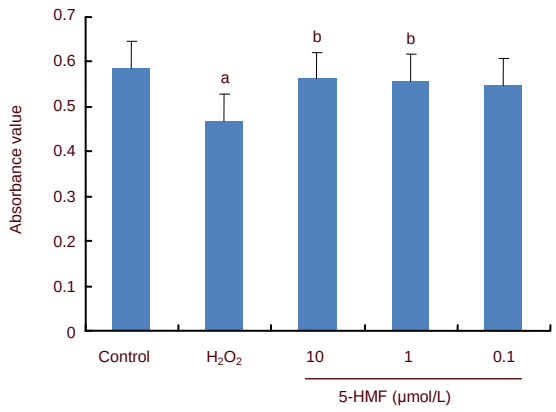

Figure 3 Protective effects of 5-hydroxymethylfurfural (5-HMF) on hippocampal neurons induced by H_2O_2.

Rat hippocampal neurons were pre-incubated with 5-HMF (0.1, 1, 10 μmol/L) for 24 hours, and then apoptosis was induced by 300 μmol/L H_2O_2 for another 24 hours. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the viability of rat hippocampal neurons.

Data are expressed as mean ± SD, $n = 8$ for triple. $^aP < 0.05$, *vs*. control group; $^bP < 0.05$, *vs*. H_2O_2 group (analysis of variance followed least significance difference test).

5-Hydroxymethylfurfural inhibited H_2O_2-induced cell apoptosis in hippocampal neurons

Based on the results of the MTT assay, we further used flow cytometry to detect the rate of apoptosis. As shown in Figure 4, approximately 19.80% apoptosis was detected in primary cultured rat hippocampal neurons induced by H_2O_2 ($P < 0.05$, *vs*. control group). Pre-treatment with 1 and 10 μM 5-hydroxymethylfurfural, however, reduced the percent of apoptosis in hippocampal neurons to approximately 14.66% and 10.09%, respectively. This observation was consistent with MTT results and morphological tests, suggesting that the high/medium dose group of 5-hydroxymethylfurfural attenuated H_2O_2-induced cell apoptosis in rat hippocampal neurons.

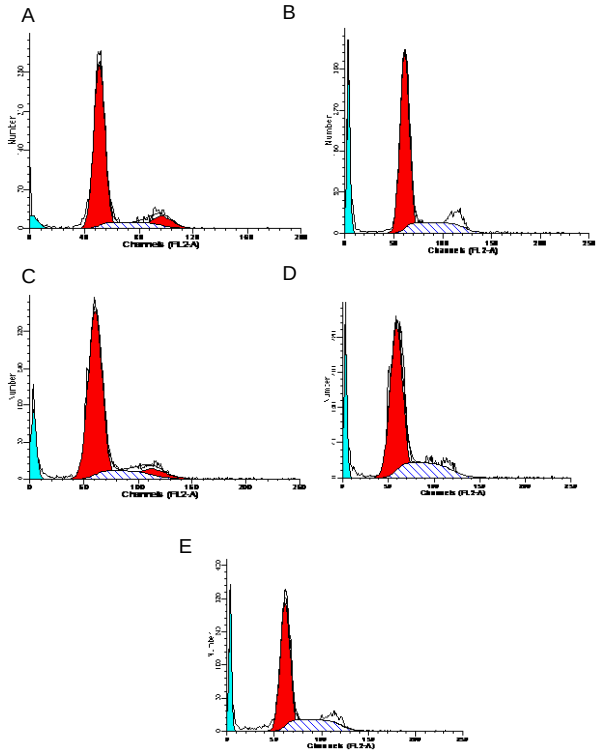

Figure 4 Effects of 5-hydroxymethylfurfural (5-HMF) on apoptosis of hippocampal neurons induced by H_2O_2.

Rat hippocampal neurons were pre-incubated with 5-HMF (0.1, 1, 10 μmol/L) for 24 hours, and then apoptosis was induced by 300 μmol/L H_2O_2 for another 24 hours. Flow cytometry results showed that the rate of apoptosis in the control group was 4.08 % (A), in the H_2O_2 group was 19.80 % (B), in the 5-HMF high, medium, low dose groups was 10.09%, 14.66%, 17.88%, respectively (C–E).

Effect of 5-hydroxymethylfurfural on bax, p53, caspase-3 and bcl-2 mRNA expression in H_2O_2-treated rat hippocampal neurons

The effects of 5-hydroxymethylfurfural on p53, caspase-3, bax and bcl-2 mRNA expression were determined by RT-PCR (Figure 5). Bax and p53 mRNA levels were significantly increased in the H_2O_2 group compared with the control group ($P < 0.01$). However, high and medium doses of 5-hydroxymethylfurfural completely prevented the increase of bax and p53 mRNA levels in a dose-dependent manner in rat hippocampal neurons. Furthermore, a significant decrease in bcl-2 mRNA levels

was observed after 24 hour exposure to H_2O_2 ($P < 0.01$) and this occurrence was significantly reversed by 5-hydroxymethylfurfural at 1 and 10 μmol/L ($P < 0.01$) in rat hippocampal neurons. However, the caspase-3 results were not significant ($P > 0.05$).

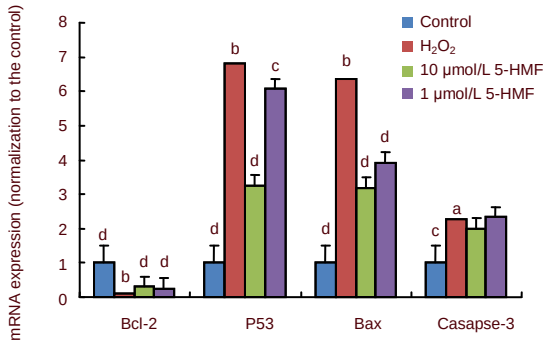

Figure 5 Effects of 5-hydroxymethylfurfural (5-HMF) on bax, p53, caspase-3 and bcl-2 mRNA expression in H_2O_2-treated rat hippocampal neurons by reverse transcription-PCR.

The rat hippocampal neurons were pre-incubated with 5-HMF (1, 10 μmol/L) for 24 hours, and then apoptosis was induced by 300 μmol/L H_2O_2 for another 24 hours. Data are expressed as mean ± SD, $n = 8$ for triple. $^aP < 0.05$, $^bP < 0.01$, *vs*. control group; $^cP < 0.05$, $^dP < 0.01$, *vs*. H_2O_2 group (one-way analysis of variance followed by least significance difference test).

Effect of 5-hydroxymethylfurfural on bax, p53, caspase-3 and bcl-2 protein expression in H_2O_2-treated rat hippocampal neurons

The effects of 5-hydroxymethylfurfural on the expression levels of some proteins in rat hippocampal neurons are shown in Figure 6. Western blot assay showed that compared with normal control cells, H_2O_2-treated cells showed an obvious increase in p53, Bax and caspase-3 expression, and a sharp reduction in Bcl-2 expression, while 1, 10 μmol/L 5-hydroxymethylfurfural pretreatment had the opposite effect. These results indicated that p53, caspase-3, Bax and Bcl-2 may all be involved in the protective action of 5-hydroxymethylfurfural on rat hippocampal neurons.

DISCUSSION

The hippocampus, which plays a significant role in important functions of the central nervous system, such as cognition, learning, and memory, is very sensitive to various neurological insults including those induced by oxidative stress[26].

In comparison with more mature neurons in culture, immature neurons are particularly susceptible to cell damage induced by oxidative stress, for example, by H_2O_2[27]. Thus, primary cultured hippocampal neurons are commonly used as a cell system for the *in vitro* study of protection against H_2O_2-induced neurotoxicity.

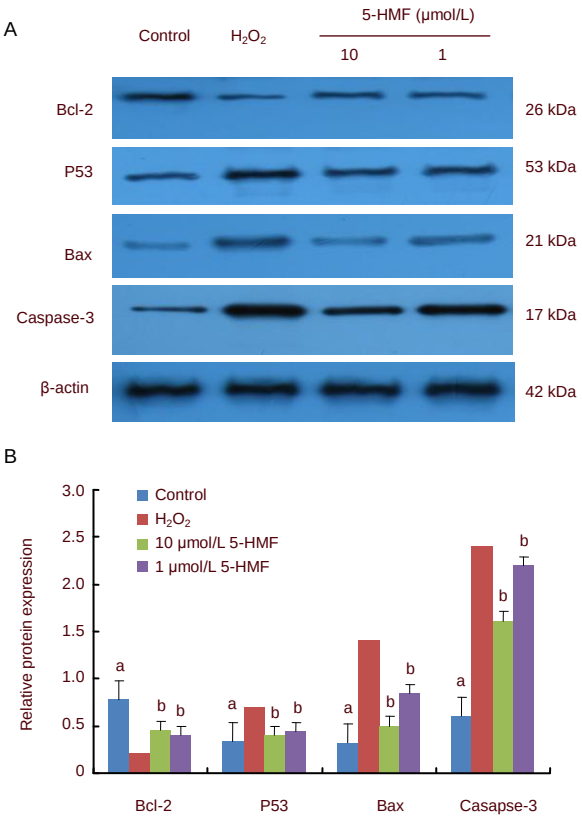

Figure 6 Effect of 5-hydroxymethylfurfural (5-HMF) on Bax, P53, caspase-3 and Bcl-2 protein expression in H_2O_2-treated rat hippocampal neurons.

Rat hippocampal neurons were pre-incubated with 5-HMF (1, 10 μmol/L) for 24 hours, and then apoptosis was induced by 300 μmol/L H_2O_2 for another 24 hours.

(A) Gel electrophoresis results of target protein expression (western bolt).

(B) Quantitative analysis of western blots. Data (absorbance value of target protein to β-actin) are expressed as mean ± SD, $n = 8$ for triple. $^aP < 0.01$, $^bP < 0.05$, *vs*. H_2O_2 group (one-way analysis of variance followed by least significance difference test).

Fructus corni contains four components including *volatiles*, *volatile components*, *tannins* and *flavones* and it also contains a large amount of small molecular compounds and organic acids; 5-hydroxymethylfurfural is a component of Fructus Corni[1]. Being once thought to have certain side effects[28], it has been found to have positive pharmacological activity[29-30]. There is evidence that wine-steamed Fructus corni exhibits stronger effects, and that the levels of 5-hydroxymethylfurfural increase

greatly after wine processing[8]. In this study, the protective effect of 5-hydroxymethylfurfural on rat hippocampal neurons was investigated.

At first, 5-hydroxymethylfurfural had a morphological protective effect on rat hippocampal neurons exposed to H_2O_2. MTT results showed that an appropriate dose of 5-hydroxymethylfurfural protected rat hippocampal neurons and reduced inhibition of neuronal cell proliferation caused by H_2O_2. In the 5-hydroxymethylfurfural high dose (10 μmol/L) and medium dose groups (1 μmol/L), cell apoptosis was obviously decreased. There was no statistical significance in neuronal apoptosis in the 5-hydroxymethylfurfural low dose group (0.1 μmol/L) before and after low dose 5-hydroxymethylfurfural treatment, so we chose 5-hydroxymethylfurfural high and medium doses in all subsequent experiments.

Second, treatment with H_2O_2 resulted in apoptosis and necrosis of rat hippocampal neurons, while 5-hydroxymethylfurfural decreased the apoptosis rate. There was a lower apoptotic peak in the control group, a robust apoptotic peak in the H_2O_2 group by flow cytometry, and the 5-hydroxymethylfurfural group had a decreased apoptotic peak. The results showed that H_2O_2 could induce cell apoptosis in primary cultured rat hippocampal neurons, while 5-hydroxymethylfurfural had visible protection on apoptotic rat hippocampal neurons. Until now, the role and precise interaction of apoptosis-related factors remained unclear in neurons. In the present study, the temporal changes of the levels of four important apoptosis-related genes were examined in primary cultures of rat hippocampal neurons. Our results show that p53, Bax, Bcl-2 and caspase-3 play important roles in H_2O_2-induced apoptosis in primary cultures of rat hippocampal neurons.

The gene p53 has recently been implicated as a mediator of the apoptotic death of neurons. The levels of p53 are known to increase in some neurons, in response to a number of stimuli and neurological disorders that can lead to neuronal cell death[31]. P53 expression is elevated in neuron models of ischemia and epilepsy, and in human brain tissue samples with chronic neurodegenerative diseases[31-32]. Signal transduction pathways associated with p53-induced neuronal cell death are being characterized, suggesting that intervention may prove effective in maintaining neuronal viability and restoring function following neural injury and disease[31]. Administration of 5-hydroxymethylfurfural was shown to suppress the H_2O_2-induced increase in p53 mRNA and protein in primary cultured rat hippocampal neurons, which suggests that the neuroprotective effects of 5-hydroxymethylfurfural treatment may be related, in part, to down-regulate p53 gene expression. The mechanism by which p53 influences neuronal cell death is poorly understood. However, currently available data suggest that the Bcl-2 family member and the caspase family are involved in p53-mediated neuronal death[33].

The Bcl-2 family of proteins functions as key regulators of apoptosis, which include pro- and anti-apoptotic factors acting at mitochondrial and microsomal membranes[34]. It was reported that the anti-apoptotic factor Bcl-2 and pro-apoptotic factor Bax play important roles in the initiation of neuronal apoptosis induced by serum deprivation[35]. Indeed, dimerization of Bcl-2 with Bax seems to be a critical interaction in the apoptotic process. When Bcl-2 is present in excess, apoptosis is inhibited, and when Bax is over-expressed, apoptosis is induced[36-37]. Thus, the Bcl-2/Bax ratio is the critical determinant for the induction or inhibition of apoptosis. In this study, a significant decrease in Bcl-2 levels was found 24 hours after H_2O_2 treatment in rat hippocampal neurons. However, 5-hydroxymethylfurfural inhibited the decrease of Bcl-2 in a dose-dependent manner. Meanwhile, the expression levels of Bax showed a significant increase 24 hours after H_2O_2 exposure, but returned to nearly normal levels following 24-hour exposure to 5-hydroxymethylfurfural at 1 and 10 μmol/L. Consequently, 5-hydroxymethylfurfural led to an increase in the Bcl-2/Bax ratio, a response that would be protective against apoptosis.

Furthermore, caspase-3 has been implicated as a key player in certain models of neuronal apoptosis[38] and in pathological conditions such as stroke[39-40]. It has been shown that caspase-3 can be activated in glutamate-induced apoptosis in cerebella granule neurons that serve as a final effector in apoptotic cell death[41-42]. Western blot data showed that H_2O_2-induced caspase-3 activation was attenuated by 5-hydroxymethylfurfural in rat hippocampal neurons, which suggests that 5-hydroxymethylfurfural inhibits apoptotic cell death through reducing caspase-3 expression. However, there was no obvious difference in mRNA expression of caspase-3 by RT-PCR detection, which may be due to delayed post- processing of caspase-3 mRNA.

In summary, our data suggest that 5-hydroxymethylfurfural may be a useful compound to prevent neuron damage during oxidative stress. The neuroprotective effects of 5-hydroxymethylfurfural might be related to resisting apoptosis. The anti-apoptotic po-

tential of 5-hydroxymethylfurfural may result from its inhibition of the down-regulation of Bcl-2 and blockade of p53, Bax and caspase-3 up-regulation induced by H_2O_2. This study reinforces the neuroprotective role of 5-hydroxymethylfurfural and indicates some possible molecular sites of activity of this compound on hippocampal neurons during oxidative stress. In a sense, this study may contribute to the development of 5-hydroxymethylfurfural as a useful agent for preventing and/or treating neuronal damage in neurodegenerative disorders.

MATERIALS AND METHODS

Design

A cytological *in vitro* parallel controlled experiment.

Time and setting

Experimental Center, College of Basic Medical Sciences, Nanjing University of Chinese Medicine, China from 2010 to 2012.

Materials

Animals

Sprague-Dawley (SD) rat embryos aged 18–19 days were obtained from the Experimental Animal Center of Nanjing University of Chinese Medicine, China (SCXK (Su) 2008-0033). All procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[43].

Drug

Fructus corni was purchased from Dogwood Pharmaceutical Company, Xixia County, Henan Province, China, identified by Professor Jianwei Chen (Laboratory for Identification and Assessment of Traditional Chinese Medicine, College of Pharmacy, Nanjing University of Chinese Medicine) and wine-pressed by Nanjing Traditional Chinese Medicine Factory, China[44]. Briefly, Fructus corni was steeped in wine, evaporated, and the sample (500 g) was extracted by methanol twice, for 45 minutes each. The extracts were mixed together, concentrated, and then dissolved in water and filtered. The dichloromethane-soluble fraction was concentrated at room temperature to provide a light yellow oil (about 3.0 g). The structure of the compound was identified by infrared spectroscopy, ultraviolet-visible spectroscopy, and gas chromatography-mass spectrometry and compared with standard samples. It was then purified to more than 98.5%[8].

Methods

Cell culture

Rat hippocampal neurons were grown in primary culture as described previously[45] with minor modifications. Primary cultures of hippocampal neurons were prepared from the hippocampi of SD rat embryos aged 18–19 days. After treatment with 0.25% (w/v) trypsin for 15 minutes in PBS without calcium or magnesium ions, the hippocampi were washed in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) with 10% (v/v) fetal bovine serum (FBS; Si-Ji-Qing Biotechnology Co., Ltd., Hangzhou, China) to stop trypsin activity. After washing with papain-free solution, the hippocampal cells were carefully dissociated by trituration through a flame-narrowed Pasteur pipette. Cells were then collected by centrifugation at low speed (1 000 × *g*, 10 minutes) and re-suspended in DMEM without glutamine, supplemented with 10% (v/v) FBS, 2% (v/v) B27 (Gibco) and 10 μmol/L cytosine β-D-arabinofuranoside (Sigma, St. Louis, MO, USA) to kill proliferating cells, including astrocytes and neuroblasts. After 24 hours, the medium was changed to serum-free neurobasal medium (Gibco) supplemented with 2% (v/v) B27, 0.5 mmol/L glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin, but without β-D-arabinofuranoside. The cultures were maintained at 37°C in 95% air/5% CO_2. Neurons remained viable up to 3 weeks in culture and were usually used 7 days after plating. At the end of incubation, the morphology of cells was monitored under an inverted light microscope (Olympus, Tokyo, Japan). Hippocampal neurons were identified on the basis of their distinct morphology (large triangular or bipolar soma, long neurotic process).

Drug incorporation

In all experiments, the cells were randomly divided into five groups: control group, H_2O_2 group, and 5-hydroxymethylfurfural high, medium, and low dose groups. The cells were seeded at a density of 1×10^6 cells/well into a 96-well plate. After 80% confluency, the cells were pretreated with 5-hydroxymethylfurfural (0.1, 1, 10 μmol/L) for 24 hours and then exposed to H_2O_2 (300 μmol/L) for another 24 hours. The hippocampal neurons that underwent neither 5-hydroxymethylfurfural pretreatment nor H_2O_2 stimulation served as a control group. The 5-hydroxymethylfurfural group was pre-incubated with 5-hydroxymethylfurfural (0.1, 1, 10 μmol/L) for 24 hours, and then apoptosis was induced by H_2O_2 (300 μmol/L; Guanghua Co., Ltd., Guangzhou, Guangdong Province, China) for another 24 hours. The H_2O_2 group was pre-incubated with normal saline for 24 hours, fol-

lowed by 24 hours of H_2O_2 (300 μmol/L) of stimulation.

Morphological examination

Cells of different groups were treated with various designated treatments. Before terminating the cell culture, rat hippocampal neurons (treated) were observed under an inverted phase-contrast microscope (Olympus) and photographed. For transmission electron microscopy, different groups of cells were collected and fixed with 2% (v/v) glutaraldehyde in PBS for 2 hours and then with 2% (v/v) OsO_4 for 2 hours. Thin sections were prepared and stained as described in a previous study[46]. The sections were observed on a JEM1230 TEM (JEOL, Tokyo, Japan).

MTT assay

Cell activity was measured by the MTT assay[47]. The cells of each group in the 96-well plate were used. Before terminating the cell culture, 20 μL of MTT solution (5 mg/mL in PBS; Sigma) was added to each well. After 4 hours of incubation at 37°C, the supernatant was discarded, 100 μL dimethyl sulfoxide (DMSO; Guanghua Co., Ltd) was added, the 96-well plate was vibrated on a micro-vibrator for an additional 10 minutes, and the absorbance value of each well was measured at 490 nm using an automated enzyme immunoassay instrument (Bio-Rad, Hercules, CA, USA).

Flow cytometry

Cell apoptosis was measured by flow cytometry. Before terminating the cell culture, cells were collected, washed twice with ice-cold PBS (pH 7.4), fixed with 75% (v/v) alcohol and stained with propidium iodide (PI; 1 mg/mL; Sigma) in the presence of 1% (v/v) RNAase A for at least 30 minutes before flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

Reverse transcription-PCR

We applied reverse transcription-PCR to quantify the expression of bcl-2, bax, p53 and caspase-3. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) from neural cells of each group. The homogenate was mixed with 200 μL chloroform and centrifuged at 12 000 × *g* for 15 minutes, 4°C. The aqueous phase (about 0.5 mL upper layer) was precipitated with an equal volume of isopropanol and then centrifuged at 12 000 × *g* for 10 minutes, 4°C. After washing with 1 mL 75% (v/v) ethanol, the final RNA total pellet was resuspended in 20 μL diethyl pyrocarbonate water. Equal quantities (2.0 μg) of total RNA were converted to first-strand cDNA using Revert Aid First Stand cDNA Synthesis kit (Fermentas Life Sciences, Vilnius, Lithuania) for RT-PCR. PCR amplification was carried out using gene-specific PCR primers synthesized by GenScript Co., Ltd (Nanjing, Jiangsu Province, China). Primer sequences are as follows:

Primer	Sequence	Product size (bp)
β-actin	Upstream: 5′-GCA GAA GGA GAT TAC TGC CCT-3′ Downstream: 5′-GCT GAT CCA CAT CTG CTG GAA-3′	136
p53	Upstream: 5′-AGA GAG CAC TGC CCA CCA-3′ Downstream: 5′-AAC ATC TCG AAG CGC TCA C-3′	109
bcl-2	Upstream: 5′-TAC CTG AAC CGG CAT CTG-3′ Downstream: 5′-GGG GCC ATA TAG TTC CAC AA-3′	75
bax	Upstream: 5′-GTG AGC GGC TGC TTG TCT-3′ Downstream: 5′-GTG GGG GTC CCG AAG TAG-3′	73
caspase-3	Upstream: 5′-CCG ACT TCC TGT ATG CTT ACT CTA-3′ Downstream: 5′-CAT GAC CCG TCC CTT GAA-3′	70

PCR was performed by denaturation at 95°C for 5 minutes, followed by 40 cycles of amplification (denaturation at 95°C for 15 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 40 seconds). The PCR products were subjected to electrophoresis on 1.5% (w/v) agarose gel and quantified by the Bio-Rad ChemiDoc XRS Gel Documentation system and Bio-Rad Quantity One 1-D analysis software. Relative quantities of expression of the genes of interest in different samples were calculated after normalization to the amount of β-actin mRNA levels.

Western blot assay

For western blot analysis, cells from each group were lysed with PBS containing 0.1% (v/v) Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mg/mL aprotinin, and 10 mg/mL leupeptin and then centrifuged at 16 000 × *g* for 10 minutes. The total protein content of each supernatant was determined with a Bio-Rad protein assay kit. Equal amounts of protein (20 μg) were separated by sodium dodecyl sulfate-polyacryla-mide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked with 2% (w/v) bovine serum albumin and then incubated with appropriate primary antibodies [rabbit anti-P53 (1: 500), Bax (1:400), Bcl-2 (1:1 000) and caspase-3 (1:500) polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA)] overnight at 4°C. After washing in PBS, the membranes were incubated with the secondary antibody, horseradish peroxidase conjugated goat an-

ti-rabbit IgG (1:5 000; Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) at room temperature for 1 hour. Protein bands on the membrane were visualized by enhanced chemifluorescence (ECF; Amersham Biosciences, Inc., Piscataway, NJ, USA) and quantified using a Storm 860 Phosphorimager (Amersham Biosciences, Inc.). All blots were reprobed taking β-actin as a control.

Statistical analysis

Each experiment was performed in triplicate. Intergroup difference were compared by one-way analysis of variance followed by least significance difference test. Data were expressed as mean ± SD and analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). A level of $P < 0.05$ was considered statistically significant.

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