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Original article

Anti-inflammatory activity and chemical composition of dichloromethane extract from *Piper nigrum* and *P. longum* on permanent focal cerebral ischemia injury in rats

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ABSTRACT

White pepper (*Piper nigrum* L.) and long pepper (*Piper longum* L.) belong to family Piperaceae and are commonly used as household spices and traditional medicine worldwide, specifically in China and Southeast Asia. In Traditional Chinese Hui Medicine, these herbs are widely used for treatment of stroke. Our present study investigated effects of these herbs on inflammation in rat model with cerebral ischemia. After subjecting the rats to permanent middle cerebral artery occlusion (pMCAO) for 6 h, at doses of 100 and 200 mg/kg, dichloromethane fraction from white pepper and long pepper, respectively, was intragastrically administered once a day for seven consecutive days. Cerebral cortical and hippocampal tissues were collected after seven days. Superoxide dismutase, malonaldehyde, tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and IL-6 were measured by spectrophotometer. Phytochemical profile of dichloromethane fraction was determined through HPLC. Dichloromethane fraction exhibited anti-inflammatory activity by suppressing expression or production of IL-1β, IL-6, and TNF-α. By contrast, dichloromethane fraction showed activity against pMCAO injury by reducing oxygen-free radicals through increased superoxide dismutase activity and decreased malonaldehyde level. HPLC analysis revealed piperine as major component of dichloromethane fraction. These results show that dichloromethane fraction provides protection against cerebral ischemia. The possible mechanism is related to anti-inflammatory activity and reduction in oxygen-free radicals.

Introduction

Traditional Chinese Hui Medicine is an integration of traditional Chinese medicine and Arab medicine and is focused on stroke treatment. Excellent achievements in stroke treatment were attained by the classics, including Hui Hui Yao Fang, Hui Yao Herbal, and Rui Zhu Tang Empirical Formula (Liu et al., 2011; Li et al., 2013a,b, 2014; Zhang et al., 2014). Our previous studies showed that in traditional Chinese Hui medicine, white pepper (*Piper nigrum* L.) and long pepper (*P. longum* L.) account for highest frequencies of 56.4% (white pepper) and 42.6% (long pepper), respectively, compared with other herbs prescribed for stroke treatment. Furthermore, these herbs are often used in similar formula (Li et al., 2013b). Therefore, we speculated that these two herbs are the main components of stroke prescriptions.

White pepper and long pepper belong to family Piperaceae and are widely cultivated in China and Southeast Asia. These plants are commonly used as household spices (such as food additives and condiments) and traditional medicine by many people worldwide, specifically in China and Southeast Asia (Kim et al., 2012). These medicines present well-documented properties, such as anti-platelet aggregation, anti-atherogenic, antioxidant, and anti-inflammatory activities (Kim et al., 2012; Son et al., 2012). A few studies were conducted on use of white pepper and long pepper for stroke treatment. However, their pharmacological effects and underlying mechanisms are poorly understood. Thus, studies should center on mechanism on how white pepper and long pepper protect the brain against cerebral ischemia. Residues (extracted by the supercritical fluid CO_2) contain ethanol extracts, including

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supercritical fluid CO_2 extract of white pepper and long pepper, dichloromethane fraction (DF), ethyl acetate fraction, butyl alcohol fraction, and water, which were used in permanent middle cerebral artery occlusion (pMCAO) rat models during our preliminary experiment. We observed that DF caused less irritation and significantly improved neurological status of the rats. Thus, DF is safe and effective for stroke prevention. However, studies should identify supporting role of this compound.

This experimental study investigated effects of DF on oxidative stress and inflammation in endovascular pMCAO rat models of embolic stroke. Chemical composition of DF was simultaneously determined with high performance liquid chromatography (HPLC). This study aids in understanding intricate material basis and mechanisms of DF in treatment of stroke and provides potential strategies for treating ischemic stroke using new drug from Traditional Chinese Hui Medicine.

Materials and methods

Drugs and chemicals

HPLC grade methanol was purchased from Fischer Scientific (Thermo Electron LLS India Pvt. Ltd., Mumbai, India). Ultrapure water was obtained from MilliQ system (Millipore Corp., Bedford, MA, USA). The following drugs and chemicals were used: ethanol, methanol, petroleum ether, dichloromethane, ethyl acetate, butyl alcohol, and chloral hydrate; the compounds were obtained from Damao Chemical Company (Tianjin, China). DF was suspended in 0.5% (w/v) dimethyl sulfoxide (DMSO)/0.5% (w/v) sodium carboxyl methyl cellulose (Na-CMC).

Herbal preparation and extraction

Fruits of white pepper (*Piper nigrum* L.) and long pepper (*P. longum* L.) were purchased form Anhui Taiyuan Chinese Herbal Pharmacology Co., Ltd. (Voucher No., SCHM 121215). Plant samples were authenticated by Lin Dong of Pharmacognosy Department, College of Pharmacy, Ningxia Medical University. A voucher specimen was deposited in the same unit (Herbarium number, 20141220).

Ethanol (70%) (solid to liquid mass ratio of 1:10) was used to extract residues of supercritical fluid CO_2 extract of white pepper and long pepper (20 kg) thrice. Extracting solution was mixed for rotatory evaporation until alcoholic taste was no longer detected. The yield was crude extract, which was then successively partitioned with petroleum ether and dichloromethane, ethyl acetate, butyl alcohol, and aqueous residue. These extractive fractions were then combined, evaporated to dryness, and stored at 4 °C until further analyses.

Experimental animals

Specific pathogen-free male Sprague–Dawley rats (260–320 g; license no., SCXK (NING) 2012-0001) were purchased from the Experimental Animal Center of Ningxia Medical University (Ningxia, China). Rats were individually housed in standard laboratory cages in barrier environment with moderate humidity (55% ± 5%) and constant temperature (22 ± 1 °C) were provided with food and water *ad libitum* under 12 h light–dark cycle prior the experiments. Experiment protocol was approved by the Ethics Committee of Ningxia Medical University, Ningxia (Ethics approval, 2015-156).

Animal pMCAO model

pMCAO was performed using a previously published method (Koizumi et al., 1986; Kogure, 1989; Luo et al., 2014) with minor modification. After food deprivation for 12 h and water *ad libitum*, animals were anesthetized with chloral hydrate (7%, 0.5 ml/100 g; intraperitoneal injection) and were placed in supine position (body temperature was maintained at 37 °C using feedback-controlled heating pad; the rats were positioned in stereotaxic frame). After performing median incision on neck skin, the right primary carotid artery (common carotid artery (CCA), external artery (ECA), and internal carotid artery (ICA)) were careful isolated. In sham-operated control rats, right ECA was ligated; a 4-0 monofilament nylon thread (A4-2026; Sunbio Biotech, China) with rounded tip coated with poly-L-lysine was inserted from ECA into ICA up to 5–10 mm. In the remaining rats, right MCA was occluded with rounded tip coated with poly-L-lysine of monofilament nylon thread (A4-2026), which was inserted from ECA into ICA up to a distance of 18–20 mm. Nylon thread was cut off from and outside the blood vessels. Before the rats wake up, body temperature was maintained at normal limits (37 ± 1 °C) using a heating pad.

Experimental groups

Piper nigrum and *P. longum* sample weighs 24 g, and DF yield is 3.95%. Normal human daily dose of DF is (24 × 3.95%) g/60 kg body weight. According to the formula $d_{rat} = d_{human} \times (6–7)$, normal dose of DF for mice should be 94.80–101.06 mg/kg/day. In the present study, we selected 200 and 100 mg/kg/day as high and low dosages for the rats, respectively. To investigate neuroprotective effects of DF, we used rat pMCAO model. Fifty-one rats were randomly divided into five groups, namely, the sham group, model group, and DF treatment groups. For DF_{LD} and DF_{HD} group, doses of 100 and 200 mg/kg body weight, respectively, were dissolved in 0.5% DMSO/0.5% Na-CMC (1 ml/100 g). All rats were intragastrically administered with the medicine every 6 h one day after inducing ischemia. Intragastrical administration continued for seven days. Seven days after reperfusion, all rats were euthanized; cerebral cortical and hippocampal tissues were quickly isolated, immediately frozen, and stored at −80 °C until further use.

Biochemical analysis

Malondialdehyde (MDA) level and total superoxide dismutase (SOD) activity of ischemic cortices and hippocampus were measured using a commercially available kit (Nanjing Jiancheng Bioengineering Institute, China) according to method of Luo et al. (2014). Assays were conducted per manufacturer's instructions. Absorbance of test solution was measured at 532 nm for MDA and 550 nm for SOD.

Enzyme-linked immunosorbent assay (ELISA)

The proteins were isolated from ischemic cortices and hippocampus. Hippocampal tissues were homogenized by ultrasonication, and homogenate was centrifuged for 30 min at 1000 × *g* at 4 °C. In culture supernatants, cytokine interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) levels were protein-quantified by ELISA kits (BIO SWAMP) per manufacturer's protocol.

Chemical compositions of DF by HPLC

China Pharmacopeia (2015) frequently uses HPLC equipped with diode array detector (DAD) detection for separation and

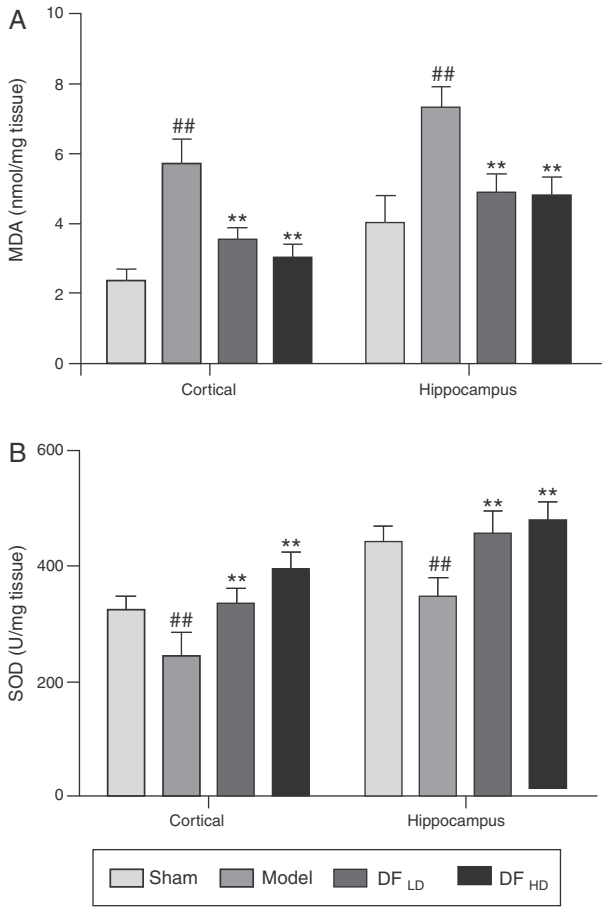

Fig. 1. DF treatment attenuated oxidative stress after cerebral ischemic injury. (A) Malondialdehyde (MDA) level. (B) Superoxide dismutase (SOD) activity. The values are expressed as the mean ± SD (n = 9–13). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with model group; $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$ compared with sham group.

detection of main compounds in DF (Commission, 2015). Analysis was performed on an Agilent Technologies 1260 HPLC system fitted with Agilent SB-18 column (length = 250 mm, diameter = 4.6 mm, and packaging size = 5 mm). Column temperature was set at 25 °C. Chromatogram was monitored at 343 nm.

Statistical analysis

Data are presented as mean values ± standard deviation (SD). Statistical analysis was conducted using one-way ANOVA, followed by Dunnett's test using SPSS Version 19.0. Differences with $p < 0.05$ were considered statistically significant.

Table 1
After 7 days of absolute numbers and percentages (%) of animals operated, animal survival, and mortality defined into sham, model and DF groups.

Groups	Animals operated	After 7 days in animal survival (%)	Animal mortality (%)
Total	51	42 (82.35)	17.76
Sham	13	13 (100.00)	0.00
Model	15	11 (73.33)	26.67
DF_{LD}	12	9 (75.00)	25.00
DF_{HD}	11	9 (81.82)	18.18

Results

Mortality of pMCAO animals

Among 51 animals with MCAO, an 82.35% survival rate was observed on seven-day period (Table 1). This finding includes 100.00% survival rate of sham-operated animals. Results also showed that mortality of model animals (26.67%) was higher than that of treatment groups; lowest death rate was observed for the DF_{HD} group (18.18%).

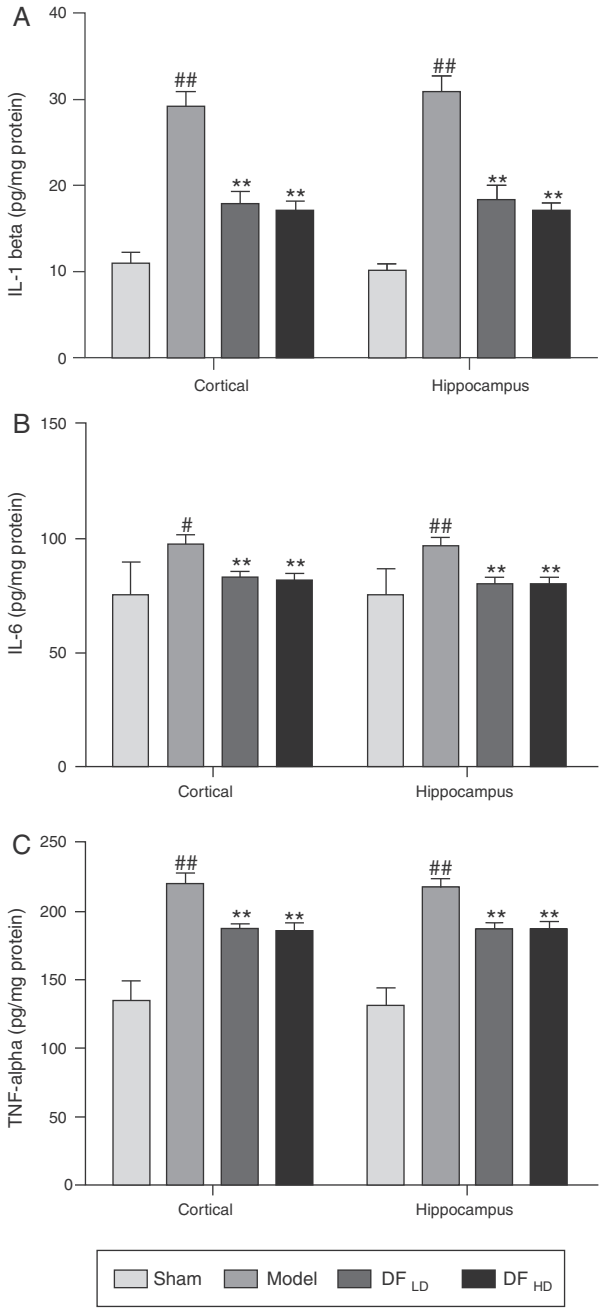

Fig. 2. DF treatment attenuated inflammation induced by cerebral ischemic injury. (A) Interleukins-1β (IL-1β) level, (B) interleukins-6 (IL-6) level, and (C) tumor necrosis factor-α (TNF-α) level. The values are expressed as the mean ± SD (n = 9–13). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with model group; $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$ compared with sham group.

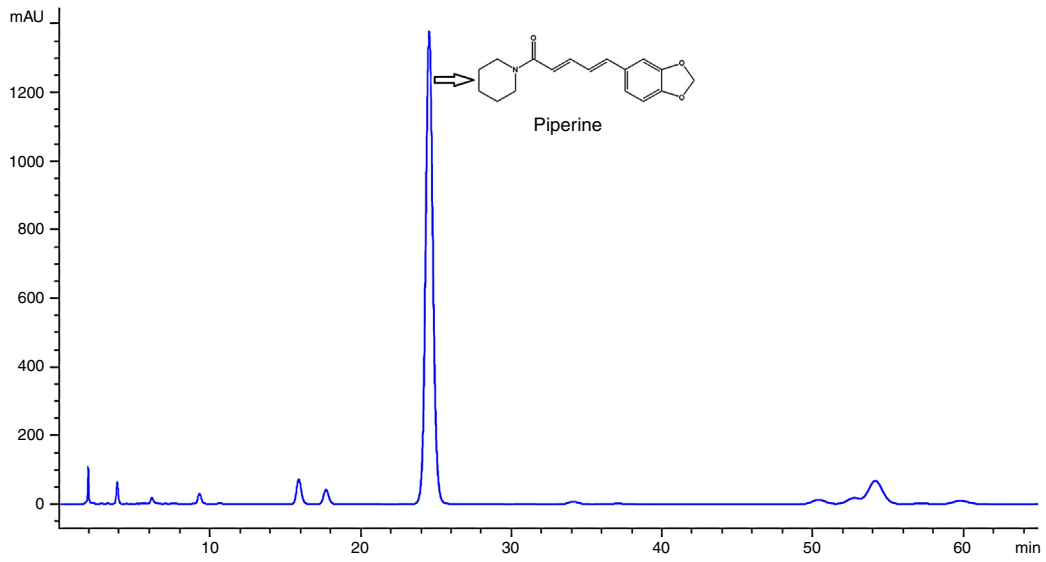

Fig. 3. HPLC chromatograms of DF from *Piper nigrum* and *P. longum* recorded at 343 nm.

Biochemical analysis

MDA content of (Fig. 1A) and SOD activity (Fig. 1B) were measured to investigate potential effects of DF on endogenous antioxidant system in cortical and hippocampal tissues. Results showed significantly increased MDA levels in the model group compared with sham-operated controls ($p < 0.01$). DF-treated rats showed significantly reduced MDA content compared with model group in a dose-dependent manner. A total of 200 mg/kg DF reduced MDA content by 46.57% at cortical tissues and 34.29% at the hippocampus (both $p < 0.01$). Conversely, the model group exhibited significantly reduced SOD activity, which was significantly attenuated by DF. Compared with the model group, DF (doses of 200 mg/kg) increased SOD levels by 61.11% at cortices and 34.22% at the hippocampus (both $p < 0.01$). SOD activity in DF-treated groups increased in a dose-dependent manner.

ELISA assay

Relative protein levels of IL-1β, IL-6, and TNF-α were significantly elevated in the animal model group (Fig. 2A–C). All protein levels increased in the model group compared with those in sham group ($p < 0.05$). Dose-dependent treatment with DF (100 and 200 mg/kg) reduced levels of IL-1β, IL-6, and TNF-α in serum with respect to the model group. DF $_{HD}$ (dose of 200 mg/kg) significantly reduced IL-1β, IL-6, and TNF-α levels in rat prefrontal cortex and hippocampus. IL-1β decreased by 41.21% at cortices ($p < 0.01$) and by 44.69% at the hippocampus ($p < 0.01$); IL-6 decreased by 16.22% at cortical area ($p < 0.01$) and by 16.67% at the hippocampus ($p < 0.01$); TNF-α decreased by 15.73% at cortices ($p < 0.01$) and by 14.22% at the hippocampus ($p < 0.01$), respectively.

Analysis of chemical constituent of DF by HPLC

In this study, DF from *P. nigrum* and *P. longum* was determined using HPLC chromatograms, which are presented in Fig. 3. Comparing retention time (RT) in Fig. 3, RT of standard compound piperine was similar to that of the main compound. Thus, piperine is the major constituent of studied herbs. As determined by HPLC, DF contained 30.18% piperine.

Discussion and conclusions

Ischemic stroke is one of the significant causes of morbidity and mortality worldwide. pMCAO is a well-characterized model and is extensively used to study mechanisms of cerebral ischemic injury (Ishrat et al., 2010; Chang et al., 2013; Tao et al., 2015; Zou et al., 2016). Research shows that changes in oxidative properties and inflammation to brain injury play vital roles in pathogenesis and exert deleterious effects on progression of tissue damage (Ye et al., 2016; Zhang et al., 2016). Therefore, reducing oxidative capacity and inhibiting inflammatory response are important in treating cerebral injury and promoting brain repair.

SOD is a key antioxidant enzyme in mitochondria (Ye et al., 2016). MDA is a product of lipid peroxidation and is an oxidative stress marker (Ahmed et al., 2014; Shu et al., 2016; Yang et al., 2016). SOD and MDA are extensively used in experimental studies on cerebral ischemia. Previous studies proved increased resistance to ischemic insults of rats over-expressing SOD and with reduced MDA (Zhang et al., 2013, 2016; Yang et al., 2016; Ye et al., 2016). Oxidative capacity is quantified by measuring levels of SOD and MDA. In our present study, SOD activity levels significantly decreased, whereas MDA levels increased after onset of cerebral ischemia. These results revealed that oxidative stress occurs following focal cerebral ischemic injury in rats. DF treatment with doses of 100 and 200 mg/kg significantly inhibited increased levels of MDA and promoted production of cellular antioxidants SOD in brain tissue of rats after pMCAO injury. Thus, we presume that DF plays a fundamental role as antioxidant against focal cerebral ischemia through amelioration of oxidative stress.

In addition, oxidative stress is closely associated to damage caused by excessive production of inflammatory cytokines (Yao et al., 2011; Yang et al., 2016); oxidative stress results in increased production of pro-inflammatory cytokines (Yang et al., 2016). Post ischemic inflammation is closely related to brain injury (Li et al., 2012; Kao et al., 2013). Microglial cells of brain serve as major inflammatory in cell populations in central nervous system; activated microglia release a wide range of soluble pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, which stimulate neurodegeneration (Xiong et al., 2009; Kao et al., 2013). Thus, IL-1β, IL-6, and TNF-α play important roles in protection against inflammatory damage of ischemia injury (Yao et al., 2011; Singh et al., 2013; Yang et al., 2013; Zhang et al., 2013; Luo et al., 2014). The present results showed that expression levels of pro-inflammatory

cytokines, IL-1β, IL-6, and TNF-α, increased in the model group. By contrast, DF treatment significantly reduced activities of IL-1β, IL-6, and TNF-α in the cortex and hippocampus. Results showed that these inflammatory molecules are involved in permanent focal cerebral ischemic injury. DF treatment prevents injury by reducing release of IL-1β, IL-6, and TNF-α.

In this study, we used HPLC method to determine major composition of DF; its primary component is piperine. Recent research suggested that piperine is a chemical *trans-trans* isomer of 1-piperoyl piperidine, an alkaloid present in pepper fruits. Piperine was established as an antioxidant, anti-inflammatory (Singh and Chopra, 2013; Hu et al., 2015), and cognitive enhancer (Banji et al., 2013). A study recently discovered that piperine plays a major role in oxidative stress (free radical damage) and anti-inflammation by inhibiting activity of NF-κB, reducing expressions of IL-1β, IL-6, TNF-α, and MDA and enhancing SOD activity (Qing et al., 2014; Daniela et al., 2016; Hao et al., 2016; Samra et al., 2016; Scott et al., 2016). Piperine also reduces brain damage induced by D-galactose (Banji et al., 2013). As major component of Mu-Xiang-You-Fang (in Hui Hui Yao Fang), piperine also plays an important role in treatment of cerebral ischemic-reperfusion injury (Zhao et al., 2016). Thus, piperine is important in treating brain damage of rats with permanent focal cerebral ischemia injury and has potential use in development of new drugs.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

Author' contributions

BW and YZ contributed in collecting plant samples and conducting the laboratory work. JH contributed to the analysis of the data. LD and TL contributed to the critical reading of the manuscript. XF designed the study, supervised the laboratory work and contributed to the critical reading of the manuscript. All of the authors have read the final manuscript and approved its submission.

Conflicts of interest

The authors declare no conflicts of interest.

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