Journal of Ethnopharmacology 144 (2012) 261–269

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Three Chinese herbal medicines promote neuroproliferation *in vitro*, and reverse the effects of chronic mild stress on behavior, the HPA axis, and proliferation of hippocampal precursor cell *in vivo*

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ARTICLE INFO

Article history:
Received 9 March 2012
Received in revised form
18 May 2012
Accepted 3 September 2012
Available online 19 September 2012

Keywords:
Neural stem cell
Corticosterone
Hippocampus
Rhizome chuanxiong
Radix phellodendri
Radix scutellaria

ABSTRACT

Ethnopharmacological relevance: The present study investigated whether Chinese herbal medicines (CHMs) could reverse the effects of chronic mild stress (CMS) in a depression-like mouse model.
Materials and methods: The effects of three Chinese herbals, Rhizome Chuanxiong, Radix Scutellaria and Radix Phellodendri on promoting neuroproliferation were evaluated *in vitro* first and followed by *in vivo* study of mice which were received by an experimental setting of CMS for 14 days. The effects of the three CHMs on depression were evaluated using a behavioral test, named a forced swimming test (FST). The possible anti-depressive mechanisms of these three CHMs, including the modulation of HPA axis and promoting the hippocampal precursor cell proliferation, were evaluated by measuring plasma corticosterone levels and BrdU incorporation.
Results: The *in vitro* results of MTS assay showed that Rhizome Chuanxiong, Radix Scutellaria and Radix Phellodendri could promote the proliferation of neural stem cells (NSCs) in a concentration-dependent manner. The oral administration of these three CHMs for 14 days reversed not only the elevation of plasma corticosterone levels and body weight loss, but also the decreasing of hippocampal precursor cell proliferation and abnormal behavior in the CMS induced depression-like mouse model.
Conclusion: These results indicated that Rhizome Chuanxiong, Radix Scutellaria and Radix Phellodendri have the potential to ameliorate depression. The possible mechanisms were the inhibition of HPA axis hyperactivity and the increasing of hippocampal precursor cell proliferation. These findings supported the multicomponent and multitargeted approach of Chinese herbal medicine.

1. Introduction

The exposure of chronic mild stress could induce depression-like behavior, and result in neuroendocrine activation and neural damage (Joels et al., 2004; Harvey et al., 2006; Martinez-Tellez et al., 2009). Stress leads to the activation of HPA axis by the suppression of neurogenesis (Schloesser et al., 2009). Downregulation of the hippocampal glucocorticoid receptor makes it difficult for hippocampus to control the glucocorticoid negative feedback, leading to hypersecretion of glucocorticoids, decreased neurogenesis, and even cell death (Watanabe et al., 1992; Sapolsky, 1996; Lee et al., 2006). Chronic antidepressant treatment could reverse the effects of stress which is resulting in the increasing of cell proliferation and neurogenesis in the hippocampus (Duman et al., 2001; Hitoshi et al., 2007). The hippocampal neurogenesis is required for the treatment of chronic antidepressant in mice (Santarelli et al., 2003). These evidences suggest that a change in neurogenesis might be crucial in both the pathogenesis and treatment of depression (Thomas and Peterson, 2008). Based on these findings, the increasing of adult hippocampal neurogenesis may be a drug target or a mechanism for the discovery of antidepressants (Malberg and Schechter, 2005).

Chinese herbal medicines (CHMs) have long been used to treat mental disorders, including depression. The applications of CHMs are based on clinical experiences, which provide the valuable clues to find new-entity drugs. However, only a few CHMs have been

Abbreviations: BrdU, Bromodeoxyuridine; CHM, Chinese herbal medicine; CMS, Chronic mild stress; FST, Forced swimming test; HPLC, High-performance liquid chromatography; HPA, Hypothalamic-pituitary-adrenal; 5-HT, 5-hydroxytryptamine, serotonin; IHC, Immunohistochemistry; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NSC, Neural stem cell; POP, Prolyl oligopeptidase; SNRI, Serotonin norepinephrine reuptake inhibitor; SGZ, Subgranular zone

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http://dx.doi.org/10.1016/j.jep.2012.09.002

validated in scientific pharmacological studies, and modern medicine has revealed that some components of CHMs have neuroprotective (Piao et al., 2004), anti-inflammatory (Yoo et al., 2008), or antidepressant effects (Kulkarni and Dhir, 2008) in recent years. Therefore, it is necessary to examine CHMs using modern pharmacological methods to develop new antidepressants.

The cultured NSC cell lines can be used as a screening model *in vitro* to determine whether a drug promotes neurogenesis. The NSCs possess the ability of self-renewal and the multipotency. Therefore, the cultured NSC cell line may serve as a convenient model at the cellular level for developing therapeutic medicines (Ma et al., 2009).

The potential effects of 90 CHMs, including 56 single herbs and 34 CHM formulae on promoting cell proliferation had been evaluated in our laboratory using cultured NSC cell line. The preliminary results indicated that Rhizome Chuanxiong, Radix Scutellaria and Radix Phellodendri were three of the most effective CHMs that promote the proliferation activity of NSCs. Therefore, the effects of these three CHMs on neurogenesis and the antidepressant-like effect in the CMS-induced stressed mice were investigated in the present study.

2. Materials and methods

2.1. CHMs

CHMs were purchased from a GMP pharmaceutical company (Sun Ten Pharmaceutical Co., Ltd., Taipei, Taiwan). The CHMs were supplied in an extracted dry powder form which was prepared by making decoction of the raw herb material in hot distilled water and then concentrated by instant spray-drying and low-temperature vacuuming. The voucher specimen number of Rhizome Chuanxiong, Radix Phellodendri, and Radix Scutellaria is 046776, 009224, and 009225, respectively. The raw herb medicines were collected from several habitats in China. The voucher specimens were identified at the herbarium of the Brion Research Institute (BRI) in Taiwan. The botanists and chemists use microscopic identification to authenticate the plant species. Classification of plant parts and phytochemical analysis of the extracts were according to Pharmacopoeia of the People's Republic of China (Pharmacopoeia Commission of People's Republic of China, 2010).

2.2. In vitro study

2.2.1. NSC culture

A mouse NSC cell line (mNSC 9601) was supplied by Dr. Shih-Hwa Chiou (Institute of Clinical Medicine, National Yang-Ming University, Taiwan) (Liu et al., 2005), and cultured in a serum-free medium of Dulbecco's modified Eagle medium (DMEM) and F-12 nutrient mixture (1:1).

2.2.2. Preparation of CHMs for in vitro study

The further extraction of CHMs was processed before *in vitro* study. The extraction of obtained CHMs was prepared in a way to simulate the physiological condition when a CHM was ingested by oral administration in humans. 100 mg of CHMs were dissolved in 25 mL saline. The pH was then adjusted to 1.2 with 1 N HCl and vortexed for 5 min at 37 °C to mimic digestion in the stomach; the pH of the above solution was further adjusted to 6.8 with 1 N NaOH and vortexed for 5 min at 37 °C to mimic the situation in the intestinal environment. The Chinese herbal medicine solutions were then centrifuged at 3000 rpm for 10 min. The supernatant was extracted and filtered through a 0.22 μm filter. Serial dilutions were performed and the solutions were finally diluted with medium to a concentration of 1:100.

2.2.3. NSCs proliferation activity assay in vitro

The cell proliferation activity of NSCs was evaluated using quantitative colorimetric assay, *i.e.* 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. The MTS compound was bioreduced by cells into a colored formazan product that remained soluble in the tissue culture medium. Neurosphere-like NSCs were dispersed into a single cell by trypsin dissociation, and viable NSCs were counted and seeded on 96 well plates at a density of 4×10^3 cells per well. The CHMs extract were diluted with medium at different concentrations of 0.04 μg/mL, 0.4 μg/mL, 4 μg/mL, and 40 μg/mL and added to each well and incubated for 48 h. 20 μL of MTS reagent was added to each of the 96 well plates and incubated for 3 h in an atmosphere of 5% CO_2 at 37 °C. The amount of MTS formazan product was determined using a microplate reader. The absorbance of formazan product was read at 490 nm (Thermo Electron Co.). Each sample was analyzed in triplicate.

2.3. In vivo study

2.3.1. Animals

Fig. 2 illustrates the experimental design adopted in this study. Experiments involved sixty-three male BALB/c mice (10 weeks of age). The animals were housed in polypropylene cages under standard laboratory conditions (12 h light/dark cycle; 22 °C, 55% humidity; food and water available *adlibitum*). All animal-use procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Care Committee of the National Defense Medical Center (Taipei, Taiwan). Two weeks after arrival, the animals were randomly assigned to either the control or stressed group. Animals in control group ($n=9$) were left undisturbed in their cages of a separate room, and received an intraperitoneal injection of 300 μL saline and oral administration of 200 μL saline daily. The experiments in this study also tested duloxetine, a new serotonin norepinephrine reuptake inhibitor (SNRI). The stressed group was randomly divided into CMS ($n=10$), CMS–fluoxetine (positive control) ($n=9$), CMS–duloxetine ($n=10$), and three CMS–CHMs groups. The CHMs tested included Radix Scutellaria ($n=7$), Radix Phellodendri ($n=9$), and Rhizome Chuanxiong ($n=9$). The stressed groups were exposed to different stressors for 14 days (Table 1). The mice in the CMS–fluoxetine group and CMS-duloxetine group were injected intraperitoneally with 10 mg/kg fluoxetine (Sigma, St. Louis, MO, USA) and 10 mg/kg duloxetine (Eli Lilly and company, Indianapolis, Indiana, U.S.A), respectively. Both groups also received a daily oral administration of 200 μL saline for 14 days in the period of CMS. The mice in the three CMS–CHMs groups were daily gavaged separately with 90 mg/kg Radix Scutellaria, 90 mg/kg Radix Phellodendri, and 150 mg/kg Rhizome Chuanxiong during the period of CMS, and received a daily intraperitoneal injection of 300 μL saline.

2.3.2. Chronic mild stress procedure

The animal model of this study had been modified from the CMS protocol proposed by Willner (2005).The adapted protocol was consisted of a series of different stressors that were administered each week in a random order. Eight stressors were applied to induce a depressive state: food or water deprivation (12 h), cage tilt (45°, 12 h), soiled cage (6 h), confinement to a restricted space (6 h), exposure to an empty bottle (6 h), reversed light/dark cycle (24 h), and continuous overnight illumination (12 h). These stressors were applied for 2 weeks. Table 1 summarizes the stress arrangements.