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Myelophil ameliorates brain oxidative stress in mice subjected to restraint stress

Jin-Seok Lee [a], Hyung-Geug Kim [a], Jong-Min Han [a], Jong-Suk Lee [b], Seung-Wan Son [c], Yo-Chan Ahn [d], Chang-Gue Son [a,*]

[a] Liver and Immunology Research Center, Oriental Medical Collage of Daejeon University, 22–5 Daehung-dong, Jung-gu, Daejeon, 301–724, Republic of Korea
[b] GyeongGi Bio-Center, GSTEP, 864–1 Iui-dong, Yeongtong-gu, Suwon, Gyeonggi-do, Republic of Korea
[c] Department of Biomedical Engineering, College of Health Science, Korea University, Seongbuk-Gu, Seoul 136-703, Republic of Korea
[d] Department of Health Service Management, Daejeon University, 96-3 Yongun-dong, Dong-gu, Daejeon, 300-716, Republic of Korea

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ABSTRACT

We evaluated the pharmacological effects of Myelophil, a 30% ethanol extract of a mix of Astragali Radix and Salviae Radix, on oxidative stress-induced brain damage in mice caused by restraint stress. C57BL/6 male mice (eight weeks old) underwent daily oral administration of distilled water, Myelophil (25, 50, or 100 mg/kg), or ascorbic acid (100 mg/kg) 1 h before induction of restraint stress, which involved 3 h of immobilization per day for 21 days. Nitric oxide levels, lipid peroxidation, activities of antioxidant enzymes (superoxide dismutase, catalase, and glutathione redox system enzymes), and concentrations of adrenaline, corticosterone, and interferon-γ, were measured in brain tissues and/or sera. Restraint stress-induced increases in nitric oxide levels (serum and brain tissues) and lipid peroxidation (brain tissues) were significantly attenuated by Myelophil treatment. Restraint stress moderately lowered total antioxidant capacity, catalase activity, glutathione content, and the activities of glutathione reductase, glutathione peroxidase, and glutathione *S*-transferase; all these responses were reversed by Myelophil. Myelophil significantly attenuated the elevated serum concentrations of adrenaline and corticosterone and restored serum and brain interferon-γ levels. Moreover, Myelophil normalized expression of the genes encoding monoamine oxidase A, catechol-*O*-methyltransferase, and phenylethanolamine *N*-methyltransferase, which was up-regulated by restraint stress in brain tissues. These results suggest that Myelophil has pharmacological properties protects brain tissues against stress-associated oxidative stress damage, perhaps in part through regulation of stress hormones.

1. Introduction

Stress is an unavoidable part of life in modern society, and uncontrolled stress has deleterious effects on mental and physical health status (Koolhaas et al., 2011). Various types of stresses are closely associated with the occurrence and progression of disorders such as depression, anxiety, and concentration disorder (Strekalova et al., 2005; Vallee et al., 1997). In addition, it is involved in the pathology of cancer recurrence, cardiovascular disturbance, post-traumatic stress disorder, idiopathic chronic fatigue (ICF), and chronic fatigue syndromes (CFS) (Kennedy et al., 2005; Schmaling et al., 2003).

Stress is closely linked to various diseases. The excessive stress can lead to alterations in the brain activity, immune system, cardiovascular system, neuroendocrine system, and sympathetic nervous system, via activation of the hypothalamus-pituitary-adrenal (HPA) axis (Dayas et al., 2001; Reyes et al., 2003). The central nervous system (CNS), especially the brain, is a target of stress and stress hormones, including glucocorticoids (Conrad, 2010). Psycho-emotional stress provokes the production of glucocorticoids and adrenaline, while glucocorticoids promote the synthesis of adrenaline and conversion of noradrenaline to adrenaline (Wong et al., 2008). Furthermore, it was found that several brain regions, including the hippocampus, can be injured as a result of excessive corticosterone release (Patel et al., 2002).

On the other hand, oxidative stress has been implicated as a major mechanisms in the pathology of brain disorders such as cerebral vascular accident, Alzheimer's disease, and Parkinson's disease (Basso et al., 2004; McGrath et al., 2001). Previous studies revealed that, in the brain, stress-induced elevation of glucocorticoid levels and glutamate triggers oxidative stress through production of reactive oxygen

Abbreviations: ANOVA, analysis of variance; COMT, catechol-*O*-methyl transferase; CNS, central nervous system; CFS, chronic fatigue syndromes; GEAC, gallic acid equivalent antioxidant capacity; GSH, total glutathione; GSH-Px, glutathione peroxidase; GSH-Rd, glutathione reductase; GST, glutathione *S*-transferase; HPA, hypothalamus-pituitary-adrenal; ICF, idiopathic chronic fatigue; iNOS, inducible nitric oxide synthase; IFN-γ, interferon-γ; MDA, lipid peroxidation; MAO-A, monoamine oxidase A; NO, nitric oxide; PNMT, phenylethanolamine *N*-methyltransferase; RIPA, radioimmunoprecipitation assay; ROS, reactive oxygen species; SOD, superoxide dismutase; TAC, total antioxidant capacity; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substance; TEP, 1, 1, 3, 3-tetraethoxypropane; TNF-α, tumor necrosis factor-alpha.

* Corresponding author at: Liver and Immunology Research Center, Daejeon Oriental Hospital of Oriental Medical Collage of Daejeon University, 22-5 Daehung-dong, Jung-gu, Daejeon, 301-724, Republic of Korea. Tel.: +82 42 257 6397; fax: +82 42 257 6398.
E-mail address: ckson@dju.ac.kr (C.-G. Son).

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species (ROS), which cause lipid peroxidation, protein oxidation, DNA damage, and cell death (Madrigal et al., 2006; Olivenza et al., 2000; Stein-Behrens et al., 1994). Furthermore, brain tissue is particularly vulnerable to oxidative damage, compared with other organs, due to its relatively high content of iron and peroxidizable fatty acids, and limited antioxidant capacity (Floyd, 1999; Herbert et al., 1994). Therefore, antioxidant therapy has been the focus of the development of medicines for various degenerative disorders in the brain (Ghosh et al., 2011; Ienco et al., 2011). Natural products, including herbal medicines, have also been highlighted as potential candidates (da Rocha et al., 2011; Ho et al., 2011).

Myelophil, an equal mix of the 30% ethanol extracts of Astragali Radix and Salviae Radix, is a commercially available supplement in Korea, which was developed based on traditional Chinese medical theory and experimental evaluation (Cho et al., 2009; Shin et al., 2008). Its two components, Astragali Radix and Salviae Radix, have been shown to have antioxidant, anti-inflammatory, and immunemodulatory effects and to increase vitality (Battaglia et al., 1989; McKenna et al., 2002; Wang et al., 1994). This supplement is used for subjects with chronic fatigue-associated diseases, concentration problems, or lack of memory. Many data indicate the existence of a link between brain oxidative stress and impairment of brain activity, including in chronic fatigue symptom (Radak et al., 2006; Singal et al., 2005).

In this study, in order to explore the brain antioxidant properties of Myelophil, we subjected C57BL/6N mice to repeated restraint stress and investigated changes in oxidative stress and antioxidant biomarkers in brain tissues, as well as changes in stress hormone levels.

2. Materials and methods

2.1. Preparation and standardization of Myelophil

Myelophil contains equal amounts of the herbs Astragali Radix (*Astragalus membranaceus*) and Salviae Radix (*Salvia miltiorrhiza*). All herbs used in this formulation complied with Korean Pharmacopoeia standards. Myelophil was manufactured by Kyung-Bang Pharmacy (Incheon, Korea), according to the approved good manufacturing practice (GMP) guidelines of the Korean Food and Drug Administration (KFDA), according to over-the-counter Korean monographs. Briefly, 100 kg of Myelophil was boiled in 1000 L of 30% ethanol for 4 h at 100 °C, and was then filtered using a 300-mesh filter (50 mm). Some parts were filtered through filter paper (Advantec, Toyo Roshi Kaisha, Tokyo, Japan) and lyophilized in our laboratory for this study. The final Myelophil product [yield 20.52% (w/w)] was stored for future use (VS No. KB-MYP-2011-01). Fingerprinting analysis of Myelophil astragaloside IV and salvianolic acid B was performed to test the Myelophil product for its content of *Astragali Radix* and *Salviae Radix*, respectively (Fig. 1). For analysis, 2 mg of Myelophil and 10 μg of each standard compound were dissolved in 1 mL of 90% methanol, and the solution was filtered and subjected to ultrahigh-performance liquid chromatography (UHPLC) mass spectrometry (MS). Liquid chromatography MS was performed using an LTQ Orbitrap XL linear ion-trap MS system (Thermo Scientific Co., San Jose, CA, USA) equipped with an electrospray ionization source. The UHPLC separation was performed on an Accela UHPLC system using an Acquity BEH C18 column (1.7 μm, 100×2.1 mm; Waters). The column was eluted at a flow rate of 0.3 mL/min using water/0.1% formic acid and acetonitrile/0.1% formic acid as mobile phases A and B, respectively, with the following gradient: 0–1 min, 10% B (isocratic); 1–10 min, 10-90% B (linear gradient); 10–12 min, 100% B (isocratic).

2.2. Chemicals and reagents

The following reagents were purchased from Sigma (St. Louis, St. Louis, MO, USA): 1,1,3,3-tetraethoxypropane (TEP), *N*,*N*-diethyl-*p*-phenylendiamine (DEPPD), ferrous sulfate, trichloroacetic acid (TCA), 5,5′-dithio-*bis*-(2-nitrobenzoic acid) (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), potassium phosphate, reduced glutathione (GSH), 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald), myoglobin, 2,2′-azino-*bis*(3-ethylbenzothiazoline −6-sulfonic acid) diammonium salt (ABTS), glutathione reductase (GSH-Rd), L-glutathione oxidized disodium salt (GSSG), β-NADPH, and tert-butyl hydroperoxide. Thiobarbituric acid (TBA) was obtained from Lancaster Co. (Lancashire, England). Hydrogen peroxide was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan), *n*-butanol was purchased from J.T.Baker (Mexico City, Mexico), and 1 M Tris–HCl solution (pH 7.4) and 500 mM ethylenediaminetetraacetic acid (EDTA) solution (pH 8.0) were purchased from Bioneer (Daejeon, Republic of Korea).

2.3. Animals and experimental design

Sixty specific pathogen-free C57BL/6N male mice (eight weeks old, 22–26 g) were procured from Koatech (Gyeonggido, Korea). The mice had free access to food pellets (Cargill Agri Furina, Gyeonggido, Korea) and water *ad libitum*, and were housed in a room whose temperature was maintained at 23±2 °C, and with a 12 h:12 h light–dark cycle. After acclimatization for one week, mice were randomly divided into six groups ($n=10$): naïve (no restraint stress and only distilled water), control (restraint stress and distilled water), Myelophil (restraint stress and 25, 50 or 100 mg/kg), and positive control (restraint stress and 100 mg/kg ascorbic acid). The Myelophil and ascorbic acid were dissolved in the distilled water. One hour after orally administration of distilled water, Myelophil or vitamin C using gavages, and mice were subjected to restraint stress through placement inside a 50 mL conical tube without access to food or water for (3 h per day for 21 days). The restraint stress model was described previously (Buynitsky and Mostofsky, 2009), and procedure was performed between 10:00 and 13:00 daily and there was a 0.5 cm air hole for breathing.

All mice were sacrificed on the last experimental day under mild ether anesthesia through removal of blood from an abdominal vein after an 8 h fast. A small portion of the blood was used for complete blood count (CBC) analysis using a HEMA VET 850 automatic hematology analyzer (CDC Technologies, CT, USA). The remaining blood was allowed to clot at room temperature, and the serum obtained was analyzed for nitric oxide (NO), hormones, and cytokines. Brains were removed whole immediately after death and were stored at −80 °C or in RNAlater (Ambion, TX, USA). The protocol was approved by the Institutional Animal Care and Use Committee of Daejeon University (DJUARB2011-017) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH).

2.4. Determination of nitric oxide (NO) levels in serum and brain tissue

NO levels in serum and brain tissue were determined by the Griess method (Green et al., 1982). Briefly, 200 mg of the mashed-whole brain tissue was homogenized in 2 mL of RIPA (radioimmunoprecipitation assay) buffer. Then, 40 μL of serum or brain homogenate was transferred to a 96-well plate, and 160 μL of Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl) ethylenediamine hydrochloride, 2.5% H_3PO_4) was added. After reaction at room temperature for 20 min, the purple azo dye product was detected at 540 nm using a spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA).

2.5. Determination of lipid peroxidation (MDA) in brain tissue

Levels of lipid peroxidation in brain tissue were determined by the thiobarbituric acid reactive substance (TBARS) method, as described previously (Mihara and Uchiyama, 1978). Briefly, a 10% (w/v) brain tissue homogenate was prepared with ice-cold 1.15% KCl, and 0.13 mL of the homogenate was mixed with 0.08 mL of 1%