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ScienceDirect
Chinese Journal of Natural Medicines 2018, **16**(3): 0175–0183
Chinese Journal of Natural Medicines

Qualitative detection of ginsenosides in brain tissues after oral administration of high-purity ginseng total saponins by using polyclonal antibody against ginsenosides

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Available online 20 Mar., 2018

[ABSTRACT] Given the limited studies and conflicting findings, the transport character of ginsenosides crossing the blood–brain barrier (BBB) remains unclear. The present study was designed to qualitatively determine the distribution of ginsenosides in brain tissues after oral administration of ginseng total saponins, using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) combined with immunohistochemistry. In brain tissue homogenates, ginsenoside Rg1 was detectable and no other ginsenosides or their metabolites were found. No ginsenosides were detected in cerebrospinal fluid. Immunohistochemistry staining of brain tissue sections by using anti-ginsenoside polyclonal antibodies revealed the localization of ginsenosides in brain tissues. Furthermore, immunofluorescence double staining revealed that ginsenosides widely existed in vascular endotheliocytes and astrocytes, and in few neurons. These results indicated that Rg1 was the main component that entered the brain after oral administration of ginseng total saponins and that ginsenosides could cross the BBB, although the transport capability of ginsenosides through the BBB may be poor.

[KEY WORDS] Ginseng; Ginsenoside; Immunostaining; Mass spectrometry; Blood–brain barrier

[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2018)03-0175-09

Introduction

In Asia, ginseng is the most valuable traditional medicinal herb that has been used for over 2000 years. Ginsenosides, the triterpenoid saponins extracted from ginseng, are the major bioactive components of ginseng [1]. Recent studies have demonstrated that ginsenosides exert protective effects against neurodegenerative disorders through attenuation of excitotoxicity and neuroinflammation, regulation of ion channels and receptors, maintenance of neurotransmitter balance, anti-apoptotic effects, and mitochondrial stabilization [2–5].

Studies have investigated the *in vivo* distribution of ginsenosides in brain tissues by using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) to determine whether the pharmacological effects of ginsenosides in the central nervous system (CNS) are caused by components that target the brain. Ten such studies are found in PubMed. For example, by using a fast HPLC-MS/MS analytical procedure, Liu *et al*. have determined the distribution behavior of ginsenosides Rg1, Re, Rb1, Rc, and Rd in brain tissues after intravenous administration of ginseng extracts [6]. Shi *et al.* and Xue *et al.* have found that subcutaneously administered Rg1 or Re is rapidly distributed in the cerebrospinal fluid (CSF) and dialysates in the hippocampus and medial prefrontal cortex [7-8]. These findings indicate that ginsenosides can cross the blood–brain barrier (BBB) and directly target the neurons, possibly causing pharmacological effects in the CNS.

However, after carefully reading these literatures, we found several limitations inherent in their experimental designs. First, the authors did not perfuse the brain with saline

[Received on] 29-Sep.-2017
[Research funding] The work was supported by the National Natural Science Foundation of China (Nos. 81303246, 81703732) and Projects of Priority-Discipline for Colleges and Universities of Jiangsu Province (integration of traditional Chinese and western medicine).
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These authors have no conflict of interest to declare.

solution before preparing brain tissue homogenate [6, 9-12]. Failure to perform such procedure possibly produced false positive results because of blood contamination in the tissue homogenate. Second, markers for BBB integrity were not used to monitor changes at the interfaces between blood, brain, and CSF while implanting dialysis probes into brain tissues or while inserting the needle of microsyringe into the cerebral ventricle [7-8, 13]. Positive detection of ginsenosides in the CSF or dialysates is likely a consequence of BBB disruption. In Wang *et al.*'s study, the ginsenoside Rg1 showed a very poor BBB permeability and failed to be detected in brain tissues [14]. Third, ginseng extracts or ginsenosides used in these studies were administered intravenously or subcutaneously instead of orally, and such approach does not represent the administration route of ginseng in the clinic. These limitations and conflicting findings render the findings on the transport and disposition of ginsenosides in CNS uncertain and thus must be clarified by using advanced analytical and detection approaches.

In addition to HPLC-MS/MS, an immunological approach using monoclonal or polyclonal antibodies can be used for quantitative or qualitative analysis of bioactive small-molecule compounds [15-16]. Antibodies against small-molecule compounds have been successfully applied to quantify small-molecule compounds in various extracts or body fluids by using Eastern blotting assay [17] or enzyme-linked immunosorbent assay [18], and bioactive constituents can be purified through a one-step immunochromatographic separation [19]. Additionally, antibodies can be used in immunohistochemistry staining, allowing visualization of small molecule compounds in a tissue section. Furthermore, immunofluorescence double staining, a specific example of immunohistochemistry, allows visualization of small-molecule compounds in certain cells by using specific biomarkers within cells. The present study aimed to elucidate the disposition of ginsenosides in CNS through immunohistochemistry by using anti-ginsenoside polyclonal antibodies.

This work included three experiments involving male rats and mice. In Experiment 1, after oral administration of ginseng total saponins, ginsenosides were qualitatively detected in brain tissue homogenates of mice using HPLC-MS/MS. In Experiment 2, we detected the presence of ginsenosides in the CSF of rats by using HPLC-MS/MS and determined whether ginsenosides can cross the BBB. In Experiment 3, we qualitatively detected ginsenosides in brain tissue sections of mice through immunohistochemistry and determined the disposition of ginsenosides in brain tissues through immunofluorescence double staining to confirm the results from Experiments 1 and 2.

Materials and Methods

Chemicals

The ginsenosides Rg1, Re, Rh1, Rb1, F1, Rd, Rh2, protopanaxatriol, protopanaxadiol, and compound K standards (purity > 98%) were purchased from the National Institute for Food and Drug Control (Beijing, China). Deionized water was prepared using a Milli-Q water purification system (Millipore, Billerica, MA, USA). Acetonitrile, methanol, and HPLC-grade formic acid were supplied by Fisher Chemicals (Loughborough, UK). All other solvents used in the present study were of analytical grade and obtained from Nanjing Chemical Reagents Co. (Nanjing, China).

Animals

Male Sprague–Dawley rats (weighing 180–210 g) and C57BL/6N mice (weighing 18–22 g) were obtained from the Laboratory Animal Center of Nanjing Medical University. The animals were housed in groups of five in home cages with sawdust on the floor, and food and water were freely available. They were maintained under a standard dark-light cycle (lights on between 7: 00 and 19: 00) at room temperature (22 ± 2 °C) and adapted to daily handling during the week after delivery. All animal treatments were approved by the IACUC (Institutional Animal Care and Use Committee) of Nanjing University of Traditional Chinese Medicine and carried out in accordance with the Guidelines of Accommodation and Care for Animals, which was formulated by the Chinese Convention for the protection of vertebrate animals used for experimental and other scientific purposes.

Preparation of high-purity ginseng total saponins (HPGTS)

HPGTS were prepared as previously reported [20]. In brief, total saponins were efficiently purified from a water decoction of Chinese ginseng root through dynamic anion-cation exchange following the removal of hydrophilic impurities by macroporous resin. Rg1, Re, Rf, F3, Rh1, Rg2, Rb1, Rc, Rb2, Rb3, Rd, F2, and Rg3 were detected in HPGTS. The total saponin content of HPGTS reached up to approximately 106.8% ± 4.5% and 74.97% ± 2.63% as determined using a colorimetric method and the determination of total analogs by single marker approach based on charged aerosol detection with post-column inverse gradient, respectively [21].

Preparation and characterization of polyclonal antibody against ginsenosides

First, ginsenoside-bovine serum albumin (BSA) conjugate was synthesized as previously described [22]. In brief, 80% ethanol solution (0.7 mL) of HPGTS (10 mg) was added dropwise into a solution (0.5 mL) containing $NaIO_4$ (4 mg); the solution was stirred at room temperature for 1 h. Carbonate buffer (100 mmol·L^{-1}, pH 9.0, 2 mL) containing BSA (10 mg) was added to the mixture, which was then stirred at room temperature for 5 h. The reaction mixture was dialyzed against water at 4 °C for 2 days and then lyophilized to obtain ginsenoside-BSA conjugate. Second, the levels of antibodies were raised by subcutaneously injecting ginsenoside-BSA conjugate emulsified with Imject^{TM} Freund's complete adjuvant (Thermo Scientific Pierce) into a New Zealand rabbit. The antiserum was collected after three injections given at a 10-day interval. Third, the immunoglobulin fractions IgG was purified through precipitation in 50% saturated $(NH_4)_2SO_4$ and