Original Article
Effects of Huanglian-Jie-Du-Tang extract on global cerebral ischemia injury via anti-inflammation reaction in mice

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Abstract: The Chinese herbal formula Huanglian-Jie-Du-Tang (HJDT) is composed of the roots of Coptis chinensis Franch., Scutellaria baicalensis Georgi., Phellodendron chinense Schneid., the fruit of Gardenia jasminoides Ellis, and is widely used in clinical. This study investigates the neuroprotective effects of HJDT extract on global cerebral ischemia (GCI) in mice. GCI was induced by occluding bilateral common carotid arteries for 30 min. HJDT (4 g/kg) and minocycline were administered orally and intraperitoneally from day 7 before until day 7 after ischemia, respectively. The behavioral tests were conducted 1 h after treatment at day 7 after GCI. The Western-Blot and ELISA methods were used to evaluate the expression of Iba-1, and the contents of TNF-α and IL-1β, respectively. HJDT had no effect on the total traveled distance, but decreased the central time during 15-30 min in locomotor activity test. In addition, HJDT significantly increased the discrimination ratio (DR) and discrimination index (DI) in NOR test, improved the morphological change of neurons, and increased the neuron density, especially in the hippocampal CA1 region. Also HJDT decreased the expression of Iba-1, the contents of IL-1β, and TNF-α at day 7 after ischemia in the hippocampal CA1 region. These results suggested that HJDT exerted neuroprotective effects on GCI via inhibiting microglia activation and decreasing the release of inflammatory cytokines.

Keywords: Huanglian-Jie-Du-Tang (HJDT), global cerebral ischemia (GCI), anti-inflammation reaction, mice

Introduction

Cerebral ischemia is a common clinical cerebrovascular disease. Restoring blood flow, called reperfusion, within 30 min after transient cerebral ischemia is vital to reduce widespread neuronal necrosis and improve functional recovery of tissue [1]. However, reperfusion may also exacerbate brain injuries, a condition called cerebral ischemia/reperfusion (I/R) injury [2]. The mechanism of I/R injury is complicated. One of the pathophysiologies of cerebral ischemia includes the actions of different cell types as well as a large number of inflammatory cytokines [3]. For example, early in ischemic injury, microglia are activated in response to cerebral ischemia injury, which synthesize and release harmful inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), reactive oxygen species, and so on [4, 5]. This reaction ultimately cause delayed neuronal damage in addition to cerebral injury [6], although the role of microglia is still being debated.

Huanglian-Jie-Du-Tang (HJDT) is a traditional Chinese herbal formula, composed of four herbs: the roots of Coptis chinensis Franch., Scutellaria baicalensis Georgi., Phellodendron chinense Schneid., and the fruit of Gardenia jasminoides Ellis. HJDT is widely used for alleviating the symptoms of gastrointestinal issues [7], liver injury [8], Alzheimer’s disease [9], ischemic brain injury [10-12], and so forth. A previous study, conducted by this group, has found HJDT had protective effects against focal cerebral ischemia [13].

Both global cerebral ischemia (GCI) and focal cerebral ischemia animal models were used to investigate the neuroprotective effects of these agents. Compared to focal cerebral ischemia,
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GCI was the model that most closely resembles clinical situations such as cardiac arrest, near drowning, or severe systemic hypotension during surgical procedures [14, 15]. As such, this model was considered a relevant and reproducible rodent model of cerebral ischemia.

Whether HJDT had neuroprotective effects on GCI has not been reported yet. Thus, this study investigated the effects of HJDT on GCI with a series of behavioral tests and further explored the effects of HJDT on microglia and inflammation cytokines.

Materials and methods

Animals

Male C57BL/6 mice, weighing 23-25 g, were purchased from Shanghai Slyke Laboratory Animal Limited Corporation (Certificate No. SCXK 2012-0002, Shanghai, China) and were housed socially in cages under a controlled temperature (22 ± 2°C) with a 12-h day/night cycle. The animals were allowed free access to food and water and were left to acclimate for 7 days, during which time they were handled repeatedly before test. All experiments were carried out in accordance to Guide for the Care and Use of Laboratory Animals provided by the National Institute of Health.

Preparation of HJDT

HJDT is consisted of the roots of *Coptis chinensis* Franch., *Scutellaria baikalensis Georgi.*, *Phe-lodendron chinense Schneid.,* and the fruit of *Gardenia jasminoides Ellis*, at a ratio of 3:2:2:3, which were purchased from Eastern China Medical Corp., Zhejiang Province, China. Raw components of the HJDT formulation were mixed and soaked in distilled water for 1 h. Then they were boiled for 2 h in distilled water, 1.5 h in 50% ethanol, and then 1.5 h in 80% ethanol. At last, all the filtrates were mixed and concentrated in a rotary evaporator under reduced pressure to a concentration of 1.0 g/ml.

Drugs and treatments

The mice were randomly divided into four groups: sham group, GCI group, HJDT (4 g/kg) group, and minocycline (MINO) (45 mg/kg) group. Each group consisted of 12 mice. HJDT was administered orally while minocycline was intraperitoneally injected to their respective groups from day 7 before until day 7 after ischemia. The control and GCI mice were administered saline orally. Minocycline was obtained from the Sigma Corporation of America.

GCI model

GCI was induced as previously described by Murakami K. [16]. Briefly, bilateral common carotid arteries were isolated and the blood flow was occluded by arterial clamps for 30 min. After 30 min, the clamps were released to restore arterial blood flow in the brain. The change of cerebral blood flow was monitored in real-time with a Laser Doppler Instrument (J&K Chemical LTD, Sweden) during the course of the operation. Cerebral blood flow decreased to less than 20% and was considered as a successful GCI model (Figure 1). The bilateral common carotid arteries of the sham group were only exposed and isolated, but did not have their cerebral blood flow blocked.

Locomotor activity test

Locomotor activity was measured and analyzed using a Spontaneous Activity Video Analysis System (JLBehv-LAG-4, Shanghai Jiliang Software Technology Co., Ltd. China). The size of the square enclosure was (40 cm × 40 cm × 60 cm) and was divided into a central region (20 cm × 20 cm) and a peripheral region. Each mouse was placed gently into the center of the enclosure 1 h after administration of the drugs. The locomotor tracks were continuously recorded by video camera for 1 h and analyzed. The following parameters: (a) total traveled distance in the central and peripheral regions, as well as (b) time in central and peripheral regions were calculated. After each test session, the enclo-

Figure 1. Change of cerebral blood flow of GCI for 30 min following reperfusion.
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Novel object recognition (NOR) test

NOR test consists of 3 phases: $T_1$ phase, interval time, and $T_2$ phase. In the $T_1$ phase, two identical objects (familiar object) were put in two opposite corners of the open field. The mice were individually placed in the open field to explore for 10 min and then removed back into their home cages. After a 1.5 h interval time, the mice were put into the open field again. $T_2$ phase started with one of the familiar objects replaced by a new object (novel object). The mice then were allowed to explore for 5 min. Tracks were recorded and analyzed by a computerized video analyzer (EthoVision XT, Noldus, Netherlands). The following parameters were analyzed: discrimination ratio (DR, \( DR = \frac{N}{(N + F)} \times 100\% \)) and discrimination index (DI, \( DI = \frac{[N - F]}{(N + F)} \times 100\% \)). (N: the time to explore a novel object, F: the time to explore familiar object). Exploration was defined as the distance between the mouse nose and the object ≤ 2 cm when it faces the object.

Histopathological examination

The mice were anesthetized after the neurological evaluation on day 7 after GCI and were perfused transcardially with 4% paraformaldehyde after pre-flushing with ice-cold saline. Brains were removed, fixed with the same fixative for 24 h, and then stored in 30% sucrose solution at 4°C for 1-2 days. The brains were cut into 8 µm coronal sections on a cryomicrotome (CM1900, Leica, Germany) -2.0 mm from bregma. The 8 µm sections were used for neuron density in the hippocampal CA1 region, temporal cortex and striatum by cresyl violet dyeing. The number of healthy-looking neurons (large cells with a pyramidal shape) was counted in sections.

Western-blot method

Samples of hippocampus were homogenized on ice in PBS containing 1% protease inhibitor and 1% phosphatase inhibitor for Western-blot. Proteins were obtained by centrifugation at 14000 rpm at 4°C for 15 min and quantified by Bradford assay (BioRad, USA). A 50 µg sample of each group was subjected to electrophoresis using 20% SDS at 80 V. The proteins were transferred to polyvinylidene fluoride membranes at 250 mA for 2 h. Antibodies for Iba-1 (1:10000, KangChen Bio-tech, Shanghai, China) were applied overnight. Then membranes were incubated with horseradish peroxidase (HRP) secondary antibody (KangChen Bio-tech, Shanghai, China). At last, the signal intensities of proteins were analyzed using Image J software.

ELISA method

Mice were decapitated and the hippocampus was separated on ice quickly at day 7 after GCI. Then it was sonicated to obtain tissue homogenates. After removing particulars by centrifugation (2000×g, 4°C, 20 min), assay was immediately detected. IL-1β and TNF-α were
measured using respective ELISA system (Shanghai Elisa Biotech Co., Ltd, China) according to the manufacturer's instructions. Finally, optical density was determined (absorbance at 450 nm) on a plate reader.

**Statistical analysis**

The data were expressed as mean ± standard error (SEM). Statistical comparisons were performed using one-way analysis of variance (ANOVA) following with Fisher’s least significant differences (LSD) post hoc analysis tests. A $P < 0.05$ was considered statistically significant.

**Results**

**Effects of HJDT on locomotor activity test**

**HJDT improved the regioselectivity in locomotor activity:** During the locomotor activity test, the sham group explored the new environment actively, and demonstrated the most activity in the first 15 min. Furthermore, the sham mice showed significant regioselectivity. They spent more time and traveled more distance in the peripheral region. Overall, the activity amount increased after GCI, but the regioselectivity was lessen. HJDT (4 g/kg) and minoclyne decreased the overall traveled distance while improving the regioselectivity (Figure 2).

**HJDT had no effect on traveled distance in locomotor activity test:** There was no significant difference in traveled distance during 0-15 min and 45-60 min among groups. After GCI, the total distance, central distance, and peripheral distance increased during 15-30 min, 30-45 min, and 0-1 h. Minoclyne decreased the central distance during 15-30 min and 30-45 min. There was no significant effect on traveled distance after treated with HJDT (Figure 3).

**HJDT decreased the central time during the 15-30 min in locomotor activity test:** During 15-30 min, the sham group spent (1.13 ± 0.14)
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In the central region and (13.87 ± 0.14) min in the peripheral region. However, in GCI group, the central time increased to (1.95 ± 0.27) min and peripheral time decreased to (13.05 ± 0.27) min during the 15-30 min duration in the test. Minocyline and HJDT (4 g/kg) reversed this effect during the 15-30 min section. Also, minocyline decreased the central time and increased the peripheral time within 1 h, but HJDT had no effect within 1 h after administration in mice (Figure 4).

\textbf{HJDT increased DR and DI in NOR test}

Performance in the NOR test for hippocampal-dependent memory was tested in this section. The sham group spent more time to explore the novel object than the familiar in mice. DR was (68.4 ± 2.6)% and DI was (29.5 ± 6.2)% in the control group. GCI significantly affected novel object recognition, as DR and DI for exploring novel object decreased to (48.4 ± 4.0)% and (15.0 ± 10.5)%, respectively. HJDT (4 g/kg) and minocyline groups significantly increased DR and DI, indicating that they recognized the novel object as the new (Figure 5).

\textbf{HJDT increased the neuron density after GCI}

In the hippocampal CA1 region, cortex, and striatum, the morphology of the neurons observed were obviously withered and the density of the apparently surviving neurons had almost disappeared at day 7 after GCI (Figure 6). HJDT (4 g/kg) and minocyline improved the morphological change of neurons and increased the neuron density, especially in the hippocampal CA1 region.

\textbf{HJDT decreased the expression of Iba-1, IL-1β and TNF-α after GCI in the hippocampus}

Compared with the sham group, the expression of Iba-1, as well as the contents of IL-1β and TNF-α both increased, at day 7 after GCI in the ischemic periphery. The results of WB showed fewer expression of Iba-1 in the hippocampus. But it significantly increased after GCI. Treatment with minocyline or HJDT reduced the expression of Iba-1 (Figure 7). The results of ELISA showed the contents of IL-1β and TNF-α increased at day 7 after GCI. HJDT treatment decreased the contents of IL-1β and TNF-α by 43.79% and 27.74% after GCI, respectively. Minocyline showed similar effects with HJDT (Figure 8).

\textbf{Discussion}

In this study, it was demonstrated that HJDT clearly improved the regioselectivity in locomotor activity test, learning-memory ability, and pathological change of neurons in the hippocampal CA1 region after GCI. In addition, HJDT decreased the expression of Iba-1, TNF-α and IL-1β in the hippocampus.

Spontaneous locomotor activity is often evaluated using the locomotor activity test and is one of the most basic behavioral tests used in psychological and pharmacological investigations (usually in rodents). The spatial and temporal organization are the most important properties of locomotor activity test in addition to the amount of activity exhibited by rodents [17]. Milot. reported [18] the total activity distance, central distance ratio, and central time ratio increased at day 5 after GCI. Our study also found the same results at day 7 after GCI. HJDT (4 g/kg) significantly increased the central time
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The NOR test has been a widely used method for the investigation into memory alterations [19-21], including GCI [22]. This test is also related with the hippocampal-dependent visual memory [23]. Hartman [22] found that GCI induces both neuronal loss in dorsal hippocampal CA1 region and learning-memory deficits using water maze, object recognition, and radial arm maze. Our study showed the same results in accordance with Hartman reported after GCI. HJDT (4 g/kg) and minocyline significantly increased the DR and DI of the novel object. They also improved the appearance of hippocampal neurons and increased the hippocampal neuron density in the hippocampal CA1 region, but not in the cortex and striatum. These results indicated that HJDT and minocyline could improve the hippocampal-dependent memory deficit after GCI.

Accumulating evidence indicated that inflammation reaction played a key role in the pathogenesis of cerebral ischemia and secondary damage [5, 24, 25]. The inflammatory responses to reperfusion were characterized by a rapid activation of resident cells (mainly microglia), leading to the production of inflammatory cytokines, such as TNF-α and IL-1β [26-29]. The results from this study showed HJDT (4 g/kg) treatment orally for 14 days after GCI reduced the expression of Iba-1, IL-1β, and TNF-α. It indicated the neuroprotective effect of HJDT may be related with inhibition of microglial activation and the induction of IL-1β and TNF-α.

Using HPLC, our previous study found the active components of HJDT are berberine, baicalin, and geniposide [13]. Berberine, baicalin, and geniposide all did show anti-inflammatory effects in different documents. Berberine is known for its anti-inflammatory, anti-oxidative, and anti-microbial effects. Jia L [30] reported berberine treatment significantly inhibited Aβ-stimulated inflammatory cytokine of IL-6 and MCP-1, and also strongly inhibited the NF-κB activation. This indicated berberine was a potent suppressor of neuroflammation. In addition, berberine inhibited the Aβ-induced inflammatory reaction in SH-SY5Y cell lines [31]. Also baicalin effectively down-regulated the expression of macrophage migration inhibitory cytokine in rats [32]. Geniposide inhibited...
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Aβ-induced microglial activation and lead to the reduction of proinflammatory cytokines, such as TNF-α and IL-1β in Alzheimer’s disease [33]. These results suggested that berberine, baicalin, and geniposide may be responsible for the anti-inflammatory effect of HJDT on GCI.

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Disclosure of conflict of interest

None.

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