Original Article

Preventive Effect of *Hwangryunhaedok-tang* on Inflammatory Responses in PHA-stimulated Peripheral Blood Mononuclear Cells from Cerebral Infarction Patients

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Objectives: *Hwangryunhaedok-tang* (HRHDT), a prescription composed of four herbs, has been wi dely used in Oriental Medicine for the treatment of cerebral infarction. However, the mechanisms by which the herbal formula affects on the production of pro- and anti-inflammatory cytokines in cerebral infarction patients remain unknown yet.
Methods: The levels of pro- and anti-inflammatory cytokines, including tumor necrosis factor (TNF)-a, interleukin (IL)-1b, and IL-6, IL-10, and TGF-β1 were determined in peripheral blood mononuclear cells (PBMCs) from cerebral infarction patients under our experimental conditions.
Results: The secretory levels of pro- and anti-inflammatory cytokines, including tumor necrosis factor (TNF)-a, interleukin (IL)-1b, and IL-6, and IL-10 were significantly increased in phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) from cerebral infarction patients. However, pretreatment with HRHDT significantly inhibited the secretion of pro- and anti-inflammatory in PBMCs. Also, HRHDT induced a significant increase of transforming growth factor (TGF)-b1 in PBMCs.
Conclusions: These data indicate that HRHDT may be beneficial in the suppression of inflammatory processes of cerebral infarct through suppression of TNF-α, IL-1β, IL-6, and IL-10 and induction of TGF-β1.

Key Words : *Hwangryunhaedok−tang* (HRHDT), cerebral infarction, pro−inflammatory cytokines, IL−10, TGF−β1

Introduction

Hwangryunhaedok-tang (HRHDT), a traditional prescription of Oriental Medicine, has been used in treatment of cerebral infarction patients to ameliorate and decrease morbidity and mortality after stroke. HRHDT contains four herbs, *Coptidis Rhizoma*, *Phellodendri Cortex*, *Scutellariae Radix*, and *Gardeniae*

Fructus, which have pharmacological and biological effects, including anti-inflammatory[1] and anti-oxidative[2-4] effects. However, the mechanism of its therapeutic benefits in cerebral infarction patients has not yet been well defined. Cerebral infarct usually causes cerebral ischemic insults with irreversible deterioration of central nervous system (CNS) behaviors. After the onset of cerebral ischemia, the inflammatory process

triggers acceleration of the early onset and functions as a determinant factor in severity of cerebral damage, morbidity and mortality in neurodegenerative brain diseases[5,6]. Usually, inflammation occurs in tissues outside the brain with features of redness, swelling, and heat. Under cerebral ischemia, the acute phase of inflammation initiates recruitment of activated inflammatory cells, including macrophages and lymphocytes, into the damaged brain lesions. Macrophages and lymphocytes, circulating immune cells, play an essential role in secreting pro-inflammatory cytokines and activating inflammatory mediators in ischemic status. Other supporting cells, including astrocytes, microglia and endothelia, are also involved in inflammatory processes after cerebral ischemic stroke[7-9]. Pro-inflammatory cytokines, including tumor necrosis factor (TNF)-a, interleukin (IL)-1b, and IL-6, are secreted in the ischemic region by activated immune cells, which drive the inflammatory process and accelerate the additional inflammatory processes by inducing inflammatory molecules, such as intercellular adhesion molecules (ICAM), vascular cell adhesion molecules-1 (VCAM-1), and selectin. These inflammatory modulators recruit more circulating leukocytes which infiltrate into ischemic region and lead to further loss of neuronal cells and brain tissue and increase of cerebral infarct size[10-12]. Interestingly, blockade of immune reaction and anti-inflammatory agents have been regarded as a potentially therapeutic benefit in cerebral ischemia over the past decade. Therefore, specific inhibition on the role of pro-inflammatory molecules has been focused in treatment of cerebral infarction patients even though molecular mechanisms are not clearly demonstrated in prevention of subsequent neuronal damages during ischemia[6,13-15]. Anti-inflammatory cytokines, such as IL-10 and transforming growth factor (TGF)-b1, have been identified to suppress the production of pro-inflammatory cytokine in protection of damaged brain tissues after ischemic stroke. Also, anti-inflammatory cytokines are associated with repairing damaged brain tissues[16-18]. To identify the functional mechanism of HRHDT on cerebral infarct, we herein investigated the regulatory roles of HRHDT on phytohemagglutinin (PHA)-induced inflammatory cytokines in peripheral blood mononuclear cells (PBMCs) from cerebral infarction patients.

Materials and Methods

1. Reagents

Ficoll-Hypaque, PHA, and 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). RPMI 1640, ampicillin, streptomycin, and fetal bovine serum (FBS) were bought from Gibco BRL (Grand Island, NY, USA). TNF-α, IL-1β, IL-6, IL-10, and TGF-β1 ELISA kits were obtained from R&D system (Minneapolis, MN, USA).

2. Patients

Patients with cerebral infarction were admitted and examined at the Department of Neurology, Wonkwang University Hospital from September 2004 to February 2005. The diagnosis of cerebral infarction was confirmed with computerized tomography (CT), magnetic resonance imaging (MRI), and specific clinical signs, including hemiparesis, hemiplegia, slurred speech, facial palsy, etc. All patients gave informed consent before participating in the research protocols, which was approved by the ethics committee of Wonkwang University Hospital.

3. Preparation of HRHDT

The ingredients of HRHDT were *Coptidis Rhizoma* (16 g), *Phellodendri Cortex* (40 g), *Scutellariae Radix* (40 g), and *Gardeniae Fructus* (40 g). An extract of HRHDT was prepared by decocting the dried prescription of herbs with distilled water (100 g/L). The decoction was filtered, lyophilized, and kept at 4℃. The yield of extraction was about 9.79% (w/w). The HRHDT powder of water extract was

dissolved in sterile saline (100 mg/ml). Final concentrations of 175, 350, and 700 μg/ml HRHDT were used for the experiments. The plant materials were obtained and identified by Professor Byung-Soon Moon from Oriental Medical Hospital, Wonkwang University, Korea.

4. Cell culture

PBMCs from heparinized venous blood of patients with cerebral infarction were isolated by Ficoll-gradient centrifugation, then washed three times in PBS and resuspended in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 10% FBS inactivated for 30 min at 56 ℃. PBMCs were adjusted to a concentration of 2×10^6 cells/ml in 30 ml falcon tubes, and 100 ml aliquots of cell-suspension were placed in a four-well cell culture plate. PBMCs were cultured with PHA (10 μg/ml) for 24 hr after pretreatment with or without HRHDT (175-700 μg/ml) in 95% humidified air containing 5% CO_2 at 37℃; the supernatants were collected by centrifugation and stored at -20 ℃.

5. Cell viability test

Cell viability was quantified using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). In brief, cells (5×10^5) were cultured in 24-well plates with varying concentrations of HRHDT for 24 h. To determine the cell viability, MTT (0.5mg) was added to 1ml of cell suspension for 4 h. After three washes of cells with phosphate-buffered saline (PBS, pH 7.4), the insoluble formazan product was dissolved in DMSO. Then, the optical density (OD) of each culture well was measured using a Microplate reader (Titertek Multiskan, Flow Laboratories) at 590 nm. The OD in control cells was taken as 100% of viability.

6. Measurement of pro-inflammatory cytokines by ELISA

Secretory level of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, and anti-inflammatory cytokines, including IL-10, and TGF-β1, in culture supernatant was determined by Quantikine ELISA kit (R&D Systems Inc, Minneapolis, MN, USA). The color generated was determined by measuring the O.D. at 450 nm of spectrophotometric microtiter plate reader (Molecular Devices Corp., Sunnyvale, CA, USA). The minimum detectable concentration of TNF-α, IL-1β, IL-6, IL-10, and TGF-β1 was estimated to be 1.6 pg/ml, 1.0 pg/ml, 0.7 pg/ml, 3.9 pg/ml, and 7 pg/ml, respectively. A standard curve was run on each assay plate using recombinant proteins, including TNF-α, IL-1β, IL-6, IL-10, and TGF-β1 in serial dilutions.

7. Statistical analysis

The data shown are a summary of the results from at least three experiments and are presented as the mean ± S.E.M. Statistical evaluation of the results was performed by one-way ANOVA. The results were considered significant at a value of $p < 0.05$.

Results

1. HRHDT significantly inhibited the production of pro-inflammatory cytokines in human PBMCs from cerebral infarction patients

We examined whether HRHDT could regulate pro-inflammatory cytokines in PBMCs from cerebral infarction patients. At first, we confirmed that the concentration of HRHDT used in this study showed no significant effect on viability of PBMCs at 700 μg/ml (Fig. 1). PBMCs from cerebral infarction patients (n=10, mean age 56±12 years; 3 female and 7 males) were treated with HRHDT for 24 hr and cell viability was tested by MTT assay. To evaluate whether
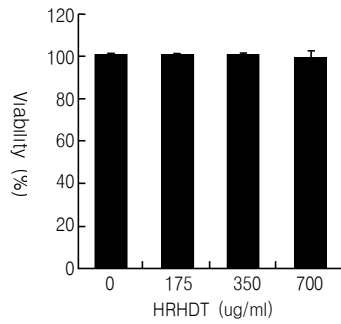
Fig. 1. HRHDT did not affect cell viability of PBMCs from CI patients.

PBMCs were treated with 0.175-0.7 mg/ml HRHDT for 24 h. Cell viability was measured by MTT assay. Results are expressed as means ± SD of three independent experiments (*p<0.05).
(PBMCs: peripheral blood mononuclear cells)

HRHDT suppresses pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, PBMCs from cerebral infarction patients were pretreated with HRHDT for 1 hr and further maintained with 10 μg/ml PHA for 24 hr. The culture supernatant was collected to measure the secretion of pro-inflammatory cytokines by ELISA
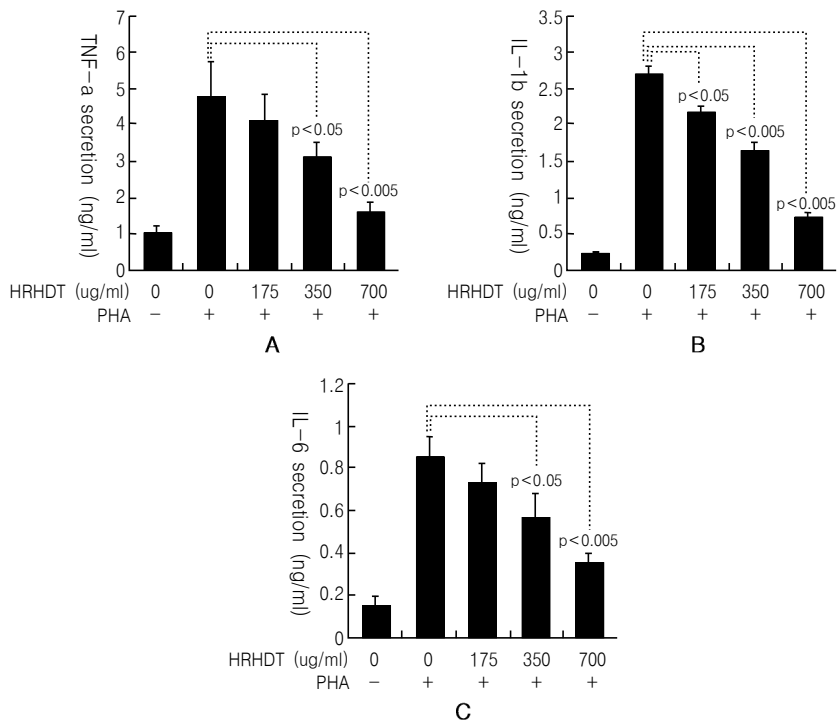
Fig. 2. HRHDT suppressed the secretion of TNF-α, IL-1β, and IL-6 in PBMCs from CI patients treated with PHA.

Amount of secreted TNF-α (A), IL-1β (B), and IL-6 (C) from the supernatant of PBMCs for 24 h was measured by ELISA method in the presence and absence of HRHDT (*p<0.05).

assay (Fig. 2). As shown in Fig. 2A, treatment with PHA resulted in a significant increase in secretion of TNF-α (control, 1.026±0.203 ng/ml; PHA-stimulated, 4.698±0.962 ng/ml). However, addition of PBMCs with HRHDT significantly suppressed TNF-α production by LPS and PHA in a concentration-dependent manner (350 μg/ml of HRHDT-pretreated, 3.068±0.406 ng/ml, $p < 0.05$; 700 μg/ml of HRHDT-pretreated, 1.602±0.251 ng/ml, $p < 0.005$).

Also, treatment with PHA resulted in a significant increase in secretion of IL-1β in PBMCs (control, 0.204±0.056 ng/ml; PHA-stimulated, 2.684±0.218 ng/ml). As shown in Fig. 2B, pretreatment with HRHDT dramatically down-regulated IL-1β secretion by PHA in PBMCs from cerebral infarction patients in a dose-dependent fashion (175 μg/ml of HRHDT-pretreated, 2.148±0.211 ng/ml, $p < 0.05$; 350 μg/ml of HRHDT-pretreated, 1.619±0.395 ng/ml, $p < 0.005$; 700 μg/ml of HRHDT-pretreated, 0.731±0.146 ng/ml, $p < 0.005$). We also measured IL-6 production in PBMCs. Treatment with PHA caused a significant increase of IL-6 production (control, 0.146±0.056 ng/ml; PHA-stimulated, 0.837±0.102 ng/ml, respectively) (Fig. 2C). HRHDT markedly suppressed IL-6 production in PBMCs treated with PHA (350 μg/ml of HRHDT-pretreated, 0.563±0.120 ng/ml, $p < 0.05$; 700 μg/ml of HRHDT-pretreated, 0.348±0.049 ng/ml, $p < 0.005$).

2. HRHDT significantly inhibited the production of IL-10 in human PBMCs of cerebral infarction patients

To evaluate whether HRHDT regulates the production of anti-inflammatory cytokines, PBMCs were pretreated with HRHDT for 1 h and maintained with 10 μg/ml PHA for 24 h. The culture supernatant was collected to measure the secretion of IL-10 by ELISA assay (Fig. 3). As shown in Fig. 3, treatment with PHA induced a significant increase in secretion of IL-10 (control, 0.08±0.012 ng/ml; PHA-stimulated, 0.447±0.071 ng/ml). Addition of PBMCs with HRHDT significantly suppressed IL-10 production by PHA in a concentration-dependent manner (350 μg/ml of HRHDT-pretreated, 0.304±0.026 ng/ml, $p < 0.05$; 700 μg/ml of HRHDT-pretreated, 0.198±0.008 ng/ml, $p < 0.005$).

3. HRHDT significantly increased the production of TGF-β 1 in human PBMCs of cerebral infarction patients

Also, to assess whether HRHDT produces TGF-β 1, an anti-inflammatory cytokine, PBMCs were treated with HRHDT for 24 h. The culture supernatant was collected to measure the production of TGF-β1 by ELISA assay (Fig. 4). As shown in Fig. 4, HRHDT markedly up-regulated TGF-β1 secretion in PBMCs
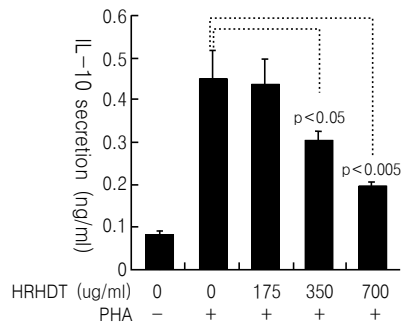
Fig. 3. HRHDT suppressed the secretion of IL-10 in PBMCs from CI patients treated with PHA.

Amount of secreted IL-10 from the supernatant of PBMCs for 24 h was measured by ELISA method in the presence and absence of HRHDT (*p<0.05).

of cerebral infarction patients in a dose-dependent fashion (control, 30.75 ± 8.26 ng/ml; 350 μg/ml of HRHDT-pretreated, 0.062 ± 0.009 ng/ml, $p < 0.005$; 700 μg/ml of HRHDT-pretreated, 0.106 ± 0.008 ng/ml, $p < 0.005$).

Discussion

In this study, we demonstrated that HRHDT suppressed the production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 in PBMCs treated with PHA (Fig. 2). The secretion of IL-10, an anti-inflammatory cytokine, was increased by PHA, which was significantly decreased by pretreatment with HRHDT in PBMC of cerebral infarction patients (Fig. 3). HRHDT also induced the production of TGF-β1, an anti-inflammatory cytokine (Fig. 4). Thus, we suggest that the one of the beneficial roles of HRHDT in treatment of cerebral infarction patients is associated with its regulatory effects on the inflammatory process.

Each herbal extract of HRHDT has been used as oriental traditional medicine and reported their pharmacological effects as follow. Berberine, an isoquinoline derivative alkaloid isolated from *Coptidis Rhizoma*, has many pharmacological and biological effects, including anti-inflammatory, antioxidant, antihypertensive, and anti-diabetic effects[19,20]. *Phellodendri Cortex* has

been demonstrated to reduce glucose level, prevent the development of diabetic nephropathy in streptozotocin-induced diabetic rats, and inhibit gene expression and production of iNOS and TNF-alpha in LPS-stimulated microglia[21,22]. Oroxylin A isolated from *Scutellariae Radix* induces G2/M phase cell-cycle arrest via inhibiting Cdk7-mediated expression of Cdc2/p34 in human gastric carcinoma BGC-823 cells[23]. *Gardeniae Fructus* has been reported to inhibit matrix metalloproteinases activity in HT1080[24], LPS- and PGE2-induced aqueous flare elevation in pigmented rabbits[25].

Under cerebral ischemia state, inflammatory cells infiltrate into the ischemic brain region and trigger to activate inherent brain cells, including astrocytes, microglia and endothelia. Inflammatory responses which affect these cells with substances, including pre-inflammatory cytokines, vasoactive substances and adhesion molecules, play an important role in the pathogenesis of cerebral lesions following cerebral ischemia[13,26].

The most important pro-inflammatory cytokines in post-ischemic inflammation include TNF-α, IL-1β, and IL-6. TNF-α and IL-1β play the major role to initiate the inflammatory response mediated by the induction of inflammatory metabolites and increased expression of adhesion molecules on the surface of endothelial cells. TNF-α and IL-1β secreted from
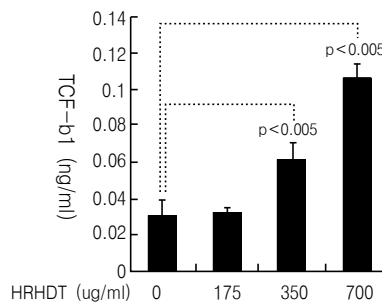
Fig. 4. HRHDT increased the secretion of TGF−β1 in PBMCs from CI patients.
Amount of secreted TGF−β1 from the supernatants of PBMCs treated with HRHDT at individual concentration for 24 h was measured by ELISA method (∗p<0.05).

brain cells share a high homology in structure and function. TNF-α and IL-1β are also responsible for the accumulation of inflammatory cells in the peripheral nucleus of cerebral infarct and induce a second inflammatory response mediated by IL-6. IL-6 is playing a central role in acute inflammatory processes exhibiting pro-inflammatory activities in many different brain pathologies including cerebral ischemia and excitotoxic brain damage.[27-30] In addition, to ameliorate brain damages followed by stroke and ischemic attack, there have been reported various therapeutic trials, including anti-TNF-α antibody and TNF-α soluble receptor type 1[31,32], IL-1β receptor antagonist[33], and recombinant IL-1ra[17,34]. Accumulated evidence has proved that the suppressive approaches of specific inflammatory mediators are believed to be one of best solutions to prevent leading to secondary ischemia and damage of brain cells under cerebral ischemia.

IL-10, a Th2-type response and an anti-inflammatory cytokine, modulates immune processes by inhibiting Th1-type response, which is associated with the function of antigen presenting cells. Especially, it reduces ischemic infarct size and the production of other pro-inflammatory mediators in the brain after stroke. Through deactivation of macrophage-like cells and astrocytes, IL-10 plays a role of inhibiting secondary inflammatory processes by them within the brain. However, cerebral infarction patients have been reported to have a significantly lower IL-10 serum level than people without.[16,35] In our results, HRHDT might prevent inflammatory signaling transduction in early stage by inhibiting the production of IL-10, which is a defensive response to inflammatory stimuli.

TGF-β1, a Th3-type response and an anti-inflammatory cytokine, regulates immune processes through the suppression of Th1-type response. TGF-β1 is implicated in tissue repair, differentiation, and various immune functions. After ischemic stroke, TGF-β1 in the brain functions on tissue repairing and angiogenesis. Wang et al. reported that it protects the cerebral hemispheres from damage induced by stroke[36-38].

In conclusion, our data demonstrate that HRHDT functions to suppress the production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 in PBMCs from cerebral infarction patients. Simultaneously, HRHDT induces a significant increase of anti-inflammatory cytokines, TGF-β1. Also, HRHDT prevents the production of anti-inflammatory cytokines, IL-10 in response to inflammatory stimuli, PHA. Taken together, HRHDT has many functions on normalization of homeostatic balance between anti- and pro-inflammatory processes mediated by mediator, including cytokines.

Furthermore, works are undergoing to identify the crucial components of herbal constituents of HRHDT and the precise signaling pathway of HRHDT on inhibitory mechanism *in vitro* and *in vivo* animal model of brain ischemia.

Acknowledgement

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