The Effects of Seonghyangjeonggisan on Cytokines Production in the Peripheral Blood Mononuclear Cells of Acute Cerebral Infarction Patients

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The Korean traditional medicine, Seonghyangjeonggisan (SHJGS) has long been used for acute cerebral infarction (CI). However, scientific investigation has been carried out a little. Cytokines, involved in the regulation of inflammatory reactions and immune responses, may play an important role in the pathogenesis of CI. The aim of this study is to investigate the effects of SHJGS on the production of various cytokines in the patients with acute CI. Peripheral blood mononuclear cells (PBMC) obtained from the patients with acute CI were cultured for 24 hr in the presence or absence of lipopolysaccharide (LPS) and phytohaemagglutinin (PHA). The amount of TNF-α, IL-1β, IL-6 and IL-8, in PBMC culture supernatant, was significantly increased in the LPS and PHA treated cells, compared with unstimulated cells ($P < 0.05$). This study showed that increased TNF-α, IL-1β, IL-6 and IL-8 level stimulated by LPS and PHA was inhibited by SHJGS (0.01-1 ㎎/㎖) in a dose-dependent manner but IL-8 level was not inhibited significantly at 1 ㎎/㎖ ($P > 0.05$). The maximal inhibition rate of TNF-α, IL-1β, IL-6 and IL-8 by SHJGS (1 ㎎/㎖) was 68% ($P < 0.05$), 53.9% ($P < 0.05$), 45.5% ($P < 0.05$), 46.7% ($P > 0.05$) respectively. These results suggest that SHJGS might have anti-inflammatory effects through cytokine modulation, which might explain its beneficial effects in the treatment of acute CI.

Key words : Seonghyangjeonggisan, acute cerebral infarction, cytokine, peripheral blood mononuclear cells

Introduction

Seonghyangjeonggisan (SHJGS), a prescription of the Korean traditional medicine, has long been used as a specific prescription for acute cerebral infarction (CI) to increase cerebral blood flow and to recover injured brain cells. It was first mentioned in Zheng Zhi Yao Jue[1], written by Dai Si Gong. It has effects of regulating vital energy and eliminating phlegm, so it has been used for stroke, zhong qi, phlegm syncope and a syncope with eating and drinking as an emergency treatment[2]. But its pharmacological mechanisms have not been well defined yet.

People with CI are frequently suffered from irreversible neurologic deficits that markedly hinder their activity of daily living. Patients may also be suffered from disturbances of motor strength, coordination, sensory discrimination, visual function, speech, memory, and other intellectual abilities. Although recovery is incomplete, partial recovery often occurs in weeks to months[3]. Recently, it has become increasingly evident that the inflammatory response plays an important role in the pathogenesis of CI. Infiltration of leukocytes and development of brain edema characterise the ischemia-induced inflammation in the early stage in the ischemic region[4].

Moreover, the resident cells of the brain (e.g. astrocytes, microglia and endothelial cells) become activated in response to the ischemic injury. Much of this inflammatory response appears to be mediated by interleukins, a multifunctional subclass of cytokines. In physiological conditions, the expression of cytokines (e.g. TNF-α, IL-1β, IL-6, IL-8) is very low. However, these cytokines are up-regulated in the brain after injury. Among the mediators involved in hemostatic and immunological imbalance leading to enlargement of ischemic brain damage, the release of pro-inflammatory cytokines, especially tumor necrosis factor-α (TNF-α) is emphasized.

TNF-α is a major inflammatory cytokine because it stimulates the synthesis of nitric oxide and other inflammatory mediators that derive chronically delayed hypersensitivity

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reaction[5-8]. The spontaneous production by mononuclear cells and the level of TNF-α in serum are significantly increased in the patients with CI.

Interleukin-1β (IL-1β) is one of the most powerful inflammatory cytokines. IL-1β is a pro-inflammatory cytokine that has been identified as an important mediator of neurodegeneration induced by experimental CI or excitatory or traumatic brain injury in rodents[9,10]. IL-1β is rapidly produced in the brain of rodents exposed to CI[11,12]. The increase of IL-1β by peripheral blood mononuclear cells (PBMC) in the serum from the patients with CI, has been described. The activity of IL-1β can be inhibited by IL-1 receptor antagonist, which binds the IL-1 receptor but does not transmit the agonist signal[13]. As the unbalanced expression of IL-1β and IL-1 receptor antagonist may be associated with CI development.

Interleukin-6 (IL-6) is a differentiation factor for B and T lymphocytes, monocytes, neural cells and hepatocytes[14-16]. It has been shown that the high level of IL-6 can occur in CI patients[13], but the possible etiopathogenetic role of IL-6 in the central nervous system (CNS) diseases is not clarified. As cytokines are involved in the regulation of immune responses and inflammatory reactions, the regulation of unbalanced cytokines is important in CI.

Interleukin-8 (IL-8), a major chemokine known to attract and activate leukocytes[17-19], has recently been under focused investigation because of its possible participation in the evolution of CI. Brain cells produce chemokines during the inflammatory process after CI both in animal models and in patients. In this study, the author investigated TNF-α, IL-1β, IL-6 and IL-8 production in lipopolysaccharide (LPS) plus phytohaemagglutinin (PHA)-stimulated PBMC from acute CI patients. The author also investigated the effects of SHJGS on LPS plus PHA-induced cytokines production in the PBMC from acute CI patients.

Materials and Methods

1. Reagents

Ficoll-Hypaque, LPS, avidin-peroxidase and 2-AZINO-bis (3-ethylbenzithiazoline-6-sulfonic acid) tablets substrate (ABTS) and PHA were purchased from Sigma (St. Louis, MO, USA). RPMI 1640, ampicillin, streptomycin and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Anti-human IL-1β, IL-6 and IL-8, biotinylated anti-human IL-1β, IL-6 and IL-8 and recombinant human IL-1β, IL-6 and IL-8 were purchased from Pharmingen (San Diego, CA, USA).

2. Patients

I analyzed the 9 patients who visited Wonkwang University Hospital within 3 hr after the onset of acute ischemic stroke from September 2002 to February 2003. For cytokine assay, blood was obtained from 9 patients (6 males and 3 females, age range 60-70) with acute CI and 10 healthy adults (5 males and 5 females, age range 50-67) with no medically diagnosable illness as a control group. All samples were collected by centrifugation and quickly frozen and stored in aliquots at -80℃ until assay.

3. Preparation of SHJGS

The ingredients of SHJGS are Herba Agastaches (6 g), Folium Perillae (4 g), Radix Angelicae Dahuricae (2 g), Pericarpium Arecae (2 g), Poria (2 g), Cortex Magnoliae Officinalis (2 g), Rhizoma Atractylodis Macrocephalae (2 g), Pericarpium Citri Reticulatae (2 g), Rhizoma Pinelliae (2 g), Radix Platycodi (2 g), Radix Glycyrrhizae (2 g), Rhizoma Arisaematis (4 g), Radix Saussurea Seu Inulae (4 g), Rhizoma Zingiberis Recens (4 g), and Fructus Jujubae (4 g). The extract of SHJGS was prepared by decocting the dried prescription of herbs with boiling distilled water.

4. PBMC isolation and culture

PBMC from heparinized venous blood (from patients with acute CI) were isolated by Ficoll-gradient centrifugation and washed three times in phophate-buffered saline (PBS) solution and resuspended in RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamin, 100 U/㎖ penicillin G, 100 ㎍/㎖ streptomycin and 10% FBS inactivated for 30 min at 56℃. PBMC were adjusted to a concentration of 3×106 cells/ml in 30 ㎖ falcon tube and 100 ㎕ aliquots of cell suspension were placed in a four-well cell culture plate. PBMC were cultured for 24 hr in 95% humidified air containing 5% CO_2 (37℃) in the presence or the absence of LPS and PHA, and the supernatants were collected by centrifugation and stored at -20℃.

5. ELISA of TNF-α, IL-1β, IL-6 and IL-8

Sandwich ELISA for TNF-α, IL-1β, IL-6 and IL-8 was carried out in duplicate in 96 well ELISA plates (Nunc, Denmark) coated with each of 100 ㎕ aliquots of anti-human TNF-α, IL-1β, IL-6 and IL-8 monoclonal antibodies at 1 ㎍/㎖ in PBS at pH 7.4 and was incubated overnight at 4℃. The plates were washed in PBS containing 0.05% Tween 20 (Sigma) and blocked with PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3 for 1 hr. After additional washes, sample or TNF-α, IL-1β, IL-6 and IL-8 standards were added and incubated at 37℃ for 2 hr. After 2 hr incubation at 37℃, the wells were washed and then each of 0.2 ㎍/㎖ of biotinylated anti-human

TNF-α, IL-1β, IL-6 and IL-8 were added and again incubated at 37℃ for 2 hr. After washing the wells, avidin-peroxidase was added and plates were incubated for 20 min at 37℃. Wells were again washed and ABTS substrate (Sigma) was added. Color development was measured at 405 ㎚ using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant TNF-α, IL-1β, IL-6 and IL-8 in serial dilutions.

6. MTT assay

The MTT colorimetric assay of cell survival was executed by the method of Trivedi et al. with minor modifications. Cell aliquots (2×10^5) were seeded in microplate wells and incubated with 20 ㎕ of a MTT solution (5 ㎍/㎖) for 4 hr at 37℃ under 5% CO_2 and 95% air. Consecutively, 250 ㎕ of DMSO was added to extract the MTT formazan and an automatic microplate reader read the absorbance of each well at 540 ㎚.

7. Statistical analysis

The shown experiments are the summary of the data from at least three experiments and are presented as the mean ± SEM. Statistical evaluation of the results was performed by independent t-test. The results were considered significant at the value of $P < 0.05$.

Results

1. Effects of SHJGS on PBMC viability

The author examined the effects of SHJGS on viability of PBMC from acute CI patients by MTT assay (Fig. 1). Cells were stimulated for 24 hr with LPS plus PHA in the absence or presence of SHJGS (0.01-1 ㎎/㎖). In the cells treated with LPS plus PHA, cell viability decreased to 94.8 ± 1.1% compared with the control value (100.0 ± 1.3%). However, SHJGS (0.01-1 ㎎/㎖) did not affect cell viability in each condition and had no toxicity on PBMC.

2. Effects of SHJGS on LPS plus PHA induced TNF-α production

The amount of TNF-α released into supernatants after stimulation with LPS plus PHA in PBMC from acute CI patients was determined by ELISA. Pre-treatment of SHJGS for 30 min inhibited the TNF-α production by 30.3 ± 0.55% (0.01 ㎎/㎖ of SHJGS), 34.5 ± 0.45% (0.1 ㎎/㎖ of SHJGS) and 68.01 ± 0.28% (1 ㎎/㎖ of SHJGS), compared with value of no treatment of SHJGS. As shown in Fig. 2, SHJGS inhibited the TNF-α production in LPS and PHA-stimulated PBMC in a concentration-dependent manner, although SHJGS treated with 0.01 and 0.1 ㎎/㎖ had no statistically significance.

3. Effects of SHJGS on LPS plus PHA induced IL-1β production

IL-1β was enhanced with stimulation of LPS and PHA in PBMC from acute CI patients (Fig. 3). The inhibitory effects of SHJGS on IL-1β production was evaluated in LPS and PHA-stimulated PBMC and pre-treatment of SHJGS for 30 min inhibited the IL-1β production (by 11.06 ± 5.2%, 20.79 ± 9.2%, 53.9 ± 1.24% for 0.01 ㎎/㎖, 0.1 ㎎/㎖ and 1 ㎎/㎖ of SHJGS respectively, compared with value of no treatment of SHJGS). Production of IL-1β by SHJGS was reduced in a dose-dependent manner. 1 ㎎/㎖ of SHJGS significantly inhibited the IL-1β production in LPS and PHA-stimulated PBMC, although SHJGS treated with 0.01 and 0.1 ㎎/㎖ had no statistically significance.

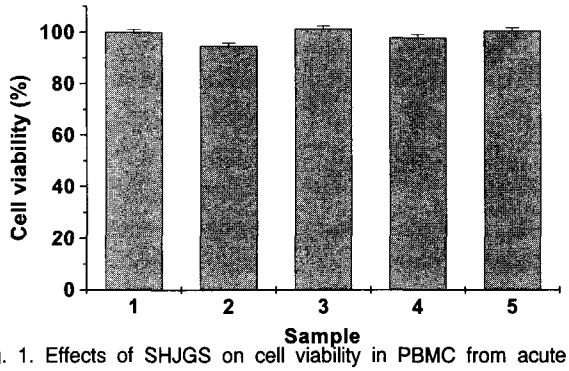

Fig. 1. Effects of SHJGS on cell viability in PBMC from acute CI patients. Cell viability was evaluated by MTT colorimetric assay for 24 hr incubation after stimulation of LPS (10 ng/㎖) plus PHA (25 ng/㎖) in the absence or presence of SHJGS (0.01-1 ㎎/㎖). The percentage of viable cells was 94.8% and SHJGS did not affect cell viability in each condition and had no toxicity on PBMC. Data represent the mean ± SEM of five independent experiments in each sample. 1. blank, 2. LPS+PHA alone, 3. LPS+PHA plus 0.01 ㎎/㎖ SHJGS, 4. LPS+PHA plus 0.1 ㎎/㎖ SHJGS, 5. LPS+PHA plus 1 ㎎/㎖ SHJGS.

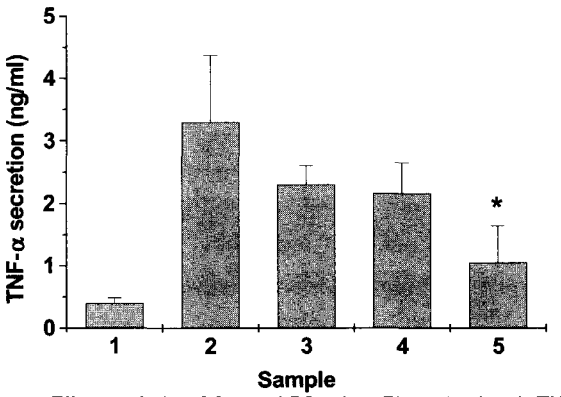

Fig. 2. Effects of SHJGS on LPS plus PHA-stimulated TNF-α production in PBMC from acute CI patients. Amount of TNF-α was measured by ELISA. PBMC (3×10^5 cells) were cultured and stimulated with LPS (10 ng/㎖) plus PHA (25 ng/㎖) for 24 hr in the absence or presence of SHJGS (0.01-1 ㎎/㎖) for 30 min prior to stimulation. Data represent the mean ± SEM of five independent experiments in each sample. *P < 0.05: significantly different from the control value. 1. blank, 2. LPS+PHA alone, 3. LPS+PHA plus 0.01 ㎎/㎖ SHJGS, 4. LPS+PHA plus 0.1 ㎎/㎖ SHJGS, 5. LPS+PHA plus 1 ㎎/㎖ SHJGS.

4. Effects of SHJGS on LPS plus PHA induced IL-6 production

The author tested inhibitory effects of SHJGS on IL-6

production in LPS plus PHA-stimulated PBMC from acute CI patients. Pre-treatment of SHJGS for 30 min inhibited the IL-6 production by 11.36 ± 1.11% (0.01 ㎎/㎖ of SHJGS), 26.19 ± 0.37% (0.1 ㎎/㎖ of SHJGS) and 45.4 ± 0.76% (1 ㎎/㎖ of SHJGS), compared with value of no treatment of SHJGS. SHJGS inhibited the IL-6 production in LPS plus PHA-stimulated PBMC in a concentration-dependent manner although SHJGS treated with 0.01 and 0.1 ㎎/㎖ had no statistically significance.

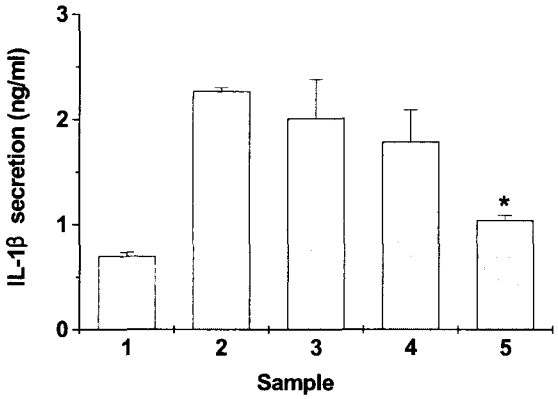

Fig. 3. Effects of SHJGS on LPS plus PHA-stimulated IL-1β production in PBMC from acute CI patients. Amount of IL-1β was measured by ELISA. PBMC (3×10^5 cells) were cultured and stimulated with LPS (10 ng/㎖) plus PHA (25 ng/㎖) for 24 hr in the absence or presence of SHJGS (0.01-1 ㎎/㎖) for 30 min prior to stimulation. Data represent the mean ± SEM of five independent experiments in each sample. *P < 0.05: significantly different from the control value. 1. blank, 2. LPS+PHA alone, 3. LPS+PHA plus 0.01 ㎎/㎖ SHJGS, 4. LPS+PHA plus 0.1 ㎎/㎖ SHJGS, 5. LPS+PHA plus 1 ㎎/㎖ SHJGS.

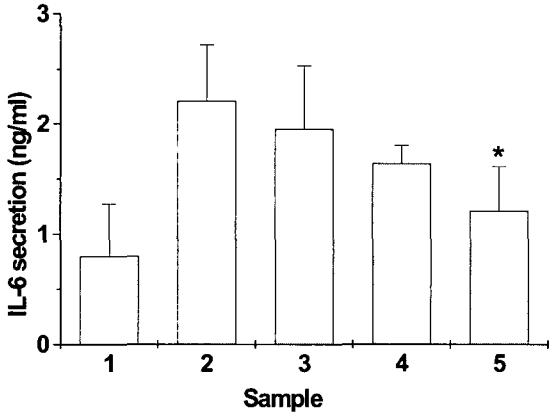

Fig. 4. Effects of SHJGS on LPS plus PHA-stimulated IL-6 production in PBMC from acute CI patients. Amount of IL-6 was measured by ELISA. PBMC (3×10^5 cells) were cultured and stimulated with LPS (10 ng/㎖) plus PHA (25 ng/㎖) for 24 hr in the absence or presence of SHJGS (0.01-1 ㎎/㎖) for 30 min prior to stimulation. Data represent the mean ± SEM of five independent experiments in each sample. *P < 0.05: significantly different from the control value. 1. blank, 2. LPS+PHA alone, 3. LPS+PHA plus 0.01 ㎎/㎖ SHJGS, 4. LPS+PHA plus 0.1 ㎎/㎖ SHJGS, 5. LPS+PHA plus 1 ㎎/㎖ SHJGS.

5. Effects of SHJGS on LPS plus PHA induced IL-8 production

The author tested inhibitory effects of SHJGS on IL-8 production in LPS plus PHA-stimulated PBMC from acute CI patients. Pre-treatment of SHJGS for 30 min inhibited the IL-8 production by 23.48 ± 0.622% (0.01 ㎎/㎖ of SHJGS), 31.92 ± 0.4% (0.1 ㎎/㎖ of SHJGS) and 46.70 ± 0.37% (1 ㎎/㎖ of SHJGS), compared with value of no treatment of SHJGS. SHJGS inhibited the IL-8 production in LPS plus PHA-stimulated PBMC in a concentration-dependent manner although SHJGS had no statistically significance.

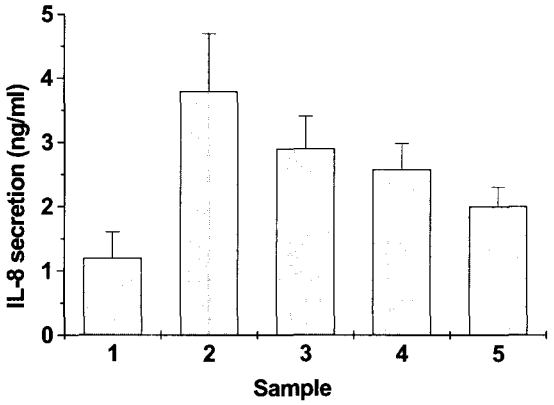

Fig. 5. Effects of SHJGS on LPS plus PHA-stimulated IL-8 production in PBMC from acute CI patients. Amount of IL-8 was measured by ELISA. PBMC (3×10^5 cells) were cultured and stimulated with LPS (10 ng/㎖) plus PHA (25 ng/㎖) for 24 hr in the absence or presence of SHJGS (0.01-1 ㎎/㎖) for 30 min prior to stimulation. Data represent the mean ± SEM of five independent experiments in each sample. 1. blank, 2. LPS+PHA alone, 3. LPS+PHA plus 0.01 ㎎/㎖ SHJGS, 4. LPS+PHA plus 0.1 ㎎/㎖ SHJGS, 5. LPS+PHA plus 1 ㎎/㎖ SHJGS.

6. Comparison of serum TNF-α and IL-6 levels of healthy control group and acute CI patients group

Finally, to study whether the level of serum cytokines are changed between healthy control group and acute CI patients group, their levels were analyzed by ELISA method. Serum TNF-α and IL-6 levels of acute CI patients group (1.344 ± 1.4 ng/㎖, 2.150 ± 1.4 ng/㎖) were higher than those of healthy control group (0.023 ± 0.05 ng/㎖, 0.068 ± 0.06 ng/㎖) (Table 1).

Table 1. Comparison of serum TNF-α and IL-6 levels of healthy control group and acute CI patients group.

	Healthy control group	Acute CI patients group
TNF-α (ng/㎖)	0.023 ± 0.05	1.344 ± 1.4
IL-6 (ng/㎖)	0.068 ± 0.06	2.150 ± 1.4

Data represent the mean ± SEM of five independent experiments in each sample.

Discussion

The interrelations between pro-inflammatory, pro-adhesive, pro-accumulative, chemotactic and pro-thrombotic/vasoactive factors play a crucial role in the development of cerebrovascular changes leading to CI. Nevertheless, the pathophysiological

mechanisms which cause the enhancement of ischemic brain damage immediately after CI onset are not fully understood.

SHJGS is the Korean tradition medicine, which has been successfully used for the treatment of acute CI but its mechanism of action still remains unknown. This study demonstrated that SHJGS inhibited the production of cytokines, TNF-α, IL-1β, IL-6 and IL-8 in LPS and PHA-stimulated PBMC from acute CI patients. These results suggest SHJGS might have effective pharmacological activities and abilities of the regulation of inflammatory responses by cytokine modulation in acute CI.

LPS and PHA can initiate cell activation and produce cytokines including interferons, TNF-α, IL-1β, IL-6, IL-8. These cytokines are involved in inflammation and exert pathophysiological effects. Most cerebrovascular disease is also related to atherosclerosis of cerebral arteries. Furthermore, the common and major pathological changes in CI are atherosclerosis and thrombogenesis in cerebral arteries. Recent attention has focused on the inflammatory component of CI[20,21]. Indeed, atherosclerosis is now described as an inflammatory disease[20] and flared plaque inflammation is considered as a cause of intimal erosion and rupture[21]. There is increasing evidence that the inflammatory response plays an important role in the potentiation of CNS ischemic injury[22]. Much of this inflammatory response appears to be mediated by ILs, a multifunctional subclass of cytokines[23]. The pro-inflammatory interleukins, including TNF-α, IL-1β and IL-6, can influence the function and synthesis of other cytokines by a complex cytokine network[23]. These pro-inflammatory interleukins are produced by a variety of cells, including microglial cells, astrocytes and leukocytes. They appear to directly modulate CNS cell apoptosis, differentiation and proliferation. Cytokines may also be involved in the activation and recruitment of leukocytes in CNS. TNF-α, IL-1β and IL-6 have been shown to activate leukocytes and increase the expression of adhesion receptors on leukocytes (CD-18), endothelial cells and astrocytes (intercellular adhesion molecule-1). Several investigators have characterized the role of TNF-α and IL-1β in experimental CNS ischemia and have found a therapeutic benefit of IL-1 receptor antagonist treatment[24-29]. Among the mediators involved in hemostatic and immunological unbalance leading to enlargement of ischemic brain damage, the release of pro-inflammatory cytokines, especially TNF-α is emphasised. Rapid increase of TNF-α levels (as soon as a few hours after middle cerebral artery occlusion) within and surrounding the focus of damaged brain in experimental animal models of cerebral ischemia has been observed. Early expression of TNF-α in ischemic neurons precedes and appears to facilitate the influx of leukocytes to the ischemic area that can exacerbate ischemic brain damage. The invasion of the inflammatory cells induced by TNF-α may deteriorate the course of brain infarction formation by several mechanisms, including edema creation and thrombotic proclivity in the surrounding blood vessels. TNF-α is known to trigger a pro-inflammatory/pro-thrombotic reaction that is produced mainly by activated mononuclear leukocytes. IL-1β is a pro-inflammatory cytokine that has been identified as an important mediator of neurodegeneration induced by experimental cerebral ischemia or excitatory or traumatic brain injury in rodents. IL-1β is rapidly produced in the brain of rodents exposed to CI and enhances ischemia and other forms of injury. IL-6 is involved in modulating the acute expression of other pro-inflammatory cytokines in the brain after ischemia. IL-6 is a multifunctional regulator of immune and inflammatory processes that has a range of biologic activities, including important roles in the development of plasma cells and stimulation of the production of acute phase response protein by hepatocytes. IL-8, a major chemokine known to attract and activate leukocytes, has recently been under focused investigation because of its possible participation in the evolution of CI. Brain cells produce chemokines during the inflammatory process after CI both in animal models and in patients.

In this study, the author confirmed that SHJGS inhibited the TNF-α, IL-1β, IL-6 and IL-8 production in a concentration-dependent manner in LPS and PHA-stimulated PBMC from acute CI patients. These results suggest that SHJGS might have beneficial effects in the treatment of acute CI. SHJGS may play a critical role in cytokine modulation but it needs to be studied whether SHJGS can regulate the other cytokines including IL-4 and IL-10 in acute CI patients.

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