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Original Article

The Effects of *Dohongsamul-tang* on Cytokine Production in Peripheral Blood Monocular Cells of Patients with Acute Cerebral Infarction

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Objectives : We investigated the effect of *Dohongsamul-tang* (DHSMT) on the production of various cytokines in lipopolysaccaride (LPS) stimulated peripheral mononuclear cells (PBMCs) from CI patients.
Methods: Cell viability was determined using MTT assay. ELISA was carried out for investigating TNF-α , IL-1β , IL-6, IL-8, IL-4, and TGF-β 1

Results : The amount of tumor necrosis factor (TNF)-α , interleukin (IL)-1β , IL-6, IL-8, IL-4, and transforming growth factor (TGF)-β 1 in PBMC culture supernatant significantly increased in the LPS treated cells compared to unstimulated cells. We show that DHSMT inhibited the production of TNF-α , IL-1β IL-6, IL-8, and IL-4 induced by LPS in a dose dependent manner. The maximal inhibition rate of the TNF-α , IL-1β , IL-6, IL-8, and IL-4 production by pretreatment of DHSMT (1.0 mg/ml) was 38.52 ± 2.5% ($P < 0.01$), 44.02 ± 3.5% ($P < 0.05$), 45.32 ± 2.3% ($P < 0.01$), 42.30 ± 3.1% ($P < 0.05$), and 49.70 ± 3.1% ($P < 0.05$), respectively. On the other hand, DHSMT significantly increased the LPS-induced TGF-β 1 production ($P < 0.05$).

Conclusions : Taken together, these results suggest that DHSMT might have regulatory effects on cytokine production, which might explain its beneficial effect in the treatment of CI.

***Key Words*:** cytokines, *Dohongsamul-tang*, cerebral infarction

Introduction

Dohongsamul-tang (DHSMT)[1,2], a traditional Korean medicine, has long been used as a specific prescription for cerebral infarction (CI) to increase cerebral blood flow and to restore injured brain cells, but its pharmacological mechanisms have not yet been well defined.

Recently, it has become increasingly evident that the inflammatory response plays an important role in the pathogenesis of CI. Much of this inflammatory response appears to be mediated by proinflammatory cytokines[3,5].

Many investigators have studied the relationship between cytokines and cerebral infarction. Some have evaluated the effect of Korean herbal medicines by cytokine production[35,37].

In this study, the author attempted to study the effect of DHSMT on tumor necrosis factor (TNF)-α , interleukin (IL)-1β , interleukin (IL)-6, interleukin (IL)-8, interleukin (IL)-4, and transforming growth factor (TGF)-β 1 production in lipopolysaccaride

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(LPS) stimulated peripheral blood mononuclear cells (PBMCs) from CI patients.

Materials and methods

1. Reagents

Ficoll-Hypaque, LPS, avidin-peroxidase, and 2-AZINO-bis (3-ethylbenzithiazoline-6-sulfonic acid) tablets substrate (ABTS) were purchased from Sigma (St. Louis, MO). RPMI 1640, penicillin G, streptomycin, and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). Anti-human TNF-α, IL-1β, and IL-4 Ab, biotinylated anti-human TNF-α, IL-1β, and IL-4 and recombinant human TNF-α, IL-1β, and IL-4 were purchased from R&D Systems (Minneapolis, MN). Anti-human IL-6, and IL-8, and TGF-β 1, biotinylated anti-human IL-6, IL-8, and TGF-β 1 and recombinant (r) human IL-6, IL-8, and TGF-β 1 were purchased from Pharmingen (San Diego, CA).

2. Patients with CI

Patients with acute cerebral infarction within hours from onset were examined at the Department of Neurology, Wonkwang University School of Medicine from July to October 2004. The diagnosis of CI was confirmed with computerized tomography (CT) and magnetic resonance imaging and clinical signs (hemiparesis, slurred speech, facial palsy, etc.). For cytokine assay, blood was obtained from 12 patients (5 males and 7 females, age range 55-70 years) with CI.

3. Preparation of DHSMT

DHSMT, which is a mixture of 6 traditional drugs as shown in Table 1, was obtained from the College of Oriental Medicine, Wonkwang University (Iksan, South Korea). Extract of DHSMT was prepared by decocting the dried prescription of herbs with boiling distilled water (72 g/L). The duration of decoction was about 3 h. The decoction was filtered, lyophilized and kept at 4°C. The yield of extraction was about 10.1% (w/w).

4. Isolation and Culture of PBMCs

PBMCs of patients with CI from heparinized venous blood were isolated by Ficoll-gradient centrifugation, washed three times in phosphate-buffered saline (PBS) solution and resuspended in RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamin, 100 U/ml penicillin G, 100 $\mu\ell$/ml streptomycin, and 10% FBS inactivated for 30 min at 56℃. PBMCs were adjusted to a concentration of 3 ×106 cells/ml in 30 ml falcon tube, and 100 $\mu\ell$ aliquots of cell suspension were placed in a four-well cell culture plate. PBMCs were cultured for 24 h in 95% humidified air containing 5% CO_2 (37°C), in the presence or

Table 1. The ratio of the component in DHSMT

Herbal name	Species		Dosage (g)
Persicae semen	*Prunus persica Batsch*	(桃仁)	16
Angelis gigantis radix	*Angelis gigas Nakai*	(當歸)	16
Rehmanniae radix	*Rehmannia glutinosa Libschitz*	(生地黃)	16
Paeoniae radix rubra	*Paeonia lactiflora var*	(赤芍藥)	8
Cnidii rhizoma	*Cnidium officinale Makino*	(川芎)	8
Carthami flos	*Carthamus tinctorius Linne*	(紅花)	8
Total amount			72

the absence of LPS, and the supernatants were collected by centrifugation and stored at -20°C. Blood samples collected from a single CI patient are not enough to get PBMC for the experiment; for this reason, blood samples from 12 patients were added together. The given data do not show the change of cytokine level in each patient.

5. MTT Assay

Cell viability was determined using MTT assay. Briefly, 500 ㎕ of PBMCs suspension (3 ×105 cells) was cultured in 4-well plates for 24 h after treatment by each concentration of DHSMT. 50 ㎕ of MTT solution (5 mg/ml) was added and then cells were incubated for 4 h at 37 ° C. After washing the supernatant out, the insoluble formazan product was dissolved in DMSO. Then, optical density of 96-well culture plates was measured using enzyme-linked immunosorbent assay (ELISA) reader at 540 nm. The optical density of formazan formed in untreated control cells was taken as 100% of viability.

6. Cytokines Assay

ELISA for TNF-α , IL-1β , IL-6, IL-8, IL-4, and TGF-β 1 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, IL-8, IL-4, and TGF-β 1 monoclonal antibodies at 1.0 μ g /ml in PBS at pH 7.4 and was incubated overnight at 4°C. The plates were washed in PBS containing 0.05% Tween 20 (Sigma) and blocked with PBS containing 1% BSA, 5% sucrose and 0.05% NaN3 for 1 h. After additional washes, sample or TNF-α , IL-1β , IL-6, IL-8, IL-4, and TGF-β 1 standards were added and incubated at 37°C for 2 h. After 2 h incubation at 37°C, the wells were washed and then each of 0.2 μ g/ml of biotinylated anti-human TNF-α , IL-1β , IL-6, IL-8, IL-4, and TGF-β 1 were added and again incubated at 37°C for 2 h. After washing the wells, avidin-peroxidase was added and plates were incubated for 20 min at 37°C. Wells were again washed and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader.

7. Statistical Analysis

Each datum represents the mean ± SEM of the different experiments under the same conditions. Student's t-test was used to make a statistical comparison between the groups. Results with $P < 0.05$ were considered statistically significant.

Results

1. Effect of DHSMT on the cell viability.

The author first examined the effect of DHSMT on the viability of PBMC using MTT assay. Cells were treated with various concentrations of DHSMT (0.01-1.0 mg/ml) for 30 min and then stimulated with LPS for 24 h. In the cells treated with LPS, cell viability decreased to 95.8 ± 4.2% compared with the control value (100.0 ± 3.2%). However, DHSMT (0.01-1.0 mg/ml) did not affect cell viability in each condition and had no toxicity on PBMC from CI patients (Fig. 1).

2. Effects of DHSMT in LPS-induced TNF-α production

To evaluate the regulatory effect of DHSMT on the TNF-α production, PBMCs were pretreated with DHSMT for 30 min and then treated LPS for 24 h. The supernatant was analyzed by ELISA method for TNF-α . The author observed that TNF-α increased by LPS was inhibited by DHSMT in a dose-dependent manner (about 14.0 ± 3.9%

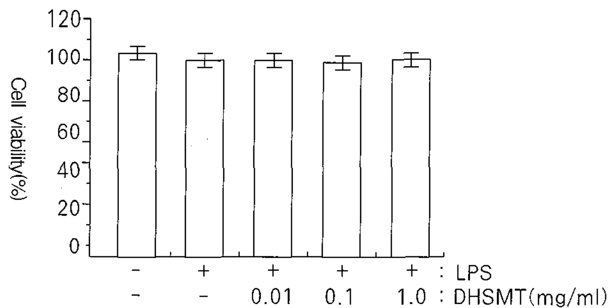

Fig. 1. Effect of DHSMT on cell viability in PBMC from CI patients.
Cell viability was evaluated by MTT colorimetric assay. Cells were pretreated with DHSMT (0.01 - 1.0 mg/ml) for 30 min, then treated with LPS (1 µg/ml) for 24 h. The percentage of viable cells was over 95%. Data represent mean ± SEM of independent six-time experiments.

at 0.01 mg/ml DHSMT, P >0.05; 29.6 ± 4.1% at 0.1 mg/ml DHSMT, P <0.05; 38.5 ± 2.5% at 1.0 mg/ml DHSMT, P <0.05) (Fig. 2).

3. Effects of DHSMT in LPS-induced IL-6 production

To evaluate the regulatory effect of DHSMT on the IL-6 production, PBMCs were pretreated with DHSMT for 30 min and then treated LPS for 24 h. The supernatant was analyzed by ELISA method for IL-6. As shown in Fig 3, IL-6 production was synergistically enhanced with stimulation of LPS. IL-6 production in response to LPS was inhibited by pre-treatment with 0.01 - 1.0 mg/ml DHSMT in a dose-dependent manner (about 14.9 ± 4.9% at 0.01 mg/ml DHSMT, P >0.05; 34.8 ± 3.1% at 0.1 mg/ml DHSMT, P <0.05; 45.3 ± 3.3% at 1.0 mg/ml DHSMT, P <0.05). (Fig. 3)

4. Effects of DHSMT in LPS-induced IL-1β production

To determine whether DHSMT can modulate LPS-induced IL-1β production, the cells were

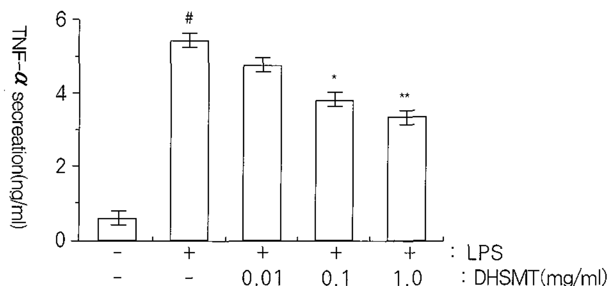

Fig. 2. Effects of DHSMT in LPS-induced TNF-α production in PBMC from CI patients.
3×105 PBMCs were pretreated with DHSMT (0.01-1.0 mg/ml) for 30 min, then stimulated with LPS (1 µg/ml) for 24 h. TNF-α concentration was measured in cell supernatants using the ELISA method. All data represent the mean ± SEM of independent four-time experiments.
# P < 0.05, significantly different from the unstimulated cells.
* P < 0.05, significantly different from the LPS -stimulated cells (non-treated with DHSMT).

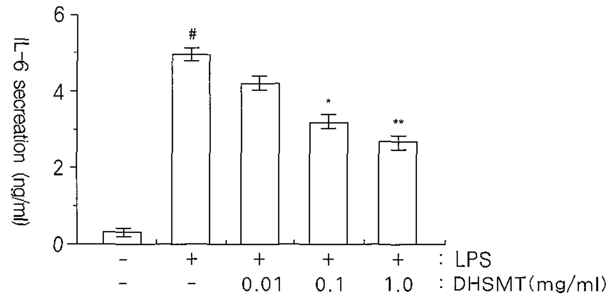

Fig. 3. Effects of DHSMT in LPS-induced IL-6 production in PBMC from CI patients.
3×105 PBMCs were pretreated with DHSMT (0.01-1.0 mg/ml) for 30 min, then stimulated with LPS (1 μg/ml) for 24 h. IL-6 concentration was measured in cell supernatants using the ELISA method. All data represent the mean ± SEM of independent four-time experiments.
# P < 0.05, significantly different from the unstimulated cells.
* P < 0.05, significantly different from the LPS-stimulated cells (non-treated with DHSMT).

pretreated with various concentrations of DHSMT for 30 min prior to LPS for 24 h. The supernatant was analyzed by ELISA method for IL-1β . The author confirmed that IL-1β increased by LPS was inhibited by DHSMT in a dose-dependent manner (about 14.3 ± 5.2% at 0.01 mg/ml DHSMT, P >0.05; 20.6 ± 4.7% at 0.1 mg/ml DHSMT, P <0.05; 44.02 ± 3.5% at 1.0 mg/ml DHSMT, P <0.05). (Fig. 4)

5. Effects of DHSMT in LPS-induced IL-8 production

The author examined the effect of DHSMT on LPS-induced IL-8 production from PBMC of CI patients. The cells were pretreated with various concentrations of DHSMT for 30 min prior to LPS for 24 h. The supernatant was analyzed by ELISA method for IL-8. The author showed that IL-8 increased by LPS was inhibited by DHSMT

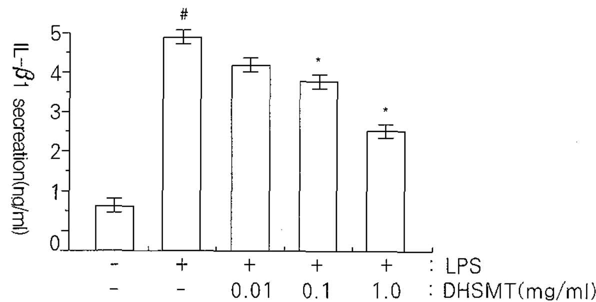

Fig. 4. Effects of DHSMT on LPS-induced IL-1β production in PBMC from CI patients.
3×105 PBMCs were pretreated with DHSMT (0.01-1.0 mg/ml) for 30 min, then stimulated with LPS (1 μg/ml) for 24 h. IL-1β concentration was measured in cell supernatants using the ELISA method. All data represent the mean ± SEM of independent four-time experiments.
# P < 0.05, significantly different from the unstimulated cells.
* P < 0.05, significantly different from the LPS-stimulated cells (non-treated with DHSMT).

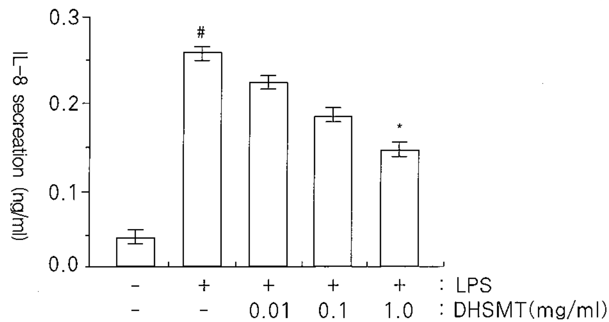

Fig. 5. Effects of DHSMT on LPS-induced IL-8 production in PBMC from CI patients.
3×105 PBMCs were pretreated with DHSMT (0.01-1.0 mg/ml) for 30 min, then stimulated with LPS (1 μg/ml) for 24 h. IL-8 concentration was measured in cell supernatants using the ELISA method. All data represent the mean ± SEM of independent four-time experiments.
# $P < 0.05$, significantly different from the unstimulated cells.
* $P < 0.05$, significantly different from the LPS-stimulated cells (non-treated with DHSMT).

in a dose-dependent manner (about 11.50 ± 6.1% at 0.01 mg/ml DHSMT, $P > 0.05$; 26.6 ± 4.9% at 0.1 mg/ml DHSMT, $P > 0.05$; 42.30 ± 3.1% at 1.0 mg/ml DHSMT, $P < 0.05$) (Fig. 5).

6. Effects of DHSMT in LPS-induced IL-4 production

The author examined the effect of DHSMT on LPS-induced IL-4 production from PBMC of CI patients. The cells were pretreated with various concentrations of DHSMT for 30 min prior to LPS for 24 h. The supernatant was analyzed by ELISA method for IL-4. The author showed that IL-4 increased by LPS was inhibited by DHSMT in a dose-dependent manner (about 15.5 ± 4.2% at 0.01 mg/ml DHSMT, $P > 0.05$; 23.4 ± 4.9% at 0.1 mg/ml DHSMT, $P > 0.05$; 49.7 ± 3.1% at 1.0 mg/ml DHSMT, $P < 0.05$) (Fig. 6)

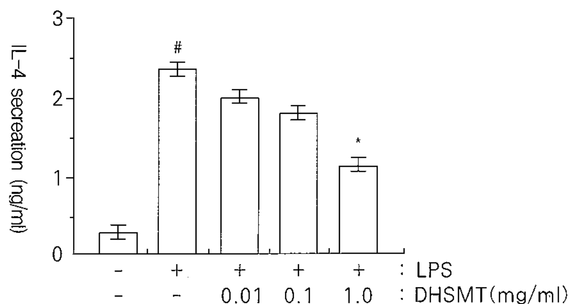

Fig. 6. Effects of DHSMT in LPS-induced IL-4 production in PBMC from CI patients.
3×105 PBMCs were pretreated with DHSMT (0.01-1.0 mg/ml) for 30 min, then stimulated with LPS (1 μg/ml) for 24 h. IL-4 concentration was measured in cell supernatants using the ELISA method. All data represent the mean ± SEM of independent four-time experiments.
# $P < 0.05$, significantly different from the unstimulated cells.
* $P < 0.05$, significantly different from the LPS-stimulated cells (non-treated with DHSMT).

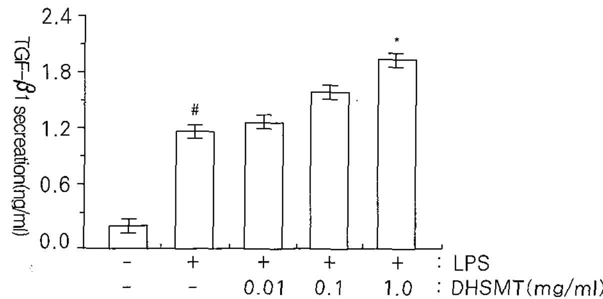

Fig. 7. Effects of DHSMT in LPS-induced TGF-β1 production in PBMC from CI patients.
3×105 PBMCs were pretreated with DHSMT (0.01-1.0 mg/ml) for 30 min, then stimulated with LPS (1 μg/ml) for 24 h. TGF-β1 concentration was measured in cell supernatants using the ELISA method. All data represent the mean ± SEM of independent four-time experiments.
# $P < 0.05$, significantly different from the unstimulated cells.
* $P < 0.05$, significantly different from the LPS-stimulated cells (non-treated with DHSMT).

7. Effects of DHSMT in LPS-induced TGF-β 1 production

The author examined the effect of DHSMT on LPS-induced TGF-β 1 production from PBMC of CI patients. The cells were pretreated with various concentrations of DHSMT for 30 min prior to LPS for 24 h. The supernatant was analyzed by ELISA method for TGF-β 1. As shown in Fig 7, the amount of TGF-β 1 was significantly higher in the DHSMT (1 mg/ml) plus LPS-treated cells than LPS-treated cells (by 1.63 ± 2.1-fold increase at 1.0 mg/ml DHSMT, P <0.05). (Fig. 7)

Discussion

Cytokines in stroke patients have been extensively studied during recent years. There are early inflammatory responses as indicated by up-regulation of pro-inflammatory cytokines in brain autopsies after acute stroke[21]. TNF-α is known to trigger a proinflammatory reaction that is produced mainly by activated mononuclear leukocytes. IL-1β is produced rapidly in the brains of rodents subjected to CI, and enhances ischaemic and other forms of injury. Several investigators characterized the role of TNF-α and IL-1β in experimental ischemia and found a therapeutic benefit of IL-1 receptor antagonist treatment[22,23]. IL-6 is involved in modulating the acute expression of other proinflammatory cytokines in the brain after ischemia. These cytokines are involved in inflammation, and exerted pathophysiological effects. In this study, the author confirmed that DHSMT inhibited TNF-α , IL-1β , and IL-6 production in LPS-stimulated PBMC from CI patients. These results suggested that DHSMT has potential effect on anti-inflammatory response through the regulation of proinflammatory cytokines production.

With proinflammatory cytokine involved in hemostatic and immunological imbalance leading to enlargement of brain damage, the release of especially TNF-α is emphasized[6,7]. TNF-α is a major inflammatory cytokine because it stimulates the synthesis of nitric oxide and other inflammatory mediator that derives a chronically delayed hypersensitivity reaction[8].

IL-1β is a proinflammatory cytokine that has

been identified as an important mediator of neurodegeneration induced by experimental cerebral ischemia (stroke) or excitatory or traumatic brain injury in rodents[9,10]. IL-1β is produced rapidly in the brains of rodents exposed to cerebral ischemia[11].

IL-6, one of the main inflammation-associated cytokines, is produced by a variety of cells in the central nervous system[12]. It was found that in patients with acute ischemic stroke, those with higher levels of IL-6 had more severe neurologic deficits on admission[13]. The spontaneous production of inflammatory cytokines by mononuclear cells and the level of cytokines in serum are significantly increased in patients with CI.

Brain cells produce chemokines during the inflammatory process after stroke both in animal models and patients. IL-8, a major chemokine known to attract and activate leukocytes[24,25] has recently been under focused investigation because of its possible participation in the evolution of CI. The author also showed that IL-8 level increased by LPS was inhibited by DHSMT pre-treatment. These results indicated that the anti-inflammatory effect of DHSMT might be through suppression of chemokine, IL-8 production in PBMC from CI patients. IL-8, as a pivotal mediator of cerebral reperfusion, was increased in brain tissues and a neutralizing anti-IL-8 antibody (Ab) significantly reduced brain edema and infarct size in comparison to rabbits receiving a control antibody[14]. These results implicate that IL-8 is a novel target for the intervention of injury.

IL-4, involved in hemostatic and immunological imbalance, leads to enlargement of various tissue damages. Other research reported that level of IL-4 was elevated in patients with CI during the acute stage[26]. *Yuldahanso-tang*, which has been developed for the purpose of preventing and treating the disease in *tàiyīnrén*, inhibited secretion of IL-4 from human astrocytes stimulated with LPS[27]. In this study, the author showed that DHSMT effectively inhibited the production of IL-4 cytokines in LPS stimulated PBMC cells. These results suggested that DHSMT might have a beneficial effect in the treatment of CI.

IL-4 is a pleiotropic cytokine derived primarily from Th2 lymphocytes and mast cells. Described originally as a B-cell growth factor, IL-4 subsequently has been proved to proliferate T lymphocytes, monocytes, endothelial cells, and fibroblasts[15,17].

TGF-β 1 has been implicated in the pathogenesis of a number of diseases including CI. TGF-β 1 protects neurons against damage[28]. Krupinski[29] reported that TGF-β 1 might be involved in angiogenesis after ischemic stroke in humans. Recent studies indicated that glial cell line- derived neurotrophic factor, a member of the TGF-β 1 superfamily, can protect the cerebral hemispheres from damage induced by middle cerebral arterial ligation[30]. In addition, TGF-β 1 is an important regulatory cytokine involved in tissues repair whose sustained production in many tissues underlies the development of fibrosis[31]. In this study, the author showed that TGF-β 1 was increased by treatment of DHSMT. Therefore, the author can speculate that DHSMT- induced TGF-β 1 expression may contribute to repairing processes, enduring cerebral infarction.

TGF-β 1 is expressed as large pro-protein (390-412 amino acid), which includes TGF-β 1 (25 kDa) at its C-terminus, and TGF-β 1 latency-associated peptide (LAP) at its N-terminus[18]. TGF-β 1 controls many diverse events such as development, differentiation, tissue repair, tumorigenesis, and many immune and endocrine functions[19]. TGF-β 1 proteins are secreted by most mammalian cells and their action is locally mediated

through an autocrine/paracrine fashion[20].

DHSMT is an oriental medication hot water-extracted from herb medicines, which consists of 6 different herbs. Other studies reported that each medicinal herb has a different effect. For example, *Rehmannia glutinosa* has an anti-inflammatory activity through inhibition TNF-α in astrocytes[32], *Paeonia lactiflora* has anti-inflammatory and cancer chemopreventive agents [33], and *Cnidium officinale* has an anti-neurotoxic effect through suppression nitric oxide production, so is a potential inhibitor in NO-mediated neuronal death[34]. DHSMT is composed on the basis of the theory of Korean medicine to maximize its efficacy.

In addition, Kwon discovered that *Dohongsamul-tang* reduced mesangial cell proliferation and fibronectin synthesis significantly. Shin revealed that DHSMT restrains the thrombus formation[38,39]

In conclusion, the author demonstrated that DHSMT inhibited proinflammatory cytokine production and increased anti-inflammatory cytokine production. These results may imply a positive CI treatment effect of DHSMT, and that it may be due to regulation of cytokine production.

Conclusion

1. In the cells treated with LPS, cell viability decreased to 95.8 ± 4.2% compared with the control value. However, DHSMT did not affect cell viability in each condition and had no toxicity on PBMC from CI patients.

2. Increased by LPS, TNF-α was inhibited by DHSMT in a dose-dependent manner.

3. IL-6 production in response to LPS was inhibited by pre-treatment with 0.01 - 1 mg/ml DHSMT in a dose-dependent manner.

4. IL-1β increased by LPS was inhibited by DHSMT in a dose-dependent manner.

5. IL-8 increased by LPS was inhibited by DHSMT in a dose-dependent manner.

6. IL-4 increased by LPS was inhibited by DHSMT in a dose-dependent manner.

7. The amount of TGF-β 1 was significantly higher in the DHSMT plus LPS-treated cells than LPS-treated cells.

In summary, the results suggest that DHSMT may have components for treatment of CI and that its effect may be due to regulation of cytokine production.

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