Regulatory Effect of Gigukjihwangtanggami on Cytokine Production in Patients with Cerebral Infarction

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The Korean genuine medicine, "Gigukjihwangtanggami (GJT)" has long been used clinically for hypertension and various cerebrovascular diseases. However, experimental study has been carried out very little. Recently cytokines involved in the regulation of inflammatory reactions and immune responses may play an important role in the pathogenesis of cerebral infarction (CI). The aim of this study is to investigate the effect of GJT on the production of pro-inflammatory cytokines and anti-inflammatory cytokines in peripheral blood mononuclear cells (PBMCs) stimulated with lipopolysaccaride (LPS) from CI patients. The amount of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-8, IL-4 and IL-10 in PBMC culture supernatant was significantly increased in the LPS treated cells compared to unstimulated cells. GJT inhibited the production of TNF-α, IL-1β, IL-6 and IL-8 induced by LPS. The maximal inhibition rate of the TNF-α, IL-1β, IL-6 and IL-8 production by pretreatment of GJT (1 mg/ml) was 57.32 ± 2.5% ($P<0.05$), 42.02 ± 3.5% ($P<0.05$), 40.02 ± 2.3% ($P<0.05$) and 48.02 ± 3.1% ($P<0.05$), respectively. In the other hand, GJT increased the production of IL-4 and IL-10. The maximal increase rate of the IL-4 and IL-10 production by pretreatment of GJT (1 mg/ml) was 42.4 ± 3.3% ($P<0.05$) and 56.4 ± 2.9% ($P<0.05$), respectively. Taken together, these results indicate that GJT may have regulatory effects on the cytokine production and suggest that GJT might use clinically for the treatment of CI.

Key words : Gigukjihwangtanggami, cerebral infarction, cytokine, regulatory effect

Introduction

Gigukjihwangtanggami (GJT), a prescription of traditional Korean medicine, has long been used as a specific prescription for cerebral infarction (CI) to increase cerebral blood flow and to recover an injured brain cell. It was first mentioned in Uigeup(醫級) written by Dong(董)[1] and it was made by adding Lycii Fructus and Flos Chrysanthemi to Yukmijihwangtang of Jeon(錢)[2] in order to increase the function of tonifying kidney and draining fire, so it has been used for treating liver-kidney yin deficiency inducing dizzness, tinnitus, dim eyesight, dryness of mouth, dry throat, the low back and knees aching and limping, redness and pain of the eye and hypertension[1-3]. But the pharmacological mechanisms of them have not been well defined yet except the study of An's reducing blood pressure[4] and Zhang's antioxidative effects of Gigukjihwangtang in neuronal cells[3].

Recently, it has become increasingly evident that the inflammatory response plays an important role in the pathogenesis of CI. Much of this inflammatory response appear to be mediated by pro-inflammatory cytokines[5-7]. Pro-inflammatory cytokines are involved in hemostatic and immunological imbalance leading to the enlargement of brain damage. Especially the release of tumor necrosis factor-α (TNF-α) is emphasized[8]. TNF-α is a major inflammatory cytokine because it stimulates the synthesis of nitric oxide and other inflammatory mediator that derives chronically delayed hypersensitive reaction[9].

Interleukin-1β (IL-1β) is a pro-inflammatory 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[10,11]. Other research reported that IL-1β is produced rapidly in the brains of rodents exposed to cerebral ischemia[12-14].

IL-6, which is one of the main inflammation-associated cytokine, is produced by a variety of cells in the central nervous system (CNS)[15]. It was found that in patients with

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acute ischemic stroke, those with higher levels of IL-6 had more severe neurologic deficits on admission[16]. The spontaneous production of inflammatory cytokines by mononuclear cells and the level of cytokines in serum are significantly increased in patients with CI.

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 Ab. These results implicate that IL-8 is a novel target for the intervention of injury[17].

Inflammatory cytokine, interleukin-4 (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 in T lymphocytes, monocytes, endothelial cells, and fibroblasts[18-20].

IL-10 is not a typical T-cell derived cytokine, but it is expressed in T lymphocytes (Th1 and Th2), monocytes and eosinophils. IL-10 has been identified as an anti-inflammatory molecule that can suppress the production of variety of pro-inflammatory molecules including TNF-α, IL-1β, and IL-8[21,22]. Administration of IL-10 protects tissue exposed to a variety of ischemia reperfusion regimens[23].

In this study, the author attempted to study the effect of GJT on TNF-α, IL-1β, IL-6, IL-8, IL-4, and IL-10 production in lipopolysaccaride (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-ethylbenzi thiazoline-6-sulfonic acid) tablets substrate (ABTS) were purchased from Sigma (St. Louis, MO, USA). RPMI 1640, penicillin G, streptomycin and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Anti-human TNF-α, IL-1β, IL-4 Ab and biotinylated anti-human TNF-α, IL-1β, IL-4 and recombinant human TNF-α, IL-1β, IL-4 were purchased from R&D Systems (Minneapolis, MN, USA). Anti-human IL-6, IL-8, IL-10 and biotinylated anti-human IL-6, IL-8, IL-10 and recombinant(r) human IL-6, IL-8 were purchased from Pharmingen (Sandiego, CA , USA).

2. Patients

Patients with CI were examined at the Department of Neurology, Wonkwang University School of Medicine from July 2004 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) with CI.

3. Preparation of GJT

GJT which is a mixture of 13 traditional drugs as shown in Table 1 was obtained from the College of Oriental Medicine, Wonkwang University (Iksan, South Korea). Extract of GJT was prepared by decocting the dried prescription of herbs with boiling distilled water (84 g/l). An extract of GJT was prepared by decocting the dried prescription of herbs with boiling distilled water. The duration of decoction was about 3 hrs. The decoction was filtered, lyophilized and kept at 4°C. The yield of extraction was about 11.8% (w/w). Amounts of the 13 traditional drugs studied in this work were shown in Table 1.

Table 1. The ratio of the component in GJT.

Herbal Name	Species(韓藥名)	Dosage (g)
Radis Rehmanniae	*Rehmannia glutino var. purpurea Makino* (熟地黃)	16
Rhizoma Dioscoreae	*Dioscorea batatas* (山藥)	8
Corni Fructus	*Cornus officinalis Sieb.et Zucc.* (山茱萸)	8
Poria cocos	*Poria cocas Wolf* (白茯苓)	4
Moutan Cortex	*Paeonia suffruticosa Andr.* (牧丹皮)	4
Alismatis Rhizoma	*Alisma orientale (Sam.) Juz.* (澤瀉)	4
Lycii Fructus	*Lycium barbarum L.* (枸杞子)	4
Flos Chrysanthemi	*Chrysanthemum indicum* (甘菊)	4
Crataegi Fructus	*Crataegus cuneata Sieb.et Zucc.* (山楂)	8
Radix Salviae Miltiorrhizae	*Salvia miltiorrhiza* (丹蔘)	8
Uncariae Ramulus cum Uncuis	*Uncari macrophylla Wall.*(釣鉤藤)	8
Gastrodiae Rhizoma	*Gastrodia elata Bl.* (天麻)	4
Ligustri Fructus	*Ligustrum lucidum Aiton* (女貞子)	4
Total amount		84

4. PBMCs Isolation and Culture

PBMCs (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 ㎕/ml streptomycin, and 10% FBS inactivated for 30 min at 56℃. PBMCs were adjusted to a concentration of 3 $\times 10^6$ cells/ml in 30 ml falcon tube, and 100 ㎕ aliquots of cell suspension were placed in a four-well cell culture plate. PBMCs were cultured for 24 hrs in 95% humidified air containing 5% CO_2 (37°C), in the presence or the absence of LPS and the supernatants were collected by centrifugation and stored at -20°C.

5. MTT Assay

Cell viability was determined by using MTT assay. Briefly, 500 ㎕ of PBMCs suspension (3 $\times 10^5$cells) was cultured in 4-well plates for 24 hrs after treatment by each concentration of GJT. 50 ㎕ of MTT solution (5 mg/ml) was added and then

cells were incubated for 4 hrs 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 IL-10 was carried out in duplicate in 96 well ELISA plates (Nunc, Denmark) coated with each of 100 $\mu\ell$ aliquots of anti-human TNF-α, IL-1β, IL-6, IL-8, IL-4 and IL-10 monoclonal antibodies at 1.0 μg/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 hrs. After additional washes, sample or TNF-α, IL-1β, IL-6, IL-8, IL-4, and IL-10 standards were added and incubated at 37°C for 2 hrs. After 2 hrs incubation at 37°C, the wells were washed and then each of 0.2 μg/ml of biotinylated anti-human TNF-α, IL-1β, IL-6, IL-8, IL-4 and IL-10 were added and again incubated at 37°C for 2 hrs. 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. The Students t-test was used to make a statistical comparison between the groups. Results with $P < 0.05$ were considered statistically significant.

Results

1. The effect of GJT on the cell viability

The author first examined the effect of GJT on the viability of PBMC using MTT assay. Cells were treated with various concentrations of GJT (0.01-1 mg/ml) for 30 min and then stimulated with LPS for 24 hrs. In the cells treated with LPS, cell viability slightly decreased to 96.36 ± 4.6% compared with the control value (100.0 ± 5.4%). GJT (0.01-1 mg/ml) did not affect cell viability in each condition and had no toxicity on PBMC from CI patients (Fig. 1).

2. The effect of GJT in LPS-induced TNF-α production

To evaluate the regulatory effect of GJT on the TNF-α production, PBMCs were pretreated with GJT for 30 min and then treated LPS for 24 hrs. The supernatant was analyzed by ELISA method for TNF-α. As shown in Fig. 2, TNF-α production increased by LPS was inhibited by GJT in a dose-dependent manner (about 12.9% at 0.01 mg/ml GJT, $P>0.05$; 40.6% at 0.1 mg/ml GJT, $P<0.05$; 57.32 ± 2.5% at 1 mg/ml GJT, $P<0.05$.

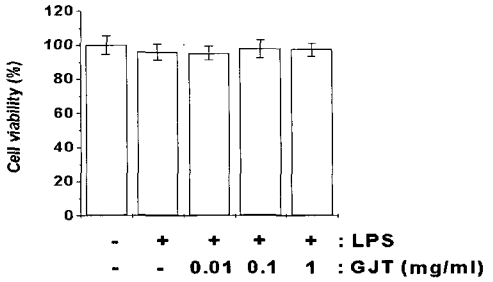

Fig. 1. The effect of GJT on the cell viability in PBMC from CI patients. Cell viability was evaluated by MTT colorimetric assay. Cells were pretreated with GJT (0.01 - 1 mg/ml) for 30 min and then treated with LPS (1 μg/ml) for 24 hrs. The percentage of viable cells was over 96%. Data represent mean ± SEM of six independent experiments.

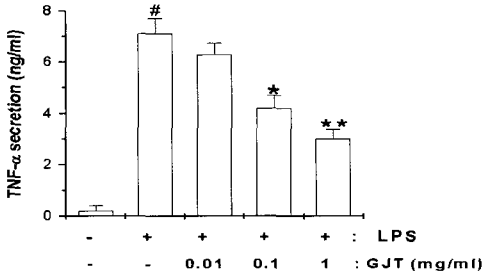

Fig. 2. The effect of GJT in LPS-induced TNF-α production in PBMC from CI patients. 3×10^5 PBMCs were pretreated with GJT (0.01-1 mg/ml) for 30 min and then stimulated with LPS (1μg/ml) for 24 hrs. TNF-α concentration was measured in cell supernatants using the ELISA method. All data represent the mean ± SEM of four independent experiments. #P<0.05, significantly different from the unstimulated cells. *P<0.05, significantly different from the LPS-stimulated cells (non-treated with GJT).

3. The effect of GJT in LPS-induced IL-1β production

To determine whether GJT can modulate LPS-induced IL-1β production, the cells were pretreated with various concentrations of GJT for 30 min prior to LPS for 24 hrs. The supernatant was analyzed by ELISA method for IL-1β. The author showed that IL-1β increased by LPS was inhibited by GJT in a dose-dependent manner (about 11.9% at 0.01 mg/ml GJT, $P>0.05$; 22.6% at 0.1 mg/ml GJT, $P<0.05$; 42.02 ± 3.5% at 1 mg/ml GJT, $P<0.05$) (Fig. 3).

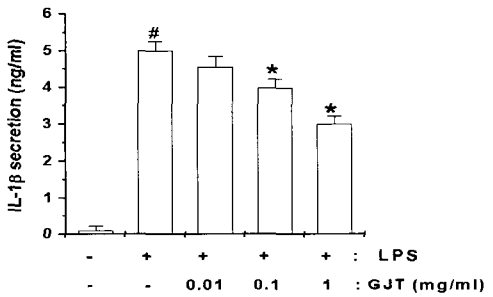

Fig. 3. The effect of GJT on LPS -induced IL-1β production in PBMC from CI patients. 3×10^5 PBMCs were pretreated with GJT (0.01-1 mg/ml) for 30 min and then stimulated with LPS (1 μg/ml) for 24 hrs. IL-1β concentration was measured in cell supernatants using the ELISA method. All data represent the mean ± SEM of four independent experiments. #P<0.05, significantly different from the unstimulated cells. *P<0.05, significantly different from the LPS-stimulated cells (non-treated with GJT).

4. The effect of GJT in LPS-induced IL-6 production

To evaluate the regulatory effect of GJT on the IL-6 production, PBMCs were pretreated with GJT for 30 min and then treated LPS for 24 h. The supernatant was analyzed by ELISA method for IL-6. As shown in Fig 4, IL-6 production was synergistically enhanced with stimulation of LPS. IL-6 production in response to LPS was inhibited by pretreatment with 0.01 - 1 mg/ml GJT in a dose-dependent manner (about 18.9% at 0.01 mg/ml GJT, $P>0.05$; 28.6% at 0.1 mg/ml GJT, $P<0.05$; 40.02 ± 2.3% at 1 mg/ml GJT, $P<0.05$).

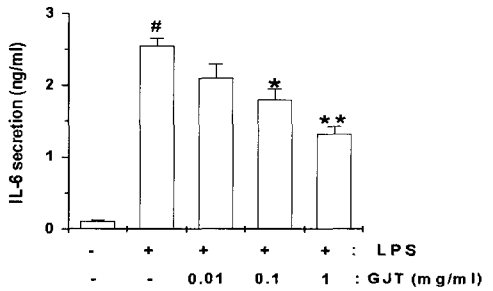

Fig. 4. The effect of GJT in LPS-induced IL-6 production in PBMC from CI patients. 3×10^5 PBMCs were pretreated with GJT (0.01-1 mg/ml) for 30 min and then stimulated with LPS (1 μg/ml) for 24 hrs. IL-6 concentration was measured in cell supernatants using the ELISA method. All data represent the mean ± SEM of four independent experiments. #P<0.05, significantly different from the unstimulated cells. *P<0.05, significantly different from the LPS-stimulated cells (non-treated with GJT).

5. The effect of GJT in LPS-induced IL-8 production

The author examined the effect of GJT on LPS-induced IL-8 production from PBMC of CI patients. The cells were pretreated with various concentrations of GJT for 30 min prior to LPS for 24 hrs. The supernatant was analyzed by ELISA method for IL-8. The author showed that IL-8 increased by LPS was inhibited by GJT in a dose-dependent manner (about 12.19% at 0.01 mg/ml GJT, $P>0.05$; 16.6% at 0.1 mg/ml GJT, $P>0.05$; 48.02 ± 3.1% at 1 mg/ml GJT, $P<0.05$) (Fig. 5).

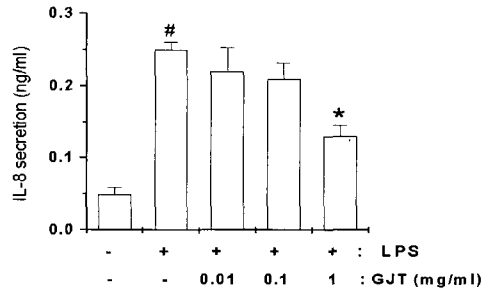

Fig. 5. The effect of GJT on LPS-induced IL-8 production in PBMC from CI patients. 3×10^5 PBMCs were pretreated with GJT (0.01-1 mg/ml) for 30 min and then stimulated with LPS (1μg/ml) for 24 hrs. IL-8 concentration was measured in cell supernatants using the ELISA method. All data represent the mean ± SEM of four independent experiments. #P<0.05, significantly different from the unstimulated cells. *P<0.05, significantly different from the LPS-stimulated cells (non-treated with GJT).

6. The effect of GJT in LPS-induced IL-4 production

The author examined the effect of GJT on LPS-induced IL-4 production from PBMC of CI patients. The cells were pretreated with various concentrations of GJT for 30 min prior to LPS for 24 hrs. The supernatants were analyzed by ELISA method for IL-4. As shown in Fig 6, the amount of IL-4 was higher in the GJT plus LPS-treated cells than LPS-treated cells in a dose-dependent manner (about 6.5% at 0.01 mg/ml GJT, $P<0.05$; 19.6% at 0.1 mg/ml GJT, $P<0.05$; 42.4 ± 3.3% at 1 mg/ml GJT, $P<0.05$).

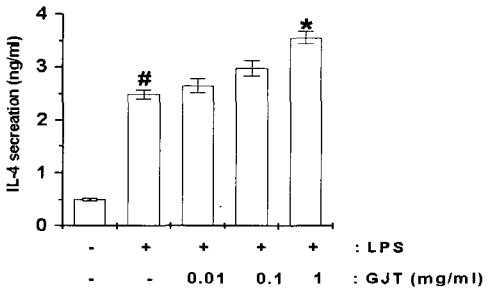

Fig. 6. The effect of GJT in LPS-induced IL-4 production in PBMC from CI patients. 3×10^5 PBMCs were pretreated with GJT (0.01-1 mg/ml) for 30 min and then stimulated with LPS (1 μg/ml) for 24 hrs. IL-4 concentration was measured in cell supernatants using the ELISA method. All data represent the mean ± SEM of four independent experiments. #P<0.05, significantly different from the unstimulated cells. *P<0.05, significantly different from the LPS-stimulated cells (non-treated with GJT).

7. The effect of GJT in LPS-induced IL-10 production

The author examined that GJT can modulate LPS-induced IL-10 production, the cell were pretreated with various concentrations of GJT for 30 min prior to LPS for 24 hrs. The supernatants were analyzed by ELISA method for IL-10. As shown in Fig 7, the amount of IL-10 was significantly higher in the GJT (1 mg/ml) plus LPS-treated cells than LPS-treated cells in a dose-dependent manner (about 23.7% at 0.01 mg/ml GJT, $P>0.05$; 35.5% at 0.1 mg/ml GJT, $P<0.05$; 56.4 ± 2.9% at 1 mg/ml GJT, $P<0.05$).

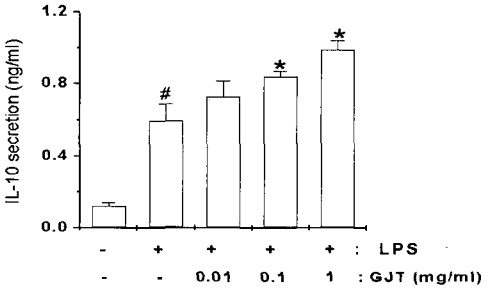

Fig. 7. The effect of GJT in LPS-induced IL-10 production in PBMC from CI patients. 3×10^5 PBMCs were pretreated with GJT (0.01-1 mg/ml) for 30 min and then stimulated with LPS (1μg/ml) for 24 hrs. IL-10 concentration was measured in cell supernatants using the ELISA method. All data represent the mean ± SEM of four independent experiments. #P<0.05, significantly different from the unstimulated cells. *P<0.05, significantly different from the LPS-stimulated cells (non-treated with GJT).

Discussion

GJT is an Oriental medicine hot water-extracted from the herb medicines, which consists of 13 different herbs. Other studies reported that each medicine herb has a different effect.

For example, Radix rehmanniae preparat has an anti-inflammatory activity in the CNS through suppression TNF-α secretion in LPS-stimulated astrocytes[24], Rhizoma dioscorea has an effect in rheumatoid arthritis through inhibition the production of TNF-α and IL-1β as well as down-regulating the expression of cyclooxygenase-2[25], Poria cocos has an anti-inflammation in arachidonic acid-induced ear inflammation in mice[26] and Fructus lycii has beneficial effects in the treatment of the immunodeficient diseases such as rheumatoid arthritis[27]. GJT composed on the basis of the theory of Korean medicine to maximize its efficacy.

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[28]. TNF-α is known to the trigger of a pro-inflammatory reaction that is produced mainly by activated mononuclear leukocytes. IL-1β is produced rapidly in the brains of rodents exposed to CI and enhances ischemic and other forms of injury. Several investigators characterized the role of TNF-α and IL-1β in experimental CNS ischemia and found a therapeutic benefit of IL-1 receptor antagonist (IL-1RA) treatment[29,30]. IL-6 is involved in modulating the acute expression of other pro-inflammatory cytokines in the brain after ischemia. These cytokines is involved in inflammation and exert pathophysiological effects. In this study, the author observed that GJT inhibited TNF-α, IL-1β and IL-6 production in LPS-stimulated PBMC from CI patients. These results suggested that GJT has potential effects on anti-inflammatory response through the regulation of pro-imflammatory cytokine production.

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[31,32], has recently been under focused investigation because of its possible participation in the evolution of CI. The author also showed that increased IL-8 level by LPS was inhibited by GJT pre-treatment. These results indicated that anti-inflammatory effects of GJT might be through suppression of chemokine, IL-8 production in PBMC from CI patients.

IL-4 has also been called as the prototypic immunoregulatory cytokine. Like many other cytokines, it can affect a variety of target cells in multiple ways. IL-4 has an important role in regulating Ab production and hematopoiesis and the development of effector T-cell responses[33]. An anti-inflammatory cytokine, IL-10, significantly inhibited the production of other pro-inflammatory mediators, such as reactive oxygen intermediates, reactive nitrogen intermediates and prostanglandins in monocyte / macrophages[34]. Stroke patients had shown significantly lowered IL-10 serum levels. Lower levels of IL-10 indicate that anti-inflammatory response is down-regulated in acute stroke patients[35]. In the present study, the author showed that GJT increased IL-4 and IL-10 production. From this, the author suggested that GJT has a beneficial effect through normalization of biologic balance on the cytokine production of CI patients.

In conclusion, the author showed that GJT inhibited the pro-inflammatory cytokine production and increased anti-inflammatory cytokines production. These results may implicate a good CI treatment effect of GJT and that it may be due to regulation of cytokine production.

Conclusion

GJT (0.01-1 mg/ml) did not affect cell viability in each condition and had no toxicity on PBMC from CI patients. TNF-α production increased by LPS was inhibited by pretreatment GJT in a dose-dependent manner. IL-1β production increased by LPS was inhibited by pretreatment GJT in a dose-dependent manner. IL-6 production increased by LPS was inhibited by pretreatment GJT in a dose-dependent manner. IL-8 production increased by LPS was inhibited by pretreatment GJT in a dose-dependent manner. The amount of IL-4 was significantly higher in the GJT (1 mg/ml) plus LPS-treated cells than LPS-treated cells. The amount of IL-10 was significantly higher in the GJT (1 mg/ml) plus LPS-treated cells than LPS-treated cells.

These results indicate that GJT may be a good prescription for CI treatment.

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