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Original Article

The Effects of *Danchunwhangagam* on LPS or DFX-induced Cytokine Production in Peripheral Mononuclear Cells of Cerebral Infarction Patients

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Objectives : This study was to investigate the effect of *Danchunwhangagam*(DCWGG) extract on the production of proinflammatory cytokines in peripheral blood mononuclear cells (PBMCs) from Cerebral infarction(CI) patients.

Methods : We examined how the inhibition rate of tumor necrosis factor (TNF)-α, interleukin(IL)-1α, IL-1β, IL-6, and IL-8 productions in DCWGG pretreatment PBMCs culture supernatant in the lipopolysaccaride(LPS)- or desferrioxamine(DFX)-treated cells compared to unstimulated cells.

Results : DCWGG inhibited the productions of TNF-α, IL-1α, IL-1β, IL-6, and IL-8 induced by LPS in a dose-dependent manner.

Conclusions : DCWGG might have regulatory effects on LPS or DFX-induced cytokine production, which might explain its beneficial effect in the treatment of CI.

Key Words: *Danchunwhangagam*, Cerebral Infarction, Cytokine

Introduction

Danchunwhan(DCW) is a prescription of traditional Korean medicine which has been used as a specific prescription for cerebral infarction(CI). *Danchunwhan* (DCW) consists of 2 different herbs, *Radix Salviae miltiorrhizae* and *Rhizoma chuanxion*, and it has an effect on promoting blood circulation and Qi, removing blood stasis and relieving pain. So this prescription is used of the treatment for arteriosclerosis, coarctation of aorta, cerebrovascular disease(stroke, dementia etc.) and coronary heart diseases[1]. *Danchunwhan*gagam (DCWGG) is added to *Caulis spatholobi, Lumbricus* and *Benth, Radix puerariae* to DCW to emphasize the effect.

We anticipated our new prescription would prohibit cerebral infarction. Inflammatory response is increased in the CI patient's brain. So we examined the cerebral infarction depression effect of DCWGG through this prescription to see what influence it makes on the change of the inflammatory reaction in a cerebral infarction patient's PBMC. During the last decade, a growing corpus of evidence has indicated an important role of cytokines in the development of brain damage.

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Recently, There is increasing evidence that the inflammatory response plays an important role in the pathogenesis of CI. Also, much of this inflammatory response appear to be mediated by proinflammatory cytokines[2-4].

Proinflammatory cytokines involved in hemostatic and immunologic imbalance leading to enlargement of brain damage, the release of especially tumor necrosis factor (TNF)-α is emphasized[5-6]. TNF-α is major inflammatory cytokine because they stimulate the synthesis of nitric oxide and other inflammatory mediators that derive chronically delayed hypersensitivity reaction[7]. Interleukin(IL)-1 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[8-9]. Both IL-1α and IL-1β are produced rapidly in the brains of rodents exposed to cerebral ischemia[10-12]. IL-6, which is one of the main inflammation-associated cytokine, is produced by a variety of cells in the central nervous system(CNS)[13]. It was found that in patients with acute ischemic stroke, and those with higher levels of IL-6 had more severe neurologic deficits on admission[14]. The spontaneous production of inflammatory cytokines by mononuclear cells and the level of cytokines in serum is 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 significantly reduced brain edema and infarct size in comparison to rabbits receiving a control antibody[15]. These results implicate that IL-8 is a novel target for the intervention of injury. Iron chelator, desferrioxamine(DFX) is capable of inducing transcription factors activation, and hypoxia dependent gene expression. Several investigators have reported that both DFX may modulate certain inflammatory mediators and regulate inflammatory processes.[16-17].

In this study, the author attempted to study the effects of DCWGG on TNF-α, IL-1α, IL-1β, IL-6, and IL-8 production in lipopolysaccaride(LPS) or desferrioxamine(DFX)-stimulated peripheral blood mononuclear cells(PBMC) from CI patients.

Materials and Methods

1. Reagents

Ficoll-Hypaque, LPS, DFX, avidin-peroxidase, and 2-AZINO-bis (3-ethylbenzithiazoline-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-1α, IL-1β antibody(Ab), biotinylated anti-human TNF-α, IL-1α, and IL-1β and recombinant human TNF-α, IL-1α, and IL-1β were purchased from R&D Systems(Minneapolis, MN, USA). Anti-human IL-6, IL-8, biotinylated anti-human IL-6, and IL-8, and recombinant (r) human IL-6, and IL-8 were purchased from Pharmingen(San Diego, CA, USA).

2. Patients with CI

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(MRI) 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 from 55 to 70 with CI.

3. Effect of SHCS on H_2O_2-induced ATP depletion

A decrease in intracellular ATP concentration precedes irreversible cell damage occurring upon

hypoxia- or ROS-induced tissue injury [1]. Thus, it was examined whether SHCS might prevent H_2O_2-induced ATP depletion. In Fig. 6, time-dependent changes of intracellular ATP content in H_2O_2-treated cells in the absence and presence of SHCS (1mg/*ml*) is depicted. ATP concentration in H_2O_2-treated cells decreased to lower than 10% of its control value in 3hr. However, in the presence of SHCS, H_2O_2-induced ATP depletion was significantly attenuated.

3. Preparation of DCWGG

DCWGG which is a mixture of 5 traditional herbs as shown in Table 1. was obtained from the College of Oriental Medicine, Wonkwang University(Iksan, South Korea). Extract of DCWGG was prepared by decocting the dried prescription of herbs with boiling distilled water(100g/1L). An extract of DCWGG was prepared by decocting the dried prescription of herbs with boiling distilled water. The duration of decoction was about 3h. The decoction was filtered, lyophilized and kept at 4℃. The yield of extraction was about 10.3% (w/w). Amounts of the 5 traditional drugs studied in this work were shown in Table 1.

4. Isolation and Culture of PBMCs

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 2mM L-glutamin, 100U/ml penicillin G, 100μl/ml streptomycin, and 10% FBS inactivated for 30min at 56℃. PBMCs were adjusted to a concentration of 3 × 10^6cells/ml in 30ml falcon tube, and 100μl aliquots of cell suspension were placed in a four-well cell culture plate. PBMCs were cultured for 24h in 95% humidified air containing 5% CO_2(37℃), in the presence or the absence of LPS or DFX, and the supernatants were collected by centrifugation and stored at -20℃.

5. MTT Assay

Cell viability was determined using MTT assay. Briefly, 500μl of PBMCs suspension(3 × 105cells) was cultured in 4-well plates for 24 h after treatment by each concentration of DCWGG. 50μl of MTT solution(5mg/ml) was added and then cells were incubated for 4h at 37℃. 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 540nm. The optical density of formazan formed in untreated control cells was taken as 100% of viability.

6. Cytokines Assay

ELISA for TNF-α, IL-1α, IL-1β, IL-6, and IL-8 was carried out in duplicate in 96 well ELISA plates(Nunc, Denmark) coated with each of 100μl aliquots of anti-human TNF-α, IL-1α, IL-1β, IL-6, and IL-8 monoclonal antibodies at 1.0μg/ml 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

Tabel 1. The Ratio of the Component in DCWGG.

Components	Amounts(g)
1. *Radix salviae miltiorrhiza* (Salvia miltiorrhiza Bung)	30
2. *Rhizoma chuanxion* (Lingustieum chuangxiong Hort)	15
3. *Caulis spatholobi* (Spatholobus suberectus Dun)	30
4. *Lumbricus* (Lumbricidae)	10
5. *Benth, Radix puerariae* (Pueraria thunbergiana (Sieb. Et Zucc.))	15

with PBS containing 1% BSA, 5% sucrose and 0.05% NaN3 for 1h. After additional washes, sample or TNF-α, IL-1α, IL-1β, IL-6, and IL-8 standards were added and incubated at 37℃ for 2h. After 2h incubation at 37℃, the wells were washed and then each of 0.2μg/ml of biotinylated anti-human TNF-α, IL-1α, IL-1β, IL-6, and IL-8 were added and again incubated at 37℃ for 2h. After washing the wells, avidin-peroxidase was added and plates were incubated for 20min at 37℃. Wells were again washed and ABTS substrate was added. Color development was measured at 405nm using an automated microplate ELISA reader.

7. HPLC Analysis

The Alliance 2695 HPLC system consisted of a pump(Waters Assoc., USA: 501 HPLC pump), a 2996 PDA detector(Water Assoc., USA: 2996 PDA detector), an autosampler(Water Assoc., USA: 746 computing integrator). YMC-Pack ODS-AQ 303 column(4.6mm × 250 nm, 5m) was used. Buffer(25mM phosphoric acid, pH 2.25): Acetonitrile (80:20) was used as the mobile phase. Detection of the peaks was made at 210nm and the sensitivity was set of 1.0AUFs. The injection volume was 20, land flow rate was 1.0. Standard solution was prepared by dissolving in distilled water(10g/100mL). The solution was filtered through 0.45L membrane filter and applied to HPLC.

8. 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. Effects of DCWGG on the cell viability

The author first examined the effects of DCWGG on the viability of PBMC using MTT assay. Cells were treated with various concentrations of DCWGG(0.01-1mg/ml) for 30min and then stimulated with LPS or DFX for 24h. In the cells treated with LPS or DFX, cell viability decreased to 95.36 ± 4.6% and 94.36 ± 2.99%, respectively compared with the control value(100.0± 5.4%). However, DCWGG(0.01-1mg/ml) did not affect cell viability in each condition and had no toxicity on PBMC from CI patients(Fig. 1).

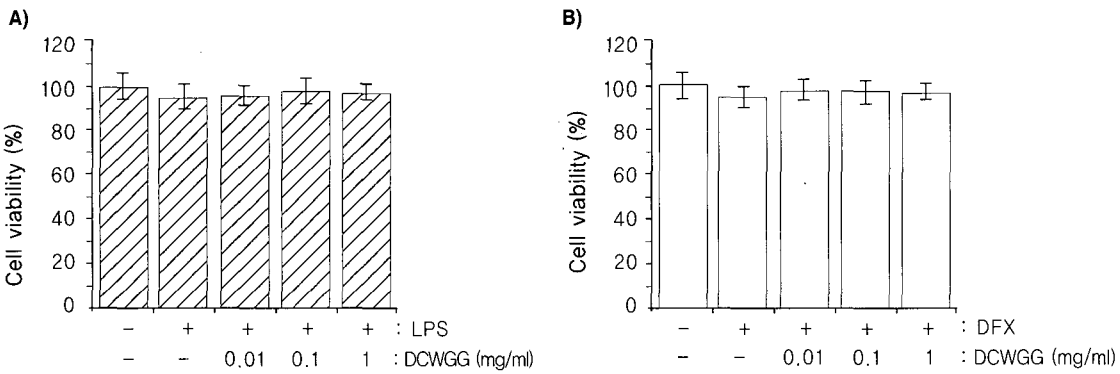

Fig. 1. Effects of DCWGG on the cell viability in PBMC from CI patients.

Cell viability was evaluated by MTT colorimetric assay for 24h incubation after stimulation of LPS(1μg/ml) or DFX(100μM) in the absence or presence of DCWGG(0.01-1mg/ml). The percentage of viable cells was over 94%. Data represent mean ± SEM of six independent experiments.

2. Effects of DCWGG on LPS- or DFX-induced TNF-α production

To evaluate the regulatory effects of DCWGG on the TNF-α production, PBMCs were pretreated with DCWGG for 30min and then treated LPS or DFX for 24h. The supernatants were analyzed by ELISA method for TNF-α. The author confirmed that TNF-α increased by LPS was inhibited by DCWGG in a dose-dependent manner(Fig. 2A). Maximal inhibition rate was 50.32 ± 2.5% at 1 mg/ml DCWGG ($p < 0.05$). The author also showed that DCWGG also inhibited TNF-α production induced DFX in PBMCs from CI patients(Fig. 2B). Maximal inhibition rate was 57.02 ± 2.5% at 1 mg/ml DCWGG ($p < 0.05$).

3. Effects of DCWGG on LPS- or DFX-induced IL-1α production

To evaluate the regulatory effects of DCWGG on the

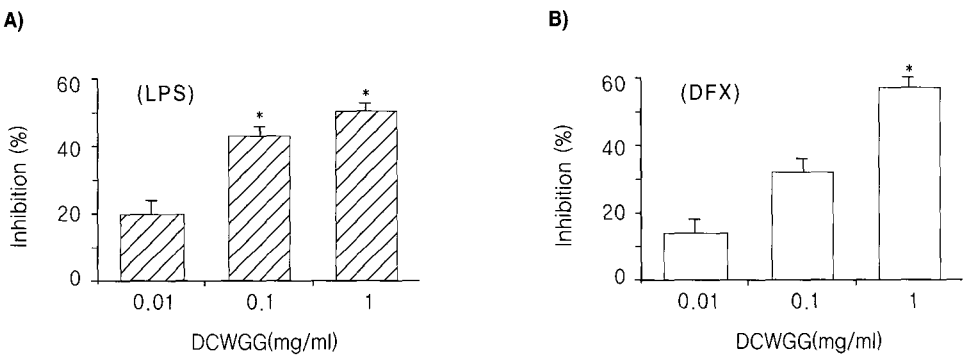

Fig. 2. Effects of DCWGG on LPS- or DFX-induced TNF-α production in PBMC from CI patients.

3×105 PBMCs were pretreated with DCWGG(0.01-1mg/ml) for 30min, and then stimulated with LPS(1μg/ml) or DFX(100μM) for 24h. 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 LPS or DFX-stimulated cells(non-treated with DCWGG).

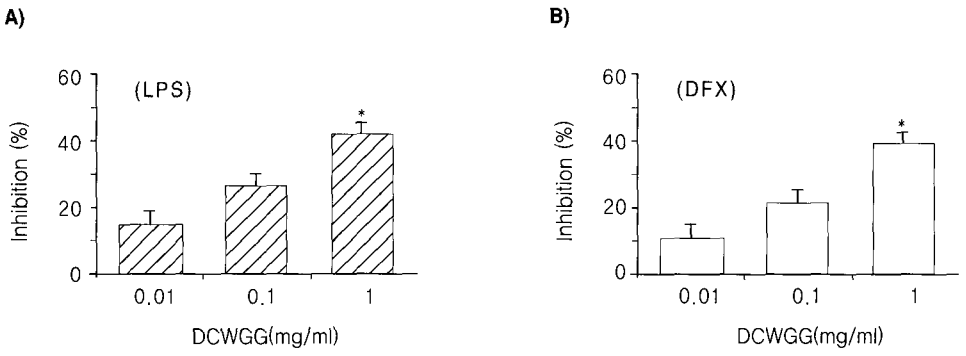

Fig. 3. Effects of DCWGG on LPS or DFX Induced IL-1α Production in PBMC from CI patients.

3×105 PBMCs were pretreated with DCWGG(0.01-1mg/ml) for 30min, and then stimulated with LPS(1μg/ml) or DFX(100μM) for 24h. IL-1α 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 LPS or DFX-stimulated cells(non-treated with DCWGG).

IL-1α production, PBMCs were pretreated with DCWGG for 30min and then treated LPS or DFX for 24h. The supernatants were analyzed by ELISA method for IL-1α. The author confirmed that IL-1α increased by LPS was inhibited by DCWGG in a dose-dependent manner(Fig. 3A). Maximal inhibition rate was 42.02 $\pm$ 3.5% at 1mg/ml DCWGG ($p < 0.05$). We also showed that DCWGG also inhibited IL-1α production induced DFX in PBMCs from CI patients(Fig. 3B). Maximal inhibition rate was 38.12 $\pm$ 3.2% at 1mg/ml DCWGG ($p < 0.05$).

4. Effects of DCWGG on LPS- or DFX-induced IL-1β production

To determine whether DCWCC can modulate LPS or DFX-induced IL-1β production, the cell were pretreated with various concentrations of DCWGG for 30min prior to LPS or DFX for 24h. The supernatants were analyzed by ELISA method for IL-1β. The author confirmed that IL-1β increased by LPS was inhibited by

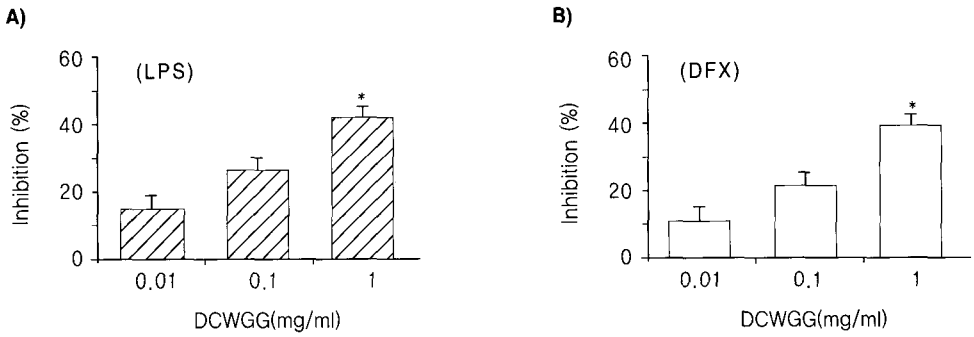

Fig. 4. Effects of DCWGG on LPS- or DFX-Induced IL-1β, production in PBMC from CI patients.

3 x 105 PBMCs were pretreated with DCWGG(0.01-1mg/ml) for 30 min, and then stimulated with LPS(1μg/ml) or DFX(100μM) for 24h. IL-1β concentration was measured in cell supernatants using the ELISA method. All data represent the mean $\pm$ SEM of four independent experiments. *$p < 0.05$, significantly different from the LPS or DFX-stimulated cells(non-treated with DCWGG).

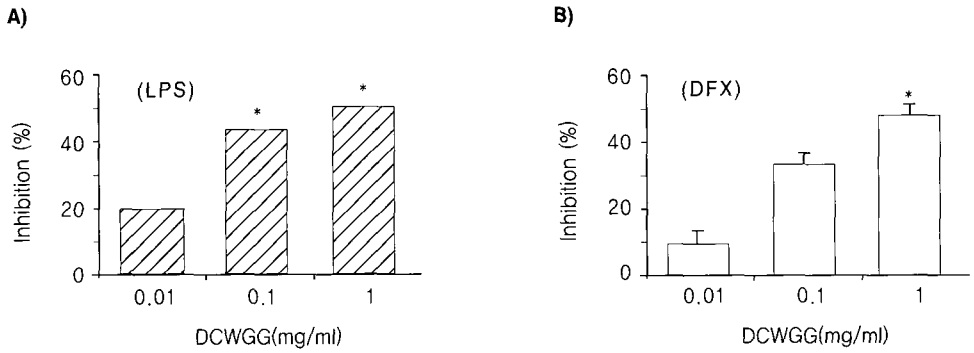

Fig. 5. Effects of DCWGG on LPS- or DFX-induced IL-6 production in PBMC from CI patients.

3 x 105 PBMCs were pretreated with DCWGG(0.01-1mg/ml) for 30 min, and then stimulated with LPS(1μg/ml) or DFX(100μM) for 24h. IL-6 concentration was measured in cell supernatants using the ELISA method. All data represent the mean $\pm$ SEM of four independent experiments. *$p < 0.05$, significantly different from the LPS or DFX-stimulated cells(non-treated with DCWGG).

DCWGG in a dose-dependent manner(Fig. 4A). Maximal inhibition rate was 43.02 ± 2.7% at 1mg/ml DCWGG ($p < 0.05$). The author also showed that DCWGG also inhibited IL-1β production induced DFX in PBMCs from CI patients(Fig. 4B). Maximal inhibition rate was 50.02 ± 3.2% at 1 mg/ml DCWGG ($p < 0.05$).

5. Effects of DCWGG on LPS- or DFX-induced IL-6 production

The author examined that DCWGG can modulate LPS or DFX-induced IL-6 production, the cell were pretreated with various concentrations of DCWGG for 30min prior to LPS or DFX for 24h. The supernatants were analyzed by ELISA method for IL-6. The author confirmed that IL-6 increased by LPS was inhibited by DCWGG in a dose-dependent manner(Fig. 5A). Maximal inhibition rate was 50.02 ± 3.1% at 1mg/ml DCWGG ($p < 0.05$). The author also showed that DCWGG also inhibited IL-1α production induced DFX

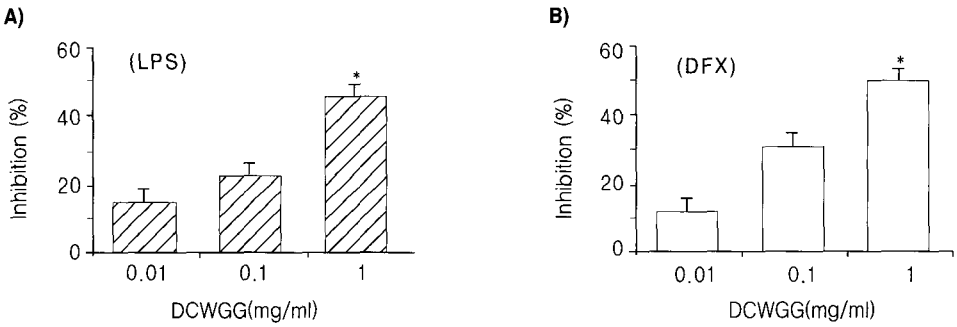

Fig. 6. Effects of DCWGG on LPS or DFX Induced IL-8 Production in PBMC from CI patients.

3×105 PBMCs were pretreated with DCWGG(0.01-1mg/ml) for 30 min, and then stimulated with LPS(1μg/ml) or DFX(100μM) for 24h. 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 LPS or DFX-stimulated cells(non-treated with DCWGG).

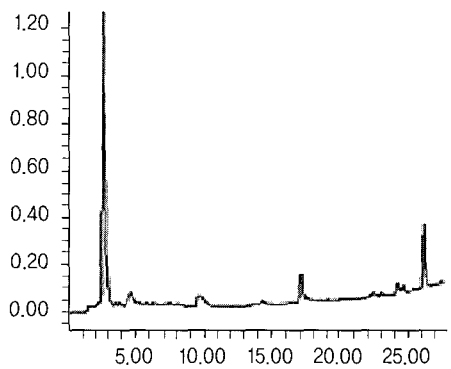

Fig. 7. HPLC chromatogram of the DCWGG.

Standard solution of DCWGG was prepared by dissolving in distilled water(10μg/100ml). The injection volume was 20μl and the detection was made at 254nm.

in PBMCs from CI patients(Fig. 5B). Maximal inhibition rate was 48.12 ± 3.2% at 1mg/ml DCWGG ($p < 0.05$).

6. Effects of DCWGG on LPS- or DFX-induced IL-8 production

The author examined the effects of DCWGG on LPS or DFX-induced IL-8 production from PBMC of CI patients. The cells were pretreated with various concentrations of DCWGG for 30min prior to LPS or DFX for 24h. The supernatants were analyzed by ELISA method for IL-8. The author showed that IL-8 increased by LPS was inhibited by DCWGG in a dose-dependent manner(Fig. 6A). Maximal inhibition rate was 46.11 ± 3.1% at 1mg/ml DCWGG ($p < 0.05$). The author also showed that DCWGG also inhibited IL-1α production induced DFX in PBMCs from CI patients(Fig. 6B). Maximal inhibition rate was 50.12 ± 3.2% at 1mg/ml DCWGG ($p < 0.05$).

7. Characterization of the principal components of DCWGG

The components of DCWGG were analyzed by HPLC. Chromatogram of the DCWGG is shown in Fig. 7. Peaks of the principal components have not yet been identified in this study.

Discussion

*Danchunwhan*gagam(DCWGG) is added three herbs to *Danchunwhan*(DCW), Caulis spatholobi which promoting circulation of blood, Lumbricus which promoting menstrual flow, and Benth, Radix puerariae which expelling pathogenic factors from muscles and skin.

Each medicine herb has a different effect. *Radix Salviae miltiorrhizae* works through the heart, pericardium, and liver meridian, promoting blood flow to regulate menstruation, promoting circulation of Qi, removing blood stasis, removing heat from the blood, relieving carbuncle, and promoting new blood. *Rhizoma chuanxion* works through liver, gallbladder, and pericardium meridian, promoting blood flow and Qi circulation, relieving stagnation, nourishing blood, expelling the wind, dispersing pathogenic wind and removing pain. *Caulis spatholobi* works through the liver, kidney meridian, promoting blood circulation, relaxing muscles and tendons and activating the flow of Qi and blood in the channels and collaterals, relieving rigidity of muscles and activating collaterals. *Lumbricus* works through liver, spleen, and bladder meridian, removing heat, calming the endopathic wind, restoring menstrual flow, inducing or increasing menstruation, promoting menstrual flow, relieving asthma, and using diuretic or hydragogue to alleviate water retention. *Benth, Radix puerariae* works through spleen and stomach meridian, relieving superficies syndrome by means of diaphoresis, expelling pathogenic factors from muscles and skin, letting out of measles, promoting the production of body fluid to quench thirst, invigorating vital function, and antidiarrhea.[18]

Radix Salviae miltiorrhizae has therapeutic effect on blood pressure in hypertensive rat[19], *Rhizoma chuanxion* has effect on cerebral blood flow regulation[20], *Caulis spatholobi* induces apoptotic cell death via inhibition of cell cycle in Jurkat human leukemia cell line[21], *Lumbricus* regulates inflammation and immunology reaction[22] and *Pueraria thunbergiana* has anti-inflammatory effect through inhibition TNF-α production in macrophage RAW264.7 cells[23].

Previous studies reported that DCW has effect of hydrogen peroxide-induced apoptosis of H9c2 cardiomyoblasts1) and effects on oxidative damage of human neural cell.[24] However, the pharmacological mechanisms of the prescription have not been defined yet.

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[25]. TNF-α is known to trigger a proinflammatory and prothrombotic reaction that is produced mainly by activated mononuclear leukocytes. IL-1 is produced rapidly in the brains of rodents exposed to CI, and enhances ischemia and other forms of injury. Several investigators characterized the role of TNF-α and IL-1 in experimental CNS ischemia and found a therapeutic benefit of IL-1 receptor antagonist(IL-1RA) treatment[26-27]. IL-6 is involved in modulating the acute expression of other proinflammatory cytokines in the brain after ischemia. These cytokines involved in inflammation, and exerted pathophysiological effects. 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 leukocytes28-[29] has recently been under focused investigation because of its possible participation in the evolution of CI. Increased IL-8 level by LPS or DFX was inhibited by DCWGG treatment. These results indicated that anti-inflammatory effect of DCWGG might be through suppression of chemokine, IL-8 production in PBMC from CI patients.

In this study, we confirmed that DCWGG inhibited the production of TNF-α, IL-1α, IL-1β, and IL-6 cytokines in LPS or DFX-stimulated PBMC cells. These results suggested that DCWGG effects on anti-inflammatory response through the regulation of cytokine production. And, these anti-inflammatory responses of DCWGG may implicate a good treatment effect on CI patient. Forward, We consider that need further study about examining in vivo, searching a specific medicine, and finding possible mechanism.

Conclusion

1. In the cells treated with LPS or DFX, cell viability decreased and respectively compared with the control value. However, DCWGG did not affect cell viability in each condition and had no toxicity on PBMC from CI patients.
2. TNF-α increased by LPS or DFX in PBMCs from CI patients was inhibited by DCWGG.
3. IL-1 increased by LPS or DFX in PBMCs from CI patients was inhibited by DCWGG.
4. IL-1β increased by LPS or DFX in PBMCs from CI patients was inhibited by DCWGG.
5. IL-6 increased by LPS or DFX in PBMCs from CI patients was inhibited by DCWGG.
6. IL-8 increased by LPS or DFX in PBMCs from CI patients was inhibited by DCWGG.
7. The profile of components of DCWGG were analyzed by HPLC.

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