Banhabaikchulcheunma-tang Down-regulates LPS-induced Production of Pro-inflammatory Cytokines

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Banhabaikchulcheunma-tang (BBCT), a prescription composed of thirteen herbal mixtures, has been widely used in the treatment of brain disorders in Oriental Medicine. However, the mechanisms by which the formula affects on the production of pro-inflammatory cytokines in cerebral infarction (CI) patients remain unknown yet. The levels of secretory protein of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-a, interlukin (IL)-1b, and IL-6, were significantly increased in lipopolysaccharide (LPS)-stimulated THP-1 differentiated macrophage-like (THP-1/M) cells and Peripheral blood mononuclear cells (PBMCs) from CI patients. However, pretreatment with BBCT significantly inhibited the secretion of pro-inflammatory cytokines, including TNF-a, IL-1b, and IL-6, in THP-1/M cells and PBMCs from CI patients with stimulus. Thus, these data indicate that BBCT may be beneficial in the cessation of inflammatory processes of cerebral infarction through suppression on the production of pro-inflammatory cytokines.

Key words : Banhabaikchulcheunma-tang (BBCT), cerebral infarction, pro-inflammatory cytokines

Introduction

Banhabaikchulcheunma-tang (BBCT), a traditional prescription of Oriental Medicine, has been widely used as a prescription of CI patients to ameliorate and decrease morbidity and mortality after stroke. BBCT is consisted of thirteen herbs, containing Pinelliae Tuber, Gastrodiae Rhizoma, Atractylodis Rhizoma Alba, Ginseng Radix, Astragali Radix, Poria, Alismatis Rhizoma, Atractylodis Rhizoma, Aurantii Nobilis Pericarpium, Massa Medicata Fermentata, Hordei Fructus Germiniatus, Phellodendri Cortex, and Zingiberis Rhizoma. However, the mechanism of its therapeutic use has not been well defined yet in CI patients.

Cerebral infarct, a neurodegenerative disease, usually induces cerebral ischemic insults with irreversible deterioration of central nervous system (CNS) behaviors. After the onset of cerebral ischemia, inflammatory process mediates the acceleration of the early onset and functions as a determinant factor in severity of cerebral damage in neurodegenerative brain diseases[1-3]. Under cerebral ischemia, acute phase of inflammation initiates to recruit the activated inflammatory cells, including as macrophages and lymphocytes, into the damaged brain area. Macrophages and lymphocytes are circulating immune cells, play an essential role to secrete pro-inflammatory cytokines and to activate inflammatory mediators in ischemic region. Other supporting cells, including astrocytes, microglia, and endothelia, are also involved in inflammatory processes after cerebral ischemic stroke[4-9].

Pro-inflammatory cytokines, including TNF-a, IL-1b, and IL-6, which are secreted in the ischemic region by activated immune cells, drive inflammatory process and accelerate the additional inflammatory process by inducing inflammatory molecules, such as intercellular adhesion molecules (ICAM), vascular cell adhesion molecules-1 (VCAM-1), and selectin. These inflammatory modulators recruit more circulating leukocytes which infiltrate into ischemic region and lead to further loss of neuronal cells in the brain tissue and increase of cerebral infarct size[8,10,11]. Also, blockade of immune reaction and anti-inflammatory agents have been regarded as potentially therapeutic candidates over the past decade. Therefore, specific inhibition on the role of pro-inflammatory molecules has been focused in the treatment of CI patients even though the mechanisms of these molecules are not clearly evidenced in the prevention of subsequent neuronal damages during ischemia[1,12-14].

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To know the functional ability of BBCT, we herein investigated the effects of BBCT on pro-inflammatory cytokines in human macrophage like THP-1 cells and PBMCs from CI patients.

Materials and Methods

1. Reagents

Ficoll-Hypaque, LPS, phorbol 12-myristate 13-acetate (PMA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). RPMI 1640, ampicillin, streptomycin, and fetal bovine serum (FBS) were bought from Gibco BRL (Grand Island, NY, USA). TNF-a, IL-1b, and IL-6 ELISA kits were obtained from R&D system (Minneapolis MN55413, USA).

2. Preparation of BBCT

The ingredients of BBCT were Pinelliae Tuber (24 g), Gastrodiae Rhizoma (16 g), Atractylodis Rhizoma Alba (12 g), Ginseng Radix (16 g), Astragali Radix (16 g), Poria (16 g), Alismatis Rhizoma (16 g), Atractylodis Rhizoma (24 g), Aurantii Nobilis Pericarpium (12 g), Massa Medicata Fermentata (24 g), Hordei Fructus Germiniatus (8 g), Phellodendri Cortex, and Zingiberis Rhizoma (8 g). An extract of BBCT was prepared by decocting the dried prescription of herbs with distilled water (100 g/L). The decoction was filtered, lyophilized, and kept at 4℃. The yield of extraction was about 9.95% (w/w). The BBCT powder of water extract was dissolved in sterile saline (100 mg/ml). The final concentration of 0.8, 0.4, 0.2 mg/ml BBCT was used for the experiments. The plant materials were obtained and identified by Professor Byoug S. Moon from Oriental Medical Hospital, Wonkwang University, Korea.

3. Patients

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

4. Cell culture

Human macrophage like THP-1 cells (ATCC, Rockville, MD, USA) were plated at a density of 1.0×10^7 cells per 10 cm dish in 13 ml RPMI-1640 medium containing 10% (v/v) FBS, supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37℃ in 95% humidified air containing 5% CO_2. Differentiation to a macrophage phenotype was induced by culturing THP-1 cells in the presence of 100 nM PMA for 2 days. The differentiated cells were washed once with phosphate-buffered saline (PBS, pH 7.4) and then exposed to RPMI-1640 medium, containing with 0.5% FCS, penicillin and streptomycin. BBCT (0.8 mg/ml) in combination with LPS (0.1 mg/ml) or alone were added in cultures.

PBMCs from heparinized venous blood of CI patients were isolated by Ficoll-gradient centrifugation, washed three times with PBS and resuspended in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin G, 100 mg/ml streptomycin, and 10% FBS inactivated for 30 min at 56 ℃. PBMCs were adjusted to a concentration of 2×10^6 cells/ml in 30 ml falcon tube, and 100 ml aliquots of cell-suspension were placed in a four-well cell culture plate. PBMCs were cultured with LPS (0.1 mg/ml) and BBCT (1 mg/ml) or LPS alone for 24 h in 95% humidified air containing 5% CO_2 at 37℃ and the supernatants were collected by centrifugation and stored at −20℃.

5. Cell viability test

Cell viability was quantified using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). In brief, cells (THP-1/M ; 1.0×10^6, PBMCs; 5×10^5) were cultured in 24-well plates with varying concentrations of BBCT for 24 h. To determine the cell viability, MTT (0.5 mg) was added to 1ml of cell suspension for 4 h. After three washes of cells with phosphate-buffered saline (PBS, pH 7.4), the insoluble formazan product was dissolved in DMSO. Then, the optical density (OD) of each culture well was measured using a Microplate reader (Titertek Multiskan, Flow Laboratories) at 590 nm. The OD in control cells was taken as 100% of viability.

6. Measurement of pro-inflammatory cytokines by ELISA

Secretory level of pro-inflammatory cytokines, including TNF-a, IL-1b and IL-6 proteins, in culture supernatants was determined by Quantikine ELISA kit (R&D Systems Inc, Mineapolis, USA). The color generated was determined by measuring the O.D. at 450 nm of spectrophotometric microtiter plate reader (Molecular Devices Corp., Sunnyvale, CA, USA). The minimum detectable concentrations of TNF-a, IL-1b and IL-6 were 1.6 pg/ml, 1.0 pg/ml, and 0.7 pg/ml, respectively. A standard curve was run on each assay plate using recombinant proteins, including TNF-a, IL-1b and IL-6, in serial dilutions.

7. HPLC analysis

The chromatographic system consisted of a pump (Waters Assoc. USA: 510 HPLC pump), a UV detector (Waters Assoc., USA: 490E detector), an autosampler (Waters Assoc., USA: 717 plus autosampler), and a date modular (Waters Assoc., USA: 746 computing integrator). A Cosmosil 5C18-AR-II columm (3.9 mm×150 mm, 5 mm) was used. Water - acetonitrile - acetic acid glacial (90:10:1) was used as the mobile phase. Detection of the peaks was made at 254 nm and the sensitivity was set of 0.5 AUFs. The injection volume was 30 ml and flow rate was 1.0 ml/min. Standard solution was prepared by dissolving in distilled water (10 mg/100 ml). The solution was filtered through 0.45 mm membrane filter and applied to HPLC. Acetonitrile-HPLC grade was purchased from Merck (Germany). Other chemicals used were all GR grade.

8. Statistical analysis

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

Results

1. Characterization of the principal components of Daesiho

BBCT was analyzed by HPLC. Chromatogram of BBCT is shown in Fig. 1 Peaks of the principal components have not yet been identified in our study.

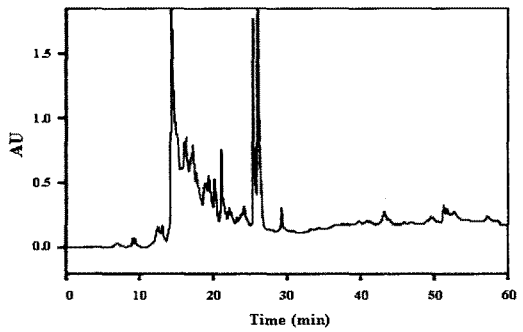

Fig. 1. Analytical HPLC chromatogram of BBCT. Standard solution of BBCT was prepared by dissolving in distilled water (10 mg/100 ml), and fractionated as described in Materials and Methods. The injection volume was 30 ml and flow rate was 1.0 ml/min. The detection was made at 254 nm.

2. Pretreatment with BBCT significantly inhibited the secretion of TNF-a in macrophage and human PBMCs after stimulus

We investigated whether BBCT could suppress LPS-induced TNF-a secretion in THP-1 differentiated macrophage-like (THP-1/M) cells and PBMCs from CI patients. At first, we decided to differentiate human monocytic leukemic THP-1 cells into macrophage-like cells showing adherent phenotype. THP-1 cells were treated with PMA (100 nM) for 48 h. To confirm cytotoxic effect of BBCT, BBCT (0.2-0.8 mg/ml) used in this study showed no significant effect on viability of THP-1/M cells through MTT assay(Fig. 2).

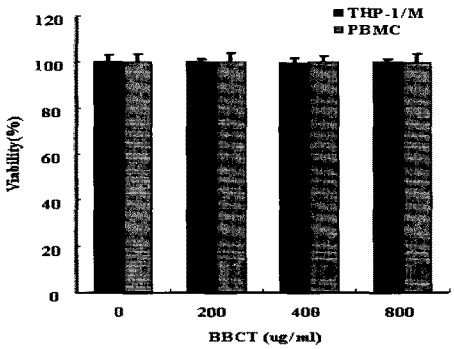

Fig. 2. BBCT did not affect cell viability of THP-1/M cells and PBMCs from CI patients. THP-1/M cells and PBMCs were treated with 0.2-0.8 mg/ml BBCT for 24 h. Cell viability was measured by MTT assay. Results are expressed as means ± SD of three independent experiments (*p<0.05).

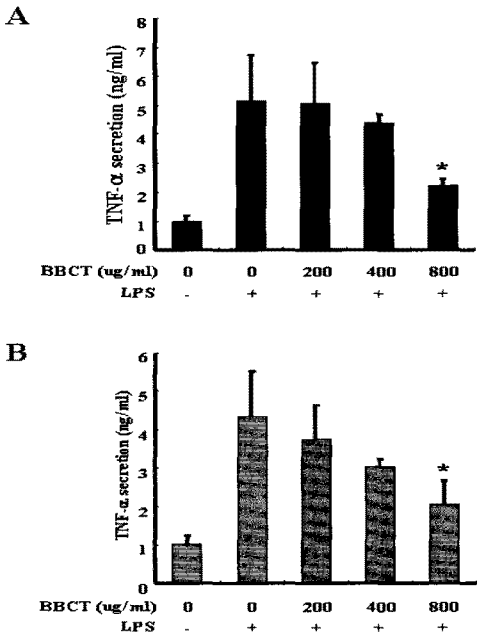

Fig. 3. BBCT suppressed the secretion of TNF-a in LPS-treated THP-1/M cells and PBMCs from CI patients. THP-1/M cells and PBMCs were stimulated with LPS (0.1 mg/ml) in the presence and absence of BBCT at individual concentrations for 24 h. Secretion of TNF-a in the supernatants of stimulus-treated THP-1/M cells (A) and PBMCs (B) was measured by ELISA. Results are expressed as means ± SD of three independent experiments (*p<0.05).

To investigate whether BBCT suppresses LPS-induced TNF-a cytokine in THP-1/M cells, THP-1/M cells were pretreated with BBCT at various concentrations for 1 h and further maintained with 0.1 mg/ml LPS, a specific activator of macrophage secretory response. The culture supernatants were collected to measure the secretion of TNF-a cytokine by ELISA

assay. As shown in Fig. 3A, treatment with LPS resulted in a significant increase in the secretion of TNF-a (control, 0.991±0.207 ng/ml; LPS-stimulated, 5.160±1.575 ng/ml) on THP-1/M cells. However, pretreatment of THP-1/M cells with BBCT significantly suppressed LPS-induced TNF-a production (2.204±0.225 ng/ml, *p < 0.05)(Fig. 3A). Next, PBMCs from CI patients (n=10, mean age 56±12 years; 3 females and 7 males) were treated with BBCT (0.2-0.8 mg/ml) for 24 h and cell viability was determined using a MTT assay. No significant cytotoxic effect on the viability of PBMCs was detected(Fig. 2). To evaluate whether BBCT suppresses TNF-a production, PBMCs from CI patients were pretreated with BBCT for 1 h and further maintained with 0.1 mg/ml LPS for 24 h. As shown in Fig. 3B, pretreatment of PBMCs from CI patients with BBCT significantly inhibited TNF-a secretion by LPS in a concentration-dependent manner (control, 1.026±0.203 ng/ml; LPS-stimulated, 4.351±1.167 ng/ml; 0.8 mg/ml of BBCT-pretreated, 2.035±0.642 ng/ml, #p < 0.05).

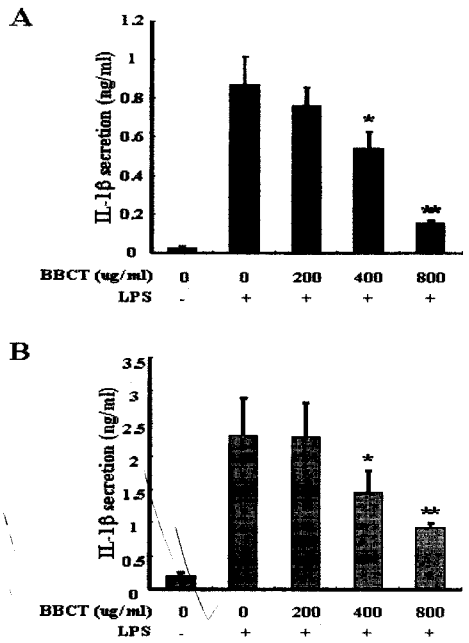

Fig. 4. BBCT suppressed the secretion of IL-1b in LPS-treated THP-1/M cells and PBMCs from CI patients. THP-1/M cells and PBMCs were stimulated with LPS (0.1 mg/ml) in the presence and absence of BBCT at individual concentrations for 24 h. Secretion of IL-1b in the supernatants of stimulus-treated THP-1/M cells (A) and PBMCs (B) was measured by ELISA. Results are expressed as means ± SD of three independent experiments (*p<0.05, **p<0.005).

2. Pretreatment with BBCT significantly inhibited the secretion of IL-1b in macrophage and human PBMCs after stimulus

Next, we measured the secretion of IL-1b in both THP-1/M cells and PBMCs from CI patients with each stimulus. To determine the effect of BBCT on the secretion of IL-1b, T/M cells were pretreated with BBCT (0.2-0.8 mg/ml) for 1 h. After exposure to LPS in THP-1/M cells, the culture supernatants were used to measure the secretion of IL-1b. As shown in Fig. 4A, addition of BBCT dramatically suppressed IL-1b secretions by LPS in THP-1/M cells in a dose-dependent manner (control, 0.025±0.011 ng/ml; LPS-stimulated, 0.868±0.146 ng/ml; 0.4 mg/ml of BBCT-pretreated, 0.541±0.089 ng/ml; 0.8 mg/ml of BBCT-pretreated, 0.156±0.016 ng/ml, *p < 0.05, **p < 0.005). Also, treatment with LPS resulted in a significant increase in secretion of IL-1b in PBMCs from (control, 0.204±0.056 ng/ml; LPS-stimulated, 2.324±0.570 ng/ml)(Fig. 4B). However, pretreatment of PBMCs from CI patients with BBCT significantly suppressed LPS-induced TNF-a production (0.4 mg/ml of BBCT-pretreated, 1.461±0.329 ng/ml; 0.8 mg/ml of BBCT-pretreated, 0.930±0.060 ng/ml, *p < 0.05, **p < 0.005)(Fig. 4B).

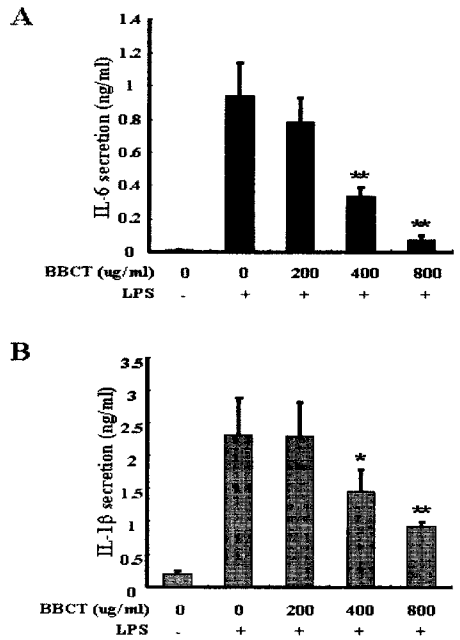

Fig. 5. BBCT suppressed the secretion of IL-6 in LPS-treated THP-1/M cells and PBMCs from CI patients. THP-1/M cells and PBMCs were stimulated with LPS (0.1 mg/ml) in the presence and absence of BBCT at individual concentrations for 24 h. Secretion of IL-6 in the supernatants of stimulus-treated THP-1/M cells (A) and PBMCs (B) was measured by ELISA. Results are expressed as means ± SD of three independent experiments (*p<0.05, **p<0.005).

3. Pretreatment with BBCT significantly inhibited the secretion of IL-6 in macrophage and human PBMCs after stimulus

We confirmed the production of IL-6 in both PBMCs and THP-1/M cells after stimulus. As a result of ELISA assay, treatment with LPS resulted in a significant increase in secretion of IL-6 in THP-1/M cells (control, 0.015±0.004 ng/ml; LPS-stimulated, 0.939±0.196 ng/ml) at 24 h(Fig. 5A). Also, the production of IL-6 was increased by treatment with LPS in PBMCs from CI patients (control, 0.146±0.045 ng/ml; LPS-stimulated, 0.771±0.088 ng/ml)(Fig. 5B). However, increased level of IL-6 by LPS was significantly inhibited by

pretreatment with BBCT in THP-1/M cells (0.4 mg/ml of BBCT-pretreated, 0.340±0.051 ng/ml; 0.8 mg/ml of BBCT-pretreated, 0.075±0.024 ng/ml)(Fig. 5A). LPS-induced IL-6 was markedly down-regulated by pretreatment with BBCT in PBMCs (0.4 mg/ml of BBCT-pretreated, 0.432±0.084 ng/ml; 0.8 mg/ml of BBCT-pretreated, 0.256±0.042 ng/ml) in a dose-dependent manner(Fig. 5B). Taken together, we demonstrated that BBCT, which is a traditional prescription of CI patients after stroke, has an anti-inflammatory effect by the suppression of pro-inflammatory cytokine productions, such as TNF-a, IL-1b, and IL-6 in human macrophage and PBMCs from CI patients.

Discussion

Although BBCT, a traditional prescription for cerebral infarct patients in oriental medicine, has effective pharmacological actions, the therapeutic mechanism has not been well defined yet. In our study, we demonstrated that BBCT suppressed the production of pro-inflammatory cytokines, including TNF-a, IL-1b, and IL-6 in LPS-stimulated THP-1/M cells and PBMCs from CI patients. Thus, we suggest that one of the functional mechanisms of BBCT in the beneficial treatment for CI patients is due to a marked suppression of inflammatory process.

BBCT is consisted of thirteen herbs, containing Pinelliae Tuber, Gastrodiae Rhizoma, Atractylodis Rhizoma Alba, Ginseng Radix, Astragali Radix, Poria, Alismatis Rhizoma, Atractylodis Rhizoma, Aurantii Nobilis Pericarpium, Massa Medicata Fermentata, Hordei Fructus Germiniatus, Phellodendri Cortex, and Zingiberis Rhizoma. These extracts of each herb have been used as an oriental traditional medicine and reported their pharmacological effects as follow. Gastrodiae Rhizoma has been reported to have the improving effect on learning and memory of senile rats[15]. Gastrodiae Rhizoma contains selina-4(14),7(11)-dien-8-one, which has been reported to inhibit melanogenesis[16]. Ginseng Radix has been reported to protect H_2O_2-induced neuronal cell damage in astrocytes primary cells[17] and 1-methyl-4-phenylpyridinium-induced apoptosis in PC12 cells[18]. Also, administration of Ginseng radix regulates type-1 cytokine production in BALB/c mice model[19,20] and decreases nitric oxide synthase expression in the hippocampus of streptozotocin-induced diabetic rats[21]. Astragali Radix has been demonstrated to suppress cardiac contractile dysfunction and inflammation in a rat model of autoimmune myocarditis[22] and elicit anti-inflammation via activation of MKP-1, concomitant with attenuation of p38 and Erk[23]. Alisol B acetate, a triterpene from Alismatis rhizoma, was reported to induce Bax nuclear translocation and apoptosis in human hormone-resistant prostate cancer PC-3 cells[24]. Phellodendri Cortex has been reported to reduce glucose level and prevent or retard the development of diabetic nephropathy in streptozotocin-induced diabetic rats[25].

Under cerebral, inflammatory cells, such as neutrophils and macrophages, have been reported to infiltrate into the ischemic brain region and trigger to activate inherent brain cells, including astrocytes, microglia, and endothelia. Inflammatory responses which affect these cells with substances, including pro-inflammatory cytokines, vasoactive substances and adhesion molecules, play an important role in the pathogenesis of cerebral lesions following cerebral ischemia[14,26,27]. The most important pro-inflammatory cytokines in post-ischemic inflammation include TNF-a, IL-1b, and IL-6. TNF-a and IL-1b have the major role to initiate the inflammatory response mediated by the induction of inflammatory metabolites and increased expression of adhesion molecules on the surface of endothelial cells. TNF-a and IL-1b secreted from brain cells share a high homology in structure and function. TNF-a and IL-1b are also responsible for the accumulation of inflammatory cells in the peripheral nucleus of cerebral infarct and induce a second inflammatory response mediated by IL-6. IL-6 is playing a central role in acute inflammatory processes exhibiting pro-inflammatory activities in many different brain pathologies including cerebral ischemia and excitotoxic brain damage[28-31]. In addition, to ameliorate brain damages followed by stroke and ischemic attack, there have been reported various therapeutic trials, including anti-TNF-a antibody and TNF-a soluble receptor type 1[32,33], IL-1b receptor antagonist[34], and recombinant IL-1ra[35,36]. Accumulated evidences have proved that the suppressive approaches of specific inflammatory mediators are one of the best solutions to prevent leading to secondary ischemia and damage of brain cells under cerebral ischemia.

In conclusion, our data suggest that BBCT functions to suppress the production of pro-inflammatory cytokines, including TNF-a, IL-1b, and IL-6 in LPS-induced THP-1/M cells and PBMCs from CI patients. Moreover, works are undergoing to identify the crucial components of herbal constituents of BBCT and the precise signaling pathway of BBCT on anti-inflammatory mechanism using in vitro and in vivo animal model of brain ischemia.

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