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Inhibitory effect of *Yongdamsagantang* water extract on IL-6 and nitric oxide production in lipopolysaccharide-activated RAW 264.7 cells

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SUMMARY

The present study was conducted to evaluate the effect of *Yongdamsagantang* (YST) on the regulatory mechanism of cytokines and nitric oxide (NO) for the immunological activities in RAW 264.7 cells. After the treatment of YST water extract, cell viability was measured by MTT assay, and NO production was monitored by measuring the nitrite content in culture medium. Inducible nitric oxide synthase (iNOS) and phospholylation of inhibitor of nuclear factor kappa B alpha (p-IκBα) were determined by Immunoblot analysis, and levels of cytokine were analyzed by sandwich immunoassays. Results provided evidences that YST inhibited the production of NO, iNOS, and interleukin-6, and the activation of p-IκBα in RAW 264.7 cells activated with lipopolysaccharide. These findings showed that YST could have some anti-inflammatory effects which might play a role in therapy in Gram-negative bacterial infections.

Key words: *Yongdamsagantang;* LPS; Nitric oxide; Cytokine

INTRODUCTION

Yongdamsagantang (YST) is composed of Gentianae Radix, Bupuleuri Radix, Alismatis Rhizoma, Plantaginis Semen, Akebiae Caulis, Scutellariae Radix, Gardeniae Fructus, Angellicae Gigantis Radix, Rehmanniae Radix and Glycyrrhizae Radix (Yang, 2001). The combination of these herbs can reduce excessive fire and clear away damp-heat without damaging blood by reducing it.

Nowadays, YST is used to treat diseases marked by excessive fire in the liver meridian, such as acute conjunctivitis, acute otitis media, acute hepatitis and acute cholecystitis; or to deal with cases with downward flow of damp-heat in the liver meridian as seen in acute pyelonephritis, cystitis, urethritis, acute pelvic inflammation, vulvitis and orchitis (Yang, 2001).

According to the literature, the therapeutic effects of many Oriental herbs are attributable to the phenolic substances (Surh, 1999). Indeed, certain phenolic compound of plants has often used as herbal anti-inflammatory remedies. COX-2 and iNOS are important enzymes that mediate inflammatory process (Wu, 1996). In recent, there have been many studies about natural products which have anti-inflammatory activities, for example, Polygonum tinctorium (Kawamata *et al.*, 2000), Melia azedarach (Lee *et al.*, 2000), Cyperus rotundus (Seo *et al.*, 2001), Chrysanthemum morifolium (Na *et al.*, 2006) Cudrania tricuspidata (Seo *et al.*, 2000), Angelicae Sinensis Radix (Jang *et al.*, 2002) and Farfarae Flos (Yoon *et al.*, 2004).

Improper up-regulation of COX-2 and/or iNOS

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has been associated with pathophysiology of certain types of inflammatory disorders. Since inflammation is closely linked to substances with potent anti-inflammatory activities, they are anticipated to exert chemopreventive effects on carcinogenesis particularly in the promotion stage. For example, there are resveratol and the green tea polyphenol epigallocathechin gallate (EGCG) that strongly suppress inflammation. Recent studies have demonstrated that nuclear factor-kappa B (NF-κB) is involved in regulation of COX-2 and iNOS expression (Yoon *et al.*, 2004).

So, we studied to evaluate the effects of YST on the regulatory mechanism of cytokines and NO for the immunological activities in RAW 264.7 cells.

MATERIALS AND METHODS

Preparation of extract

The composition of YST originated from collection of prescriptions with notes, but the dose of it was not recorded in it. So we have experimented on this prescription in accordance with the dose of Chinese medical formulae (Yang, 2001).

YST was prepared by boiling 3 pack of YST in water (234 g in 5 L) for 3 h. The YST was filtered through a 0.2 μm filter (Nalgene, New York, NY, USA) and stored at -20°C until use. The amount of YST was estimated by the dried weight of lyophilized YST. The yield of lyophilized YST was 12.24%.

The composition of YST is described in Table 1. These plants materials were purchased from Youngnam pharm (Daegu, Korea).

Table 1. Composition of *Yongdamsagantang* (YST; per one pack)

Pharmacological name	Dose (g)
Gentianae Radix	6
Bupuleuri Radix	6
Alismatis Rhizoma	12
Plantaginis Semen	9
Akebiae Caulis	9
Scutellariae Radix	9
Gardeniae Fructus	9
Angellicae Gigantis Radix	3
Rehmanniae Radix	9
Glycyrrhizae Radix	6
Total	78

Cell culture

RAW 264.7 cells, a murine macrophage cell line (KCLRF, Korean Cell Line Research Foundation, Seoul, Korea), were cultured in Dulbecco's modified Eagle's medium (DMEM, Cambrex Bio Science, MD. USA) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. RAW 264.7 cells were plated at a density of 2 - 3×10^6 cells/ml and pre-incubated for 24 h at 37°C. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. For all experiments, cells were grown to 80 - 90% confluency, and were subjected to no more than 20 cell passages. RAW 264.7 cells were incubated in the medium without 10% FBS for 12 h and then exposed to lipopolysaccharide (LPS) or LPS+YST for the indicated time periods (6 - 24 h). YST dissolved in medium (EMEM, Cambrex Bio Science, MD. USA) was added to the incubation medium 1h prior to the addition of LPS.

Reagents

LPS (Escherichia coli 026:B6; Difco, Detroit, MI, USA) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoleum (MTT) were obtained from Sigma (St. Louis, MO, USA). FBS and antibiotics were purchased from Gibco/BRL (Eggenstein, Germany). Antibodies were obtained from BD Bioscience (USA), Cayman (USA) and Zymed (USA), and NC paper was obtained from Schleicher & Schuell (USA). Tumor Necrosis Factor-alpha (TNF-α) and Interlukine-6 (IL-6) ELISA Kits were purchased from Pierce endogen (Rockford, IL, USA).

Cell viability

RAW 264.7 cells were plated at a density of 5 × 10^4 cells/well in a 96 well plate to determine cytotoxic

concentrations of YST. Cells were exposed to YST at the concentrations of 0.1 through 3 mg/ml at 37°C under 5% CO_2. After incubation of the cells in the presence of YST, viable cells were stained with MTT (0.5 mg/ml) for 4 h. The media were then removed and produced formazan crystals in the wells were dissolved by addition of 200 μl of dimethylsulfoxide (DMSO). Absorbance was measured at 540 nm using a Titertek Multiskan Automatic ELISA microplate reader (Model MCC/340, Huntsville, AL, USA). Cell viability was defined relative to untreated control cells [viability (% control) = 100 × [(absorbance of treated sample)/(absorbance of control)].

Assay of nitrite production

NO production was monitored by measuring the nitrite content in culture medium. This was performed by mixing the samples with Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylenediamine dihydrochloride and 2.5% phosphoric acid). Absorbance was measured at 540 nm after incubation for 10 min.

Immunoblot analysis

Cells were lysed in the buffer containing 20 mM Tris HCl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1mM sodium orthovanadate, 25 mM b-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride and 1mg/ml leupeptin. Cell lysates were centrifuged at 10,000 × g for 10 min to remove debris. Expression of iNOS was immunochemically monitored in the lysate fraction of RAW 264.7 cells using anti-mouse iNOS antibody. Polyclonal anti p-IκBα antibody was used to assess p-IκBα protein in cytosol. The secondary antibodies were alkaline phosphatase conjugated anti-mouse and anti-goat antibodies. The bands of iNOS and p-IκBα proteins were visualized using ECL western blotting detection reagents (Amersham, USA) according to the manufacturer's instruction.

Measurement of cytokine production

For cytokine immunoassays, the cells (1×10^6/ml) were pre-incubated 1 h with YST and further cultured for 6 or 12 h with 1 μg/ml of LPS in 6-well plates. Supernatants were removed at the allotted times and TNF-α and IL-6 productions were quantified by ELISA Kit (Pierce endogen, Rockford, IL, USA) according to the manufacturer's instructions, respectively. Briefly describing, 50 μl of Bionylated Antibody Reagent and samples was added to anti-mouse TNF-α and IL-6 precoated 96-well strip plates. The plates were covered at room temperature for 2 h, washed three times in prepared washing buffer, and added with 100 μl of streptavidin-HRP concentrate. After 30 min incubation at room temperature, the wells were washed three times, and then 100 μl of tetramethylbenzidine (TMB) substrate solution was added and developed in the dark at room temperature for 30 min. And 100 μl of TMB stop solution was added to stop the reaction, and the plates were measured with absorbance at 450 nm minus 550 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant TNF-α and IL-6 in serial dilutions.

Statistical evaluations

Data are expressed as mean ± S.D. of results obtained from number (n) of experiments. One-way analysis of variance (ANOVA) procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman-Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at $P < 0.05$.

RESULTS

Inhibitory effect of YST on LPS-stimulated NO production

To investigate the inhibition of NO production by YST in RAW 264.7 cells, we measured NO production in culture medium of RAW 264.7 cells

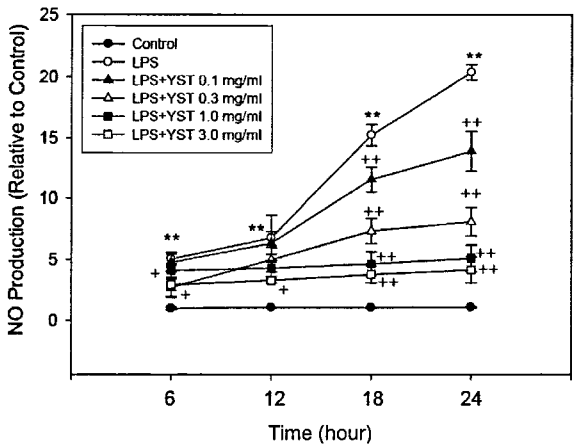

Fig. 1. Inhibitory effects of YST on the production of NO in LPS stimulated RAW 264.7 cells. RAW 264.7 cells were treated with 0.1 - 0.3 mg/ml concentrations of YST dissolved in EMEM for 1 h prior to the addition of LPS (1 μg/ml), and the cells were further incubated for 6 - 24 h. Control cells were incubated with vehicle alone. The concentrations of nitrite and nitrate in culture medium were monitored as described in the Experimental procedures. Data represent the mean ± S.D. with eight separate experiments. One-way ANOVA was used for comparisons of multiple group means followed by Newman-Keuls test (significant as compared to control, $^{**}P < 0.01$, significant as compared to LPS alone, $^{+}P < 0.05$, $^{++}P < 0.01$).

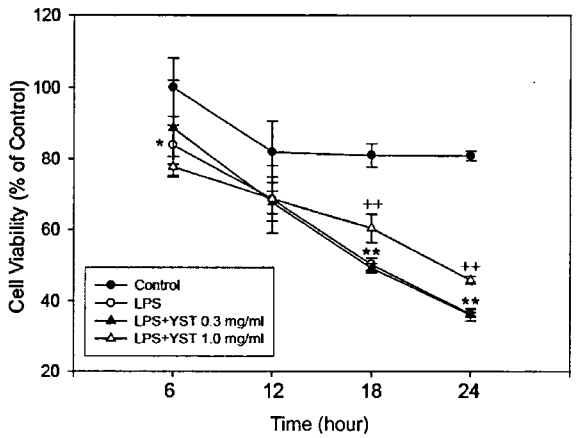

Fig. 2. Effect of YST on the cell viability in LPS stimulated RAW 264.7 cells. Each bar shows the mean ± S.D. of three independent experiments performed in triplicate (significant as compared to control, $^{*}P < 0.05$, $^{**}P < 0.01$, significant as compared to LPS alone, $^{++}P < 0.01$).

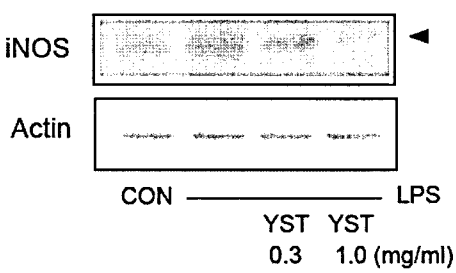

Fig. 3. Inhibitory effect of YST on the induction of iNOS by LPS. The level of iNOS protein was monitored 18 h after treatment of cells with LPS (1 μg/ml) with or without YST pretreatment (i.e. 1 h before LPS).

treated with the concentration of 0.1 - 3.0 mg/ml YST. As shown in Fig. 1, in LPS plus YST groups, NO production was decreased in a concentration dependent manner as compared with LPS group. In 0.1 - 0.3 mg/ml of YST group, NO production was significantly inhibited at 18 h and 24 h (Fig. 1).

Effects of YST on cell viability

To investigate whether the reduction of NO production is contributed to the decrease of cell population by YST, we measured cell viability by MTT assay at the degree of concentration and at the time manner. The results suggested that the used dosage of YST did not exhibit any cell toxicity during 6 - 24 h compare with LPS alone (Fig. 2).

Inhibitory effect of YST on LPS-stimulated iNOS expression

We examined iNOS protein expression in cytosol fraction using immunoblotting analysis. iNOS protein strongly induced by LPS. The groups of 0.3 and 1.0 mg/ml of YST with LPS showed the reduction of iNOS protein expression at the concentration dependent manner (Fig. 3).

Effects of YST on LPS-stimulated p-IκBα expression

We measured the phosphorylated form of IκBα. To assess whether YST could directly affect p-IκBα expression in macrophage cell, the level of p-IκBα protein expression was immunochemically assessed in RAW 264.7 cells incubated with or without YST. LPS induced the p-IκBα expression, however, 0.3 mg/ml of YST reduced LPS-induced p-IκBα

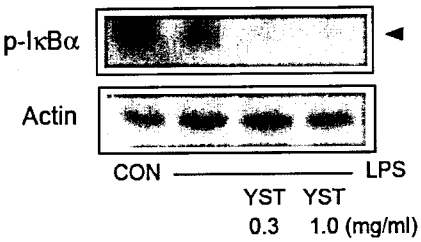

Fig. 4. Inhibitory effect of YST on the induction of p-IκBα by LPS. The level of p-IκBα protein was monitored 15 min after treatment of cells with LPS (1 μg/ml) with or without YST pretreatment (i.e. 1 h before LPS).

expression, and 1.0 mg/ml of YST markedly reduced the protein levels of p-IκBα expression in a dose dependent manner (Fig. 4).

Effects of YST on LPS-stimulated TNF-α production

TNF-α, interleukin-1β (IL-1β), interleukin-2 (IL-2), and IL-6 are frequently considered as pro-inflammatory cytokines. These cytokines are involved in a variety of immunological functions as well as interaction with a variety of target cells (Delgado *et al.*, 2003). As shown in Fig. 5, LPS

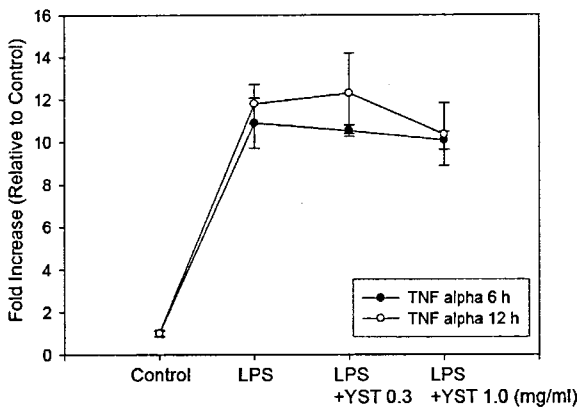

Fig. 5. Effect of YST on LPS-stimulated TNF-α production. Production of TNF-α was measured in the medium of RAW 264.7 cells cultured with LPS (1 μg/ml) in the presence or absence of YST for 6 and 12 h. The amount of TNF-α was measured by immunoassay as described in Experimental procedures. Data represent the mean ± S.D. with three separate experiments. One-way ANOVA was used for comparisons of multiple group means followed by Newman-Keuls test.

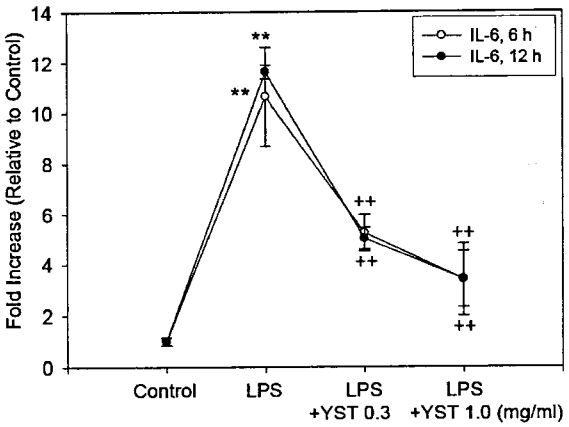

Fig. 6. Inhibitory effect of YST on LPS-stimulated IL-6 production. Production of IL-6 was measured in the medium of RAW 264.7 cells cultured with LPS (1 μg/ml) in the presence or absence of YST for 6 and 12 h. The amount of IL-6 was measured by immunoassay as described in Experimental procedures. Data represent the mean ± S.D. with three separate experiments. One-way ANOVA was used for comparisons of multiple group means followed by Newman-Keuls test (significant as compared to control, $^{**}P < 0.01$, significant as compared to LPS alone, $^{++}P < 0.01$).

increased the TNF-α production, however 0.3 or 1.0 mg/ml of YST did not affect the levels of LPS-induced TNF-α production (Fig. 5).

Effects of YST on LPS-stimulated IL-6 production

IL-6, secreted primarily by monocytes and macrophages, is always found with increased levels at the sites of inflammation (Delgado *et al.*, 2003). Then we measured the effect of YST on LPS-induced IL-6 production. In this experiment, LPS significantly increased the secretion of IL-6, and 1.0 and 3.0 mg/ml of YST significantly reduced IL-6 production in 6 and 12 h (Fig. 6).

DISCUSSION

Yongdamsagantang is composed of Gentianae Radix, Bupuleuri Radix, Alismatis Rhizoma, Plantaginis Semen, Akebiae Caulis, Scutellariae Radix, Gardeniae Fructus, Angellicae Gigantis Radix, Rehmanniae Radix and Glycyrrhizae Radix (Hu *et al.*, 1999).

In the prescription, Gentianae Radix extremely bitter in flavor and cold in nature, is used to purge excessive fire in the liver and gallbladder and clear damp-heat in the lower-warmer, providing the principal curative efficacy. Scutellariae Radix and Gardeniae Fructus, being bitter in flavor and cold in nature, are used in combination to reinforce the effect of the Gentianae Radix, playing the role of an assistant drug. Functioning together as adjuvant and guiding drugs, Alismatis Rhizoma, Akebiae Caulis and Plantaginis Semen have the effect of removing heat and inducing diuresis to dispel damp-heat while Angellicae Gigantis Radix and Rehmanniae Radix possess the effect of nourishing yin and blood so as to soothe the liver. Bupuleuri Radix and Glycyrrhizae Radix work in combination as guiding drugs, with the former having the effect of soothing the liver and regulation the circulation of qi in the liver and gallbladder, and inducing the efficacy of all the medicines into the liver and gallbladder, and the latter having the effect of coordination the effect of various ingredients in the recipe so as to prevent the stomach from being hurt by the bitter and cold property of the drugs (Hu *et al.*, 1999).

Clinically and experimentally, it is ascertained that the recipe has the effect of bringing down the fever, tranquilizing the mind, relieving inflammation, inducing diuresis, resisting bacteria, promoting the function of gallbladder, protecting the liver, arresting bleeding and decreasing blood pressure. But, since most of the drugs in the recipe are bitter in flavor and cold in property, constant and long-term administration of it is not advisable (Hu *et al.*, 1999).

Nowadays, this YST is used to treat diseases marked by excessive fire in the liver meridian, such as acute conjunctivitis, acute otitis media, acute hepatitis and acute cholecystitis; or to deal with cases with downward flow of damp-heat in the liver meridian as seen in acute pyelonephritis, cystitis, urethritis, acute pelvic inflammation, vulvitis and orchitis (Yang, 2001).

NO plays a dual role as a beneficial or detrimental molecule in the inflammatory process. iNOS produces a high output of NO during inflammation, whereas constitutively expressed NOS (cNOS) generates a physiologically low level of NO (MacMicking *et al.*, 1997; Kubes, 2000; Bogdan, 2001).

iNOS-catalyzed oxidation of L-arginine leads to generation of the free radical gas NO, one of the most versatile modulators of cellular function with potent cytotoxic and immunomodulatory properties (Palmer *et al.*, 1998; Miljkovic *et al.*, 2004).

Although iNOS-derived NO primarily acts as a potent antimicrobial and tumoricidal agent, its uncontrolled release has also been implicated in inflammatory destruction of the target tissue in infection, autoimmunity or during transplant rejection (MacMicking *et al.*, 1997; Bogdan, 2001). At lower concentrations, NO can generate or modify intracellular signals, profoundly affecting the function of the immune cells, as well as tumor cells and resident cells of different tissues and organs (MacMicking *et al.*, 1997; Bogdan, 2001). For these reasons, modulation of iNOS-mediated NO release appears as plausible candidate for therapeutic optimization of protective immunity and prevention of detrimental effects of inflammation (Miljkovic *et al.*, 2004).

Here, we demonstrated that YST inhibited production of NO and IL-6 and expression of iNOS in activated macrophages by LPS. These inhibiting effects are mediated through the inhibition of phosphorylation of IκBα. To investigate the relation of iNOS and NO production, we examined iNOS protein expression by using immunobloting analysis. iNOS protein strongly induced by LPS. The groups of 0.3 and 1.0 mg/ml of YST with LPS showed the reduction of iNOS protein expression at the concentration dependent manner.

NF-κB, AP-1 and C/EBP have been well defined, to be associated with iNOS and COX-2 expression. Among these, the NF-κB is a functionally transcriptional factor (Park *et al.*, 2002; Castranova, 2004). NF-κB is involved in the inhibition of cell

apoptosis, cell cycle regulation and oncogenesis (Chen *et al.*, 2001; Sanlioglu *et al.*, 2004). The NF-κB plays an important role in the regulation of immune and inflammation response, such as MHC-I, MHC-II, interferon regulatory factor-1 (IRF-1), and diverse cytokines (TNF-α, IL-1, IL-6, IL-8, GM-CSF, G-CSF, M-CSF, MCP-1 and RANTES) (Ghosh *et al.*, 1998; Ravi *et al.*, 2004). Wide stimuli including toxic materials like virus and bacteria activate NF-κB. Activated NF-κB translocates into nucleus (Ravi *et al.*, 2004), and modulates the expression of iNOS or TNF-α in nucleus (Bolli *et al.*, 2002; Lee *et al.*, 2003; Miljkovic *et al.*, 2004). In resting cells, NF-κB is sequestered in the cytoplasm as an inactive form through association with one of several inhibitory molecules like IκBα, IκBβ, IκBε, p105 and p100 (Shishodia *et al.*, 2004). Activation of the signaling cascade of NF-κB results in a complete degradation of IκB or partial degradation of the carboxyl termini of p105 and p100 precursors. So the translocation of NF-κB to the nucleus induces transcription of COX-2, iNOS, Bcl-$_{XL}$ and cIAPs in nucleus. The kinds of IκB protein have been known to IκBα, IκBβ and IκBε. Among them IκBα is the most abundant inhibitory protein for NF-κB (Chen *et al.*, 2001).

Phosphorylation of IκBs at two critical serine residues (Ser^{32} and Ser^{36} in IκBα, Ser^{19} and Ser^{23} in IκBβ) in their N-terminal regulatory domain by the IκB kinase (IKK) complex, targets them for rapid ubiquitin-mediated proteasomal degradation (Karin *et al.*, 2000; Senftleben *et al.*, 2002).

In the p-IκBα level induced by LPS, 0.3 mg/ml of YST reduced p-IκBα expression, and 1.0 mg/ml of YST reduced more than 0.3 mg/ml.

TNF-α, IL-1β, IL-2 and IL-6 are frequently encountered pro-inflammatory cytokines. These cytokines are involved in immunological functions and interactions with various target cells (Delgado *et al.*, 2003).

In mammals, TNF-α is a well-known pro-inflammatory cytokine with a wide range of biological functions. The actions of these factors are diverse and profound involving inflammation, apoptosis, cell proliferation and the stimulation of various aspects within the immune system. TNF-α is known to affect the growth, differentiation, survival and physiological function of a variety of different cells, including cells outside of the immune system (Beutler *et al.*, 1988; Beutler *et al.*, 1989; Vassalli, 1992). TNF-α is a true pleiotropic factor which plays an important role in the immune response as well as in other physiological processes, such as metabolism and reproduction (Goetz *et al.*, 2004).

IL-6 is one of a family of cytokines that act through the gp130 receptor, and is an extremely important cytokine in the regulation of inflammation and immunity (Bravo *et al.*, 2000). IL-6 stimulates lymphocyte activation and proliferation (Hedger *et al.*, 2003).

As results, LPS induced the TNF-α and IL-6 production. And 0.3 and 1.0 mg/ml of YST reduced the level of IL-6 production, however the YST did not affect the level of TNF-α production. These findings showed that YST could produce some anti-inflammatory effects which might play a role in adjunctive therapy in Gram-negative bacterial infections.

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