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The Effects of *Cninnier(L)Cuss*(蛇床子) on the Esscntial Elements to the Activated Macrophages

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國文抄錄

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外治에 응용되는 蛇床子의 抗炎症效果를 살펴보고자 NO의 生成濃度, 細胞 生存度, iNOS의 합성 및 TNF-α의 生成濃度를 實驗하였다. 그 結果 NO의 濃度는 顯著하게 減少하였고, 細胞 生存度는 蛇床子에 의하여 影響을 받지 않았으며, iNOS의 합성에 있어서 iNOS의 濃度가 顯著하게 낮아졌고, TNF-α의 濃度는 顯著하게 減少하였다.

以上을 살펴볼 때 蛇床子가 iNOS및 TNF-α의 생성자체의 억제를 통하여 NO의 合成을 조절함으로써 蛇床子가 충분한 抗炎症效果가 있음을 알 수 있었다. 향후 蛇床子의 성분분석등 다양한 연구가 진행되어야 할 것으로 생각되며, 이를 통하여 앞으로 抗炎症效果가 우수한 약제의 개발이 가능할 것으로 思料된다.

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I . Introduction

SaSangja(蛇床子: SSJ), a seed of *Cnidium monnieri(L.) Cuss*, has been used for centuries in oriental medicine. Properties and flavors of SSJ are bitter and warm in nature(1).

Pharmacologic function of SSJ is anti-fungus, anti-virus, expel intestinal parasites, anti-tricomonas etc(2). This herbs has traditionally been used to treat itching sensation, urticaria, eczema, gynecological diseases, destroy intestinal parasites, viginitis, labor pains, pharyngolaryngitis etc, by the oral administration or external treatment method (3, 4). This herbs principles and methods of treatments are expel wind and destroy intestinal parasites (祛風殺蟲) and warm the kidneys to reinforce its vital function (溫腎助陽)(1, 2, 4, 5).

Inflammation is a complex localized response to foreign substances such as bacteria or in some instance to internally produced substances and Macrophages perform specific immunologic and nonspecific inflammatory functions(6). And also activated macrophages are generate large amount of Nitric oxide(NO) and tumor necrosis factor-alpha(TNF-α), etc.

Nitric oxide(NO) and tumor necrosis factor-alpha(TNF-α) have been suggested to play an important role in endotoxin shock and inflammation.

No, particularly synthesized by iNOS(inducible Nitric oxide synthase), is involved in the regulation of many cell functions and in the expression of several diseases including trauma(7), sepsis(8,9), atherosclerosis, multiple sclerosis(10), arthritis(11,12). And accumulating evidences indicate that excessive production of NO plays a pathogenic role in both acute and chronic inflammations(13).

In the present study, we aimed anti-inflammatory effects of SSJ, so we investigated effects of NO and TNF-α secretion on the murine macrophages.

II. Materials and Methods

2-1. *Reagents*

Thioglycollate(TG) broth(Brewer) was purchased from DIFCO(Detroit, MI). Murine rIFN-(1106 U/mg), Murine rTNF-(1106 U/ml), rabbit anti-murine TNF- polyclonal antibody (neutralizing), and hamster anti-mutine TNF- monoclonal antibody were purchased from Genzyme(Munchen, Germany). Anti-murine iNOS polyclonal antibody was purchased from Santa Cruz Biotechnology(Santa Cruz, CA). LPS(phenol extracted *Salmonella enteritidis*), Tween-20, BSA, phosphatase-conjugated goat anti-rabbit IgG, and *p*-nitrophenyl phosphate, nitrate reductase(from *Aspergillus* species), -NADPH, sodium pyruvate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Lactate dehydrogenase was purchased from Amresco(Solon, OH). 24, 96-well tissue culture plates and 60 mm culture dishes were purchased from Nunc, Inc. (North Aurora Road,

IL). RPMI containing L-arginine(200 mg/L), FBS, and other tisue culture reagents were purchased from Life Technologies(Gaithersburg, MD). All reagents and media for tissue culture experiments were tested for LPS content with use of colorimetric Limulus amoebocyte lysate assay(detection limit, 10 pg/ml; Whittaker Bioproducus, Walkersville, MD).

2-2. *Preparation of extracts*

An extract of SSJ was the dried prescription of herbs with boiling distilled water. The duration of decoction was about 3h. The decoction was filtered with 0.45㎛-filter, and concentrated through a evapolator resulting in a semifluid. Then lyophilized through freeze-dried and kept at 4℃.The dried extract was dissolved in phosphate buffered saline(PBS) and through 0.22m filter before use. This herb was obtained from Dong-Eui Oriental Medicine Hospital (Pusan, Korea).

2-3. *Macrophage culture*

The original stock of C57BL/6 mice were purchased from Dae Han Animal Center(DHAC, Korea) and the mice were maintained in an airconditioned room with lighting from 7 a.m. to p.m. The room temperature(about 23℃) and humidity(about 60%) were controlled automatically. A labolatory pellet chew(Sam-Yang Co.) and water given freely. The mouse was adapted themselves for 2 weeks to lab circumstances before being used.

TG-elicited macrophages were harvested 3days after i.p. injection of 2.5ml of 3% TG into mice 8-12 weeks of age, as reported previously(14).Peritoneal lavage was performed twice by using 10ml of HBSS, which contained 10 U/ml heparin. Then, the cells were distributed in RPMI, which was supplemented with 10%(v/v) FBS, in either 24-well tissue culture plates(2×105 cells/well) or 60-mm diameter plastic petri dishes(1×107 cells/dish), and incubated for 3h at 37℃ in an atmosphere at 5% CO2. Nonadherent cells were removed by suction, and then freshly prepared complete media were added with the indicated experimental reagents.

2-4. *Measurement of nitric oxide(Griess assay)*

Nitric oxide synthesis in cell was measured by a microplate assay method as a Griess reaction(15). Briefly, 100㎕ of cell culture supernatant were removed and combined with an equal volume of the Griess reagent(1:1 mixture of 0.1% *N*-(1-naphthyl)-ethylenediamine and 1% sulfanilamide) in a 96well plate, followed by spectrophotometric measurement at 540nm using a microplate reader(Molecular Devices Co., Melno park, CA, USA). The Nitrite(NO2-) concentration in the supernatants were determined by comparison with sodium nitrite standard curve. And the cell-free medium alone contained 5-8μM of nitrite. This value was determined in each experiments and substracted from the value obtained from the medium cells. Data are expressed as the total μM nitrite produced by 1×105cell for 24hr. Data are expressed as the mean±S.E.

2-5. *Cell viability assay*

The cytotoxicity of SSJ was assessed using microculture tetrazolium(MTT, 3-(4,50dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Chemical Co., St. Louis, MO, USA)-based colorimetric assay. The remaining cells after Griess reaction were used for cell viability. The 10㎕ of MTT solution(5㎎/㎕) was added to each well. After incubation for 3hr at 37℃ and 5% CO2, the formed formazan crystals in viable cells were solubilized with 100㎕ of DMSO. The absorbance of each well was then read at 570nm using microplate reader.

2-6. *Assay of TNF-α secretion*

TNF-α secretion was measured by modification of an enzyme linked immunosorbent assay(ELISA), as previously described(16). The ELISA was sensitive to TNF-α concentrations in medium above 40pg/ml. The ELISA was devised by coating 96-well plates with 6.25ng/well of murine monoclonal antibody with specificity for murine TNF-α. Before use and between subsequent steps in the assay, coated plates were washed twice with PBS containing 0.05% Tween 20 and twice with PBS alone. All reagents used in this assay were incubated for 1h at room temperature with coated wells. For the standard curve, TNF-α was added to serum previously determined to be negative for endogenous TNF-α. After exposure to the medium, assay plates were sequentially exposed to rabbit anti-TNF-α, phosphatase-conjugated goat anti-rabbit IgG, and *p*-nitrophenyl. Optical density reading at 410nm were taken using a Emax 96-well microtest plate spectrophotometer(Molecular Devices, Menlo Park, CA, USA). Appropriate specificity controls were included.

2-7. *Western blot analysis*

Peritonial macrophages were incubated with or without various concentration of SSJ and stimulated 1㎍/㎖ of LPS, IFN-γ. Cells were washed three times with ice-cold PBS(calcium and magnesium-free phosphate buffered saline), and lysed in 0.2㎖ of ice-cold lysis buffer(50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride, 1㎍/㎖ leupeptin, 150mM Nacl) and homogenized to obtain total cell lysate. An aliquot of lysate was used to determine protein concentration by the Bradford method(17). 30㎍/lane of protein was loaded onto 10% SDS-polyacrylamide gels to detection iNOS. After running at 20mA for 1h, the sized-seperated proteins were transferred a Nitrocellulose membrane to 0.45μM at 60V for 2h. The blot was blocked with 5% skim milk for 3h, washed with 0.1% TPBS(PBS containing 0.1% Tween 20) and then incubated with specific iNOS(Upstate Biotechnology, Inc., Lake Placid, NY, USAI) monoclonical antibodies diluted 1:1000 for 1hr. After washing three times in 0.1% TPBS, the anti-rabbit secondary antibodies conjugated with horseradish peroxidase(Amersham, Buckinghamshire, UK) were diluted 1:1000 and incubated for 1hr. An Amersham ECL system(Amersham, Buckinghamshire, UK) was used for detection and exposed to x-ray film(Agfa, Belgium).

2-8. *Statistical analysis*

Data collected were expressed as means±S.E.

Statistical analysis was performed by the Student's t-test to the difference between two groups.

Ⅲ. Results

3.1. Effect of SSJ on NO production in LPS-stimulated murine macrophages.

To investigate the molecular mechanisms of anti-inflammatory activity of SSJ, we tested the effects of SSJ on nitrite production in LPS-stimulated murine macrophages. TG-elicited murine macrophages were incubated with SSJ for 2h. Then, murine macrophages were stimulated with LPS(1 ng/ml). And were incubated for 24 hr. The production of nitrite concentration was determined by measuring the culture medium with Griess reagent. As shown in Fig.1, unstimulated murine macrophages produced 4.97±0.43 mM NO. LPS-stimulated cells without SSJ produced 24.55±2.96 mM NO. When murine macrophages were treated with 0.01, 0.1, 0.2, 0.5 mg/ml SSJ, the cells produced 19.83±2.96, 15.96±3.88, 11.17±2.34, 4.37±1.58 mM NO, respectively. SSJ suppressed NO release into culture supernatant in a dose-dependent manner. And NO products treat with 0.5mg/ml SSJ decreased dramatically to the basal level. But cell viability was not affected by SSJ as measured by MTT assay(Fig.2). These results suggest SSJ inhibits LPS-induced NO production in murine macrophages without effect on the cell death.

3.2. Effect of SSJ on expression of iNOs in LPS-stimulated murine peritoneal macrophages

Since the expression of iNOS is primarily responsible for the NO overproduction in LPS-stimulated murine peritoneal macrophages, we analyzed the amount of iNOS by western blot analysis. TG-elicited murine peritoneal macrophages were incubated with SSJ for 2h, 0.2mg/ml, 0.5mg/ml, respectively. Then, murine peritoneal macrophages were stimulated with LPS(1 ng/ml) for 24h. The level of iNOS was reduced by SSJ in a dose-dependent manner(Fig. 3). The result of western blot analysis indicated that SSJ inhibits NO release by modulating the iNOS expression level in the LPS-stimulated murine peritoneal macrophages.

3.3. Effect of SSJ on the TNF-α secretion in the LPS-stimulated murine peritoneal macrophages

IFN-g and/or LPS-stimulated induced TNF-α secretion is known to be crucial for synergistic induction of NO synthesis murine peritoneal macrophages(18). So, we investigated TNF-α secretion in the LPS-stimulated murine peritoneal macrophages. To examine whether the SSJ evokes reduction of NO synthesis by the regulation of TNF-α production, we assessed quantitatively the amount of TNF-α secretion. TG-elicited murine peritoneal macrophages were incubated with SSJ for 2h, 0.01, 0.5mg/ml, respectively. Then, murine peritoneal macrophages were stimulated with LPS(1 ng/ml) for 20h. After, TNF-α in culture medium was assayed by capture ELISA(Fig. 4).

As shown in Fig. 4 instimulated murine peritoneal macrophage produced 0.970 ± 0.09 ng/ml. LPS-stimulated cells without SSJ produced 14.26 ± 0.60 ng/ml. When murine peritoneal macrophage were treated with 0.01, 0.1, 0.25, 0.5 mg/ml SSJ, the cells produced 9.57 ± 0.18, 7.02 ±0.17, 5.70 ± 0.24 , 6.60 ± 0.25 ng/ml, respectively, TNF-α secretion treat with SSJ was significantly decreased in a dose-dependent manner.

Ⅳ. Discussion

Oriental medicine views the body as an Ki (氣) and emphasizes abnormality in the balance or flow of Ki(氣) as the sources of disease. Thus, the basic principle of the Oriental medicine therapy is to regulate the homeostasis of the whole body and to normalize the physical disorders.

One mainstay of Oriental medicine is the administration of medicinal herbs with a combination of a number of ingredients. But some Oriental medicine have been treated by spreading on the skin(19).

SSJ has been used for centuries in oriental medicine. The one of the purposes are that treat the dermatitis by the oral administration or external treatment method. This herbs principles and methods of treatments is expel wind and destroy intestinal parasites (祛風殺蟲) and warm the kidneys to reinforce its vital function (溫腎助陽)(1, 2, 4, 5). Their major constituents are known to be essential oil like as l-pinene, camphene, isovaleric acid, borneol-iso-va-larate and methoxy stenol (1, 2), etc.

Lipopolysaccharides(LPS), also termed endotoxins, are a family of toxic phosphorylated glycolipids derived from the cell envelope of gram-negative bacteria(20, 21). They are essential for the physical integrity and function of the outer membrane, from which they can be released by multiplication or lysis of bacteria(22). LPS are endowed with a broad spectrum of toxic properties, such as pyrogenity, hypotension, and shock, resulting in impaired perfusion of essential organs, including the death from massive multiorgan dysfunction(23, 24).

These protective effects of LPS have been attributed to the stimulation of certain protective cytokines such as interleukins-1(25) or augmentation of isoform inducible of nitric oxide synthase(iNOS)(26).

Macrophages perform specific immunologic and nonspecific inflammatory functions(15), Macrophages are relatively quiescent cells unless they are stimulated, however, if it is stimulated by the number of bacterial products including Gram-negative endotoxin(LPS) cause macrophage to activate in vivo. If macrophages are activated, results in increased oxygen consumption increased functional ability (including phagocytosis and tumor cell killing), change in the surface markers(including MHC classⅡ) and the secretion of numerous biologically active products including both IL-1 and TNF-(27). Also, activated macrophages are generate large amounts of nitric oxide(NO) from

L-arginine by action of iNOS.

It is known that NO is synthesized by a number of cell types ranging in diversity from neurons to hepatocytes(28,29). NO is derived from the amino acid L-arginine by the enzyme nitric oxide synthase(NOS). Three isoforms of NOS are known to exist and can be classified into two categories: constitutive and inducible. Constitutive NOS enzymes are continually present in cells such endothelial cells(28,29), neurons(30,31), or cardiac myocytes(32) and can be immediately activated to produce small amounts of NO. The inducible NOS(iNOS) isoform must first be synthesized in cells such as hepatocytes(33,34), vascular smooth muscle cells(35,36), fibroblasts(37), or murine macrophages(38, 39), but then generates large amounts of NO.

Nitric oxide, particularly synthesized by iNOS, is involved in the regulation of many cell functions and in the expression of several diseases including trauma(7), sepsis(8,9), atherosclerosis, multiple sclerosis(10), arthritis (11,12). And accumulating evidences indicate that excessive production of NO plays a pathogenic role in both acute and chronic inflammations(13). Therefore, inhibition of NO overproduction could be beneficial in various forms of inflammatory diseases. Isozyme specific inhibitors of NOS are essential for therapeutic purposes and drugs that specifically inhibit iNOS could be useful in treating diseases mediated by NO overproduction(40). In fact, administration of the selective inhibitors of iNOS has been reported to attenuate periodonititis(41), osteoarthritis(42), experimental autoimmune myocarditis(43), inflammation and demyelination produced by virus infection(44), carrageenan-induced models of inflammation(45), multiple sclerosis(46) and shock(47). So we investigated whether the SSJ exhibit the anti-inflammatory effect by suppressing the NO synthesis in vitro.

Tumor necrosis factor is mainly produced by activated T lymphocytes, monocytes, and macrophages and plays critical roles in the regulation of immune responses against infectious agents(48). However, inappropriate production of TNF is implicated in mainly pathologic conditions such as septic shock(49,50), rheumatoid arthritis(51,52), cerebral malaria(53), and multiple sclerosis(54,55).

In this study, SSJ profoundly inhibited the production of NO, iNOS expression and TNF-α secreation in TG-elicited mouse peritoneal macrophages(Figs. 1, 3 and 4).

In addition, inhibition of NO synthesis is not likely due to cell death by SSJ because cell viability was not affected, while NO production was reduced to control level at 0.5 mg/ml of SSJ(Fig. 2).

SSJ also influences the production of TNF-α involved in the activation of macrophages, including their NO formation. TNF-α has been found to act synergistically on NO production and blocking of TNF-α by a specific antibody to indirectly decrease NO synthesis, impairing cells defense against parasites. So SSJ may inhibit the production of NO by suppressing TNF-α secretion.

As the effect of SSJ is mediated via inhibition of iNOS, the drug could perhaps be

used in pathological processes in which induction of NO is involved.

In conclusion, the aqueous extract of SSJ inhibited the production of NO and TNF-α in LPS-stimulated mouse peritoneal macrophages and reduced NO level in LPS-induced mouse peritoneal macrophages. This suggests that SSJ could be used as an anti-inflammatory medicinal herbs. Further studies on the precise mechanism of action and the isolation and characterization of the active chemical constituents are in progress.

V. Conclusion

To evaluate of SSJ on the anti-inflammatory effects, we observed NO production, Cell viability, iNOS expression, TNF-α concentration.

The results were as followed.

1. SSJ inhibits LPS-induced NO production in murine macrophages without effect on the cell death.
2. Cell viability was not affected by SSJ .
3. SSJ inhibits the iNOS expression in the LPS-stimulated murine peritoneal macrophages.
4. TNF-α secretion treat with SSJ was significantly decreased in a dose-dependent manner.

These results imply that SSJ has anti-inflammatory effects.

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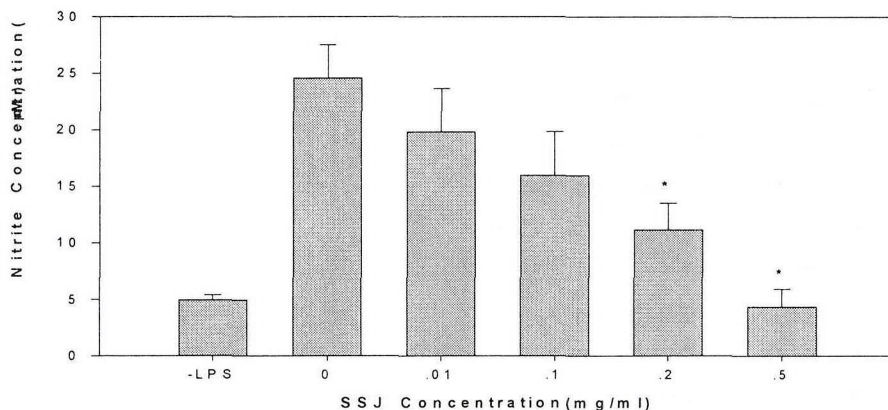

Fig. 1. Inhibition effect of *SSJ* on the nitrite production on the LPS-stimulated macrophages. TG-elicited murine macrophage(2105 cells/well) were incubated for 2 hr in the presence of the indicated concentrations of *SSJ*. Then, the cells were stimulated with LPS(1 ng/ml). After cells were incubated for 24 hr. The production of nitrite concentration was determined by measuring the culture medium with Griess reagent.
* P<0.005, significantly different from the LPS.

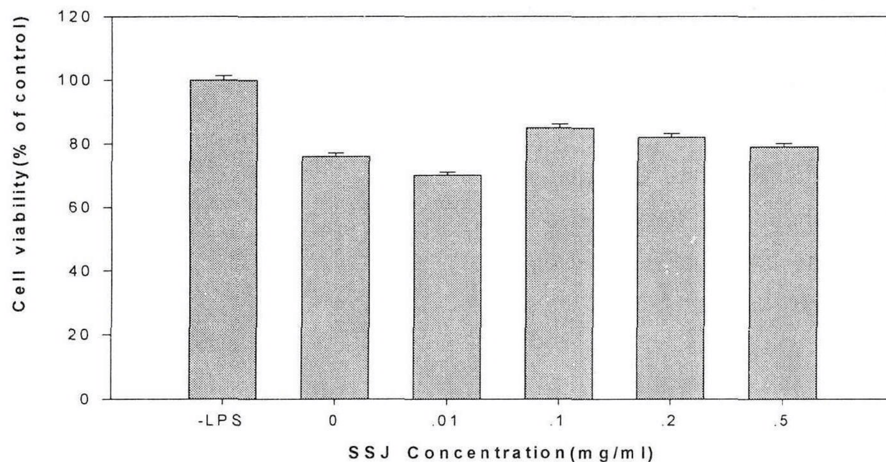

Fig. 2. Cell cytotoxicity effects of *SSJ* on the LPS-stimulated macrophages. TG-elicited murine macrophage(2105 cells/well) were incubated for 2 hr in the presence of the indicated concentrations of *SSJ*. Then, the cells were stimulated with LPS(1 ng/ml). After cells were incubated for 24 hr. No effect on cell viability was detected in the tested concentration as measured by the MTT assay.

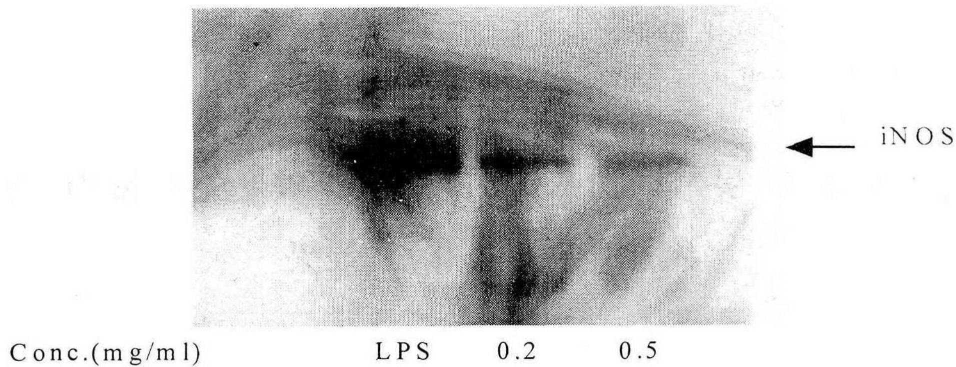

Fig. 3. Effects of *SSJ* on the synthesis of iNOS in the LPS-stimulated macrophages. TG-elicited murine macrophage(2105 cells/well) were incubated for 2 hr in the presence of the indicated concentrations of *SSJ*. Then, the cells were stimulated with or without LPS(1 ng/ml) and incubated for 24 hr. After, the cellular proteins were collected and then 10% SDS-PAGE was performed. Western blot was probed with a monoclonal antibody to iNOS. The position of m.w. marker and iNOS are indicated.

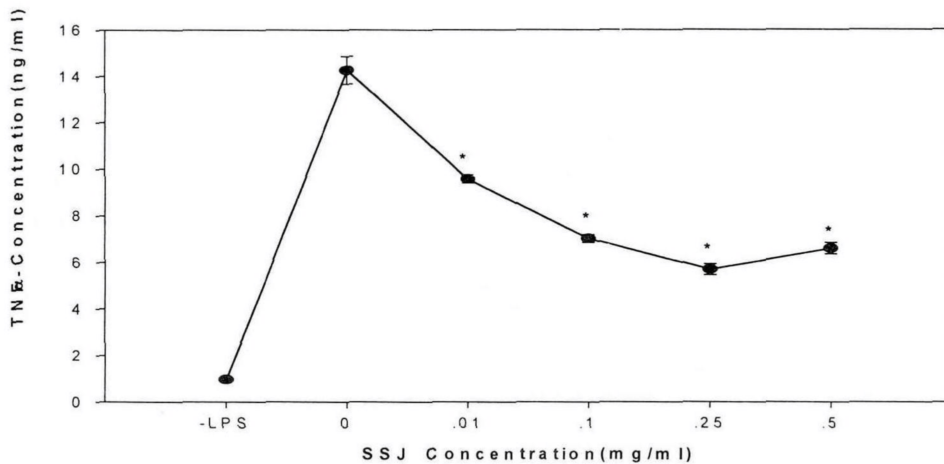

Fig. 4. Effects of *SSJ* on the LPS plus IFN--stimulated TNF- secretion in the murine macrophages. TG-elicited murine macrophage(2105 cells/well) were incubated for 2 hr in the presence of the indicated concentrations of *SSJ*. Then, the cells were stimulated with LPS(1 ng/ml) and incubated for 24 hr. The supernatants were collected 24 hr after stimulation. TNF-secretion was assayed by specific ELISA.

* P<0.005, significantly different from the LPS