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원 저

The Anti-Inflammatory Effects of *Takrisodok-eum* in the Ong-Juh(옹저)

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癰疽에 사용되는 托裏消毒飮의 抗炎症效果

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托裏消毒飮이 癰疽에 있어 抗炎症 效果를 살펴보고자 NO의 濃度, 細胞 生存度, iNOS의 表現 및 TNF-α의 濃度를 實驗한 結果, NO의 濃度는 顯著하게 減少하였고 細胞 生存度는 托裏消毒飮에 依하여 影響을 받지 않았으며 iNOS의 表現에 있어서 iNOS의 高度가 顯著하게 낮아졌고 TNF-α의 濃度는 顯著한 變化가 없었다.

以上을 살펴볼 때 托裏消毒飮이 癰疽에 있어 抗炎症效果가 있음을 알 수 있었다. 또한 托裏消毒飮이 充分히 大食細胞의 役割을 代身하며 이 以外의 信號經路가 炎症機轉에 作用한 것으로 생각되며 많은 研究가 必要할 것으로 思料된다. (J Korean Oriental Med 2000;21(1):45-52)

Introduction

Takrisodok-eum(托裡消毒飮: TRSDE) is a traditional orient herbal medicine formula consisting of twelve ingredients, formulated by Gong-Sin(龔信) in the Chinese Myong Dynasty(1578)[1]. This formula has traditionally been used to treat inflammatory diseases such as abscess, carbuncle and phlegmon, etc. by resolving inflammation (化膿), promoting tissue regeneration(生肌), dispersing stagnated blood(散瘀), inducing detumescence(消腫)[2-4,5].

Ong-Juh(癰疽) is one of the oriental medical name of disease, including all inflammatory disease of skin. In the view of western medicine, Ong-Juh(癰疽) is very similar to various acute and chronic inflammatory disease[6].

Inflammation is localized response to foreign substance such as bacteria or in some instance to internally produced substances and has relation with immunity system[7].

The macrophage plays a role in the development of the lymphohaemopoietic system before and after birth, as well as in the natural and acquired immune responses of the adult to immunogens, including infectious agents[8].

If macrophages that were activated by LPS are inactivated by oriental medicine, we will imply that this medicine has effects of anti-inflammation, but we cannot find studies about effects of the Takrisodok-eum that is widely used in the inflamm ation traditionally to the macrophages.

The aim of this study is to investigate the anti-inflammatory effects of the Takrisodok-eum on the macrophages.

After treating Takrisodok-eum on the macrophages, We observed NO concentration, cell viability, expression of iNOS, TNF-α concentration, and have obtained some significant results.

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So we reported this paper.

Materials and Methods

1. Reagents

Murine rIFN-(1×10^6 U/mg), Murine rTNF-(1×106 U/ml), rabbit anti-murine TNF- polyclonal antibody(neutralizing), and hamster anti-murine TNF-monoclonal antibody were purchased from Genzyme(Munich, Germany). Anti-murine iNOS polyclonal antibody was purchased from Santa Cruz Biotechnology(Santa Cruz, CA). LPS(phenol extracted Salmonella enteritidis), Thioglycollate(TG), broth (Brewer) were purchased from DIFCO(Detroit, MI). Tween-20, BSA, phosphatase-conjugated goat anti-rabbit IgG, and p-nitrophenyl phosphate, nitrate reductase(from Aspergillus species), β-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. Preparation of extracts

An extract of TRSDE 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 0.22㎛ filter before use. The composition and ingredients of TRSDE is described in Table 1. These plants materials were obtained from Dongeui Oriental Medicine Hospital (Pusan, Korea).

Table 1. Composition of Takrisodok-eum(托裡消毒飮)

Herbs	Scientific Name	Dose(g)
金銀花	Lonicerae Flos	12
陳皮	Auranti nobilis Pericarpium	12
黃芪	Astragali Radix	8
天花粉	Trichosanthis Radix	8
防風	Ledebouriellae Radix	4
當歸	Angelicae gigantis Radix	4
川芎	Cnidii Rhizoma	4
白芷	Angelicae Radix	4
桔梗	Platycodi Radix	4
厚朴	Magnoliae officinalis Cortex	4
穿山甲(炒黑)	Manitis Squaama	4
皂角刺	Gleditisiae Fructus	4
Total		72

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[9].

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(2×10^5 cells/well) or 60-mm diameter plastic petri dishes(1×10^7 cells/dish), and incubated for 3h at 37℃ in an atmosphere at 5% CO_2. Nonadherent cells were removed by suction, and then freshly prepared complete media were added with the indicated experimental reagents.

4. Measurement of nitric oxide(Griess assay)

Nitric oxide synthesis in cell was measured by a microplate assay method as a Griess reaction[10]. 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 1×10^5cell for 24hr. Data are expressed as the mean$\pm$S.E.

5. Cell viability assay

The cytotoxicity of TRSDE 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 10μl of MTT solution(5mg/μl) was added to each well. After incubation for 3hr at 37℃ and 5% CO_2, the formed formazan crystals in viable cells were solubilized with 100μl of DMSO. The absorbance of each well was then read at 570nm using microplate reader.

6. Assay of TNF-α secretion

TNF-α secretion was measured by modification of an enzyme linked immunosorbent assay(ELISA), as previously described[11]. 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.

7. Western blot analysis

Peritonial macrophages were incubated with or without various concentration of TRSDE and stimulated 1$\mu g/ml$ of LPS, IFN-γ. Cells were washed three times with ice-cold PBS(calcium and magnesium-free phosphate buffered saline), and lysed in 0.2ml of ice-cold lysis buffer(50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride, 1$\mu g/ml$ leupeptin, 150mM Nacl) and homogenized to obtain total cell lysate. An aliquot of lysate was used to determine protein concentration by the Bradford method[12].

30μg/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).

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.

Results

3.1 Effect of TRSDE on NO production in LPS-stimulated murine peritoneal macrophages

To investigate the molecular mechanisms of anti-inflammatory activity of TRSDE, we tested the effects of TRSDE on nitrite production in LPS-stimulated murine peritoneal macrophages. TG-elicited murine peritoneal macrophages were incubated with TRSDE for 2h. Then, murine peritoneal macrophages were stimulated with LPS(1 ng/ml) or LPS(1 ng/ml) plus IFN-γ(5 U/ml). And

were incubated for 24 hr. The production of nitrite concentration was determined by measuring the culture medium with Griess reagent. First, as shown in Fig.1, unstimulated murine peritoneal macrophages produced 5.45±2.45μM NO. LPS-stimulated cells without TRSDE produced 28.64±2.33μM NO. When murine peritoneal macrophages were treated with 0.01, 0.1, 0.5, 1mg/ml TRSDE, the cells produced 23.32±0.95, 18.89±1.68, 16.17±2.28, 11.35±0.13 μM NO, respectively. TRSDE suppressed NO release into culture supernatant in a dose-dependent manner. Particularly, NO production was significantly decreased in 0.2, 0.5, 1mg/ml concentration. Second, as shown in Fig.2, unstimulated murine peritoneal macrophages produced 5.45±2.45 μM NO. LPS plus IFN-stimulated cells without TRSDE produced 23.39± 1.21μ M NO. When murine peritoneal macrophages were treated with 0.01, 0.1, 0.2, 0.5mg/ml TRSDE, the cells produced 17.57±1.52, 15.15±1.80, 13.59±1.49, 10.39±1.31μM NO, respectively. TRSDE suppressed NO release into culture supernatant in a dose-dependent manner. Particularly, NO production was significantly decreased in 0.1, 0.2, 0.5mg/ml concentration. But cell viability was not affected by TRSDE as measured by MTT assay(Fig.3). These results suggest TRSDE inhibits LPS-induced NO production in murine peritoneal macrophages without effect on the cell death.

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

Since the expression of iNOS is primarily responsible for the NO over production in LPS-stimulated murine peritoneal macrophages, we analyzed the amount of iNOS by western blot analysis. TG-elicited murine peritoneal macrophages were incubated with TRSDE for 2h, 0.5, 1mg/ml, respectively. Then, murine peritoneal macrophages were stimulated with LPS(1 ng/ml) for 24h. The level of iNOS was dramatically reduced by TRSDE in a dose-dependent manner(Fig. 4). The result of western blot analysis indicated that TRSDE inhibits NO release by modulating the iNOS espression level in the LPS-stimulated murine peritoneal macrophages

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

IFN-γ and/or LPS-stimulated induced TNF-α secretion is known to be crucial for synergistic induction of NO synthesis murine peritoneal macrophages. So, we

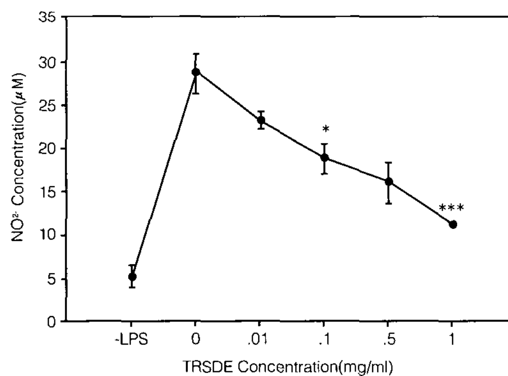

Fig. 1. Inhibition effect of TRSDE on the nitrite production on the LPS-stimulated macrophages. TG-elicited murine macrophage(2×10^5 cells/well) were incubated for 2 hr in the presence of the indicated concentrations of TRSDE. Then, the cells were stimulated with LPS(1 ng/ml). After cells were incubated for 24 hr. The production of nitrite concentration was determined by measuring the culture medium with Griess reagent.
* P<0.05, ** P<0.01, ***P<0.001 significantly different from the LPS.

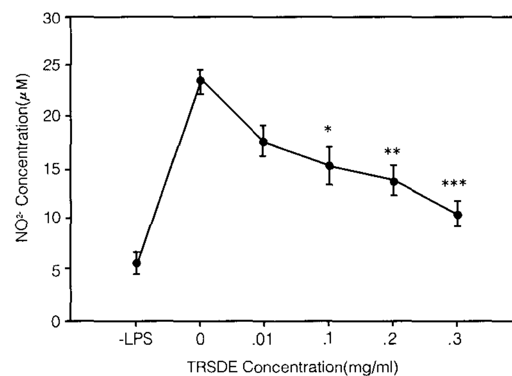

Fig. 2. Inhibition effects of TRSDE on the nitrite production on the LPS plus IFN-stimulated macrophages. TG-elicited murine macrophage(2×10^5 cells/well) were incubated for 2 hr in the presence of the indicated concentrations of TRSDE. Then, the cells were stimulated with LPS(1 ng/ml) plus IFN(5 U/ml) . After cells were incubated for 24 hr. The production of nitrite concentration was determined by measuring the culture medium with Griess reagent.
* P<0.05, ** P<0.01, *** P<0.001, significantly different from the LPS.

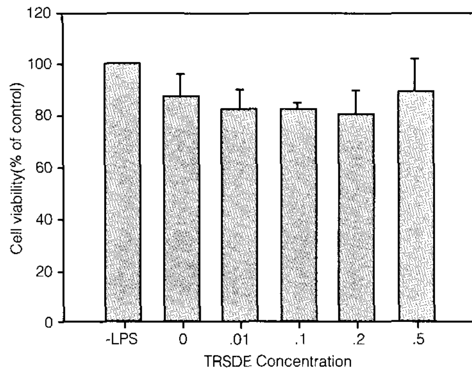

Fig. 3. Cell cytotoxicity effects of TRSDE on the LPS-stimulated macrophages. TG-elicited murine macrophage(2×10^5 cells/well) were incubated for 2 hr in the presence of the indicated concentrations of TRSDE. Then, the cells were stimulated with LPS(1 ng/ml). After cells were incubated for 24 hr. No effect on cell viability was detected in the tested concentration as measured by the MTT assay.

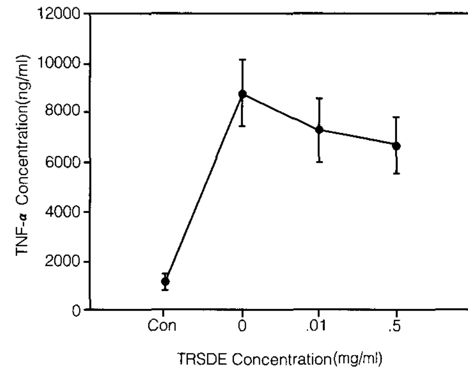

Fig. 5. Effects of TRSDE on the LPS plus IFN-stimulated TNF- secretion in the murine macrophages. TG-elicited murine macrophage(2×10^5 cells/well) were incubated for 2 hr in the presence of the indicated concentrations of TRSDE. Then, the cells were stimulated with LPS(1 ng/ml) and incubated for 24 hr. The supernatants were collected 24 hr after stimulation. TNF- secretion was assayed by specific ELISA.

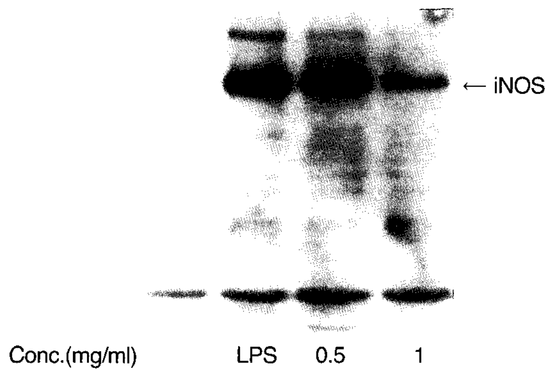

Fig. 4. Effects of TRSDE on the synthesis of iNOS in the LPS-stimulated macrophages. TG-elicited murine macrophage(2×10^5 cells/well) were incubated for 2 hr in the presence of the indicated concentrations of TRSDE. Then, the cells were stimulated with or without LPS(1 ng/ml) and incubated for 24 hr. 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.

investigated TNF-α secretion in the LPS-stimulated murine peritoneal macrophages. To examine whether the TRSDE 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 TRSDE 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. 5). TNF-α secretion was not significantly changed.

Discussion

The application of medicinal herbs dates back to the beginning of civilization. Interestingly, medicinal herbs are still routinely used by most of world' s population. Medicinal herbs have been used for the improvement of diseases in Asian countries for many centuries. Over time, many information has been accumulated by using these herbs in practice, and it is experientially proved that these herbs have the effect of pain alleviation, life extension and disease prevention, such as their efficacy and adverse reactions when used long term[13].

Oriental medicine views the body as an Ki(氣) system and emphasizes abnormality in the balance or flow of Ki(氣) as the sources of disease. Thus, the basic principle of the traditional 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[14].

Takrisodok-eum, a traditional oriental medicine, has been used for patient with cutaneous diseases, and it was

consisted of twelve ingredients, formulated by Gong-Sin(龔信) in the Chinese Myong Dynasty(1578)[5].

Doctors have applied Takrisodok-eum for treating inflammatory diseases such as abscess, carbuncle and phlegmon, etc. by resolving inflammation(化膿), promoting tissue regeneration(生肌), dispersing stagnated blood(散瘀), inducing detumescence(消腫)[2-4,5].

There have been many experimental studies on the Takrisodok-eum, and Choi reported "the effect of Takrisodok-eum extract on anti-tumor activity and immune-function[1].

Ong-Juh(癰疽) is one of the oriental medical name of disease, and include all inflammatory disease of skin. Distinguishing Ong(癰) and Juh(疽) strictly, Ong(癰) attacks between skin and flesh and there are flare, swelling, feeling of heat, pain and this disease is acute. Juh(疽) attacks between muscle and bone and flare, swelling, feeling of heat and pain are not severe and this disease is chronic[2,3,15]. Important distinction of Ong(癰) and Juh(疽) is acute and chronic. If inflammatory disease like Ong-Juh(癰疽) become occur, macrophage plays a important role in healing.

The macrophage plays a role in the development of the lymphohaemopoietic system before and after birth, as well as in the natural and acquired immune responses of the adult to immunogens, including infectious agents. General features of the distribution of resident macrophages throughout the lymphoid and non-lymphoid tissues are well known, as are some of the pheno-typic characteristics of 'elicited' macrophages recruited to sites of inflammation and 'activated' macrophages involved in specific immune responses. Also, activated macrophages are generate large amounts of nitric oxide(NO) from L-arginine by action of inducible NO synthase(iNOS)[8].

It is known that nitric oxide(NO) is synthesized by a number of cell types ranging in diversity from neurons to hepatocytes[16,17]. 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, neurons[18,19], or cardiac myocytes[20] 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[21,22], vascular smooth muscle cells[23,24], fibroblasts[25], or murine macrophages[26,27], but then generates large amounts of NO.

NO has very short life span in oxygenated, aqueous solutions and further oxidized to nitrite(NO2) and nitrate(NO3), which are the stable and inactive end products of NO formation[27].

Nitric oxide, particularly synthesized by iNOs, is involved in the regulation of many cell functions and in the expression of several diseases including trauma[28], septis[29], multiple sclerosis[30], arthritis[31]. And accumulating evidences indicate that excessive production of NO plays a pathogenic role in both acute and chronic inflammation[32]. 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[33]. In fact, administration of the selective inhibitors of iNOs has been reported to attenuate periodonititis[34], osteoarthritis[35], experimental autoimmune myocarditis[36], inflammation and demyelination produced by virus infection[37], carrageenan-induced models of inflammation[38], multiple scerosis[39] and shock[40].

According to present study, as shown in Fig.1, Fig.2, Particularly, NO production was significantly decreased in 0.2, 0.5, 1mg/ml concentration. But cell viability was not affected by TRSDE as measured by MTT assay(Fig.3). This experimental results imply that TRSDE efforts the effects of anti-inflammation through decrease the NO production, without cell viability affects.

And, to analyze the amount of iNOs, TG-elicited murine peritoneal macrophages were incubated with TRSDE for 2h, 0.5, 1mg/ml, respectively. Then, murine peritoneal macrophages were stimulated with LPS(1 ng/ml) for 24h. The level of iNOS was dramatically reduced by TRSDE in a dose-dependent manner(Fig. 4). We thought that TRSDE efforted anti-inflammatory effects, as reducing NO production through inhibite iNOS expression.

To examine whether the TRSDE evokes reduction of NO synthesis by the regulation of TNF-α production, TNF-α in culture medium was assayed by capture ELISA(Fig. 5). TNF-α secretion was not significantly

changed.

From this study, we have identified the effectiveness of TRSDE on the anti-inflammatory effect. And We think that TRSDE takes the place of macrophage' s role sufficiently and other signal pathway acts on to the inflammatory system.

Conclusions

To evaluate the effect of TRSDE on the anti-inflammatory effects in the Ong-Juh(癰疽), we observed NO concentration, cell viability, expression of iNOS, TNF-α concentration. The results were as followed.

1. NO production was significantly decreased.
2. Cell viability was not affected by TRSDE.
3. In the expression of iNOS, the level of iNOS was dramatically reduced.
4. TNF-α concentration was not significantly changed.

These results imply that TRSDE has anti-inflammatory effects in the Ong-Juh(癰疽).

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