Comparative Study of the Methanol and Water Extracts of Dangguisoo-san in Suppressing Inflammatory Reaction

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Dangguisoo-san (DGSS), a traditional Korean herbal formula, has been prescribed to patients who suffer from various symptoms related with blood stagnation. Since inflammation can cause blood stagnation, we hypothesized that DGSS suppresses inflammation, relieving the symptoms associated with blood stagnation. In this study, given that DGSS is prepared in a mix of alcohol and water, we prepared the methanol (mDGSS) and water extract (wDGSS) of DGSS and compared their activities in suppressing inflammatory reaction. Western blot analyses show that mDGSS was more effective than wDGSS in activating Nrf2, a key factor that suppresses inflammation. Semi-quantitative RT-PCR shows that mDGSS activating Nrf2 resulted in the induction of Nrf2-dependent genes expression. However, mDGSS was not effective in suppressing the nuclear translocation of NF-kB, a key factor that promotes inflammation, and the expression of NF-kB-dependent genes such as TNF-a and IL-1b. When comparing with wDGSS, mDGSS was less effective in suppressing luciferase activity driven by NF-kB. Therefore, our results show that mDGSS has the anti-inflammatory function by mainly activating Nrf2, while wDGSS does by both activating Nrf2 and suppressing NF-kB. Our results suggest that preparing DGSS in a mix of water and methanol is a better way to achieve a strong anti-inflammatory efficacy of DGSS.

keywords : Dangguisoo-san, Water and methanol extraction, Anti-inflammation, Nrf2, NF-κB

Introduction

Inflammation is an underlying mechanism for various diseases, including acute lung injury, sepsis, rheumatoid arthritis, heart diseases, and even cancer[1,2]. Therefore, understanding and regulating inflammation could provide an important tool to treat those diseases. It has been reported that macrophages play an important role in regulating inflammation[3]. When a bacterial infection occurs, macrophages sense the presence of the pathogen by using Toll-like receptor 4 (TLR4). TLR4 binding to lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, triggers a series of signaling cascades, resulting in the activation of NF-κB[4]. NF-κB promotes inflammation by inducing the expression of various pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-1β[5]. On the other hand, inflammation has mechanisms to limit the inflammatory reactions[6]. For instance, active inflammatory reaction activates nuclear factor-E2-related factor 2 (Nrf2), a member of the cap'n'collar family of basic leucine zipper transcription factors[7], via reactive oxygen species (ROS) that are generated during the inflammatory reaction[8]. Once activated, Nrf2 moves to the nucleus and binds to the antioxidant responsive element (ARE) to induce the expression of phase 2 detoxification and antioxidant enzymes such as glutamate-cysteine ligase catalytic subunit (GCLC), NAD(P)H:quinine oxidoreductase-1 (NQO1), and heme oxygenase-1 (HO-1)[9,10]. These proteins participate in the removal of ROS, by which they protect from the damage incurred by ROS, contributing to the suppression of inflammation. Because of the key roles of NF-kB and Nrf2 in regulating inflammation, these proteins have been proposed as therapeutic targets for the treatment of various inflammatory diseases[11,12].

Dangguisoo-san (DGSS) is an herbal formula that has long been used in traditional Korean medicine to treat the patients who have symptoms associated with blood stagnation, "Eo Hyeol" in Korean[13]. Blood stagnation is implicated in various forms of diseases. For instance, tissue destruction inflicted by physical injury causes hemostasis that restricts a local blood circulation[14]. While inflammation results in blood stagnation[15], infection in tissue elicits inflammation, which interferes with normal blood

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·Received : 2016/12/26 ·Revised : 2017/02/22 ·Accepted : 2017/02/23

pISSN 1738-7698 eISSN 2288-2529 http://dx.doi.org/10.15188/kjopp.2017.02.31.1.75

Available online at https://kmpath.jams.or.kr

circulation, causing hyperemia[16], a type of blood stagnation. As a result, it is rather subjective to diagnosis "Eo Hyeol", a blood stagnation syndrome[13]. Given that inflammation can cause blood stagnation, we hypothesized that DGSS suppresses inflammation, which contributes to relieving the symptom associated with blood stagnation. Indeed, our previous study showed that the water extract of DGSS (wDGSS) suppresses inflammation, which is mediated by activating Nrf2 and suppressing NF-&B[17]. However, given that DGSS is routinely prepared in a mix of alcohol and water, it needs to be determined whether DGSS prepared with alcoholic media suppresses inflammation. To determine the efficacy of an alcoholic extract of DGSS in suppressing inflammation, we prepared the methanol (mDGSS) and water (wDGSS) extracts of DGSS and examined whether mDGSS has an anti-inflammatory potential. By comparing the effect of mDGSS on inflammatory reactions with that of wDGSS, we suggest that DGSS needs to be prepared in a traditional way for effectively suppressing inflammation.

Materials and Methods

1. Preparation of the water and methanol extracts of DGSS

The medicinal herbs comprising DGSS (Table 1)[18] were purchased from Kwang Myung Dang Natural Pharmaceutical Co. Ulsan, Korea, and were authenticated by Dr. Su-In Cho at the School of Korean Medicine. A voucher specimen number is pnukh002. For the preparation of wDGSS, DGSS (60g) was boiled in 1L of distilled water in a Herb Extractor (Dae-Woong Co, Korea) for 2 h, yielding final 200 ml of DS extract. For the preparation of mDGSS, DGSS (900 g) was mixed with 3L of methanol at ambient temperature for 24 h. After centrifugation in a sterile condition, the supernatant was collected and lyophilized through evaporation under reduced pressure at -80 ℃. The final amount of wDGSS was 4.6 g and that of mDGSS was 92.41 g. The lyophilized wDGSS or mDGSS was dissolved in sterile phosphate-buffered saline (PBS) prior to the experiment.

Table 1. Constituents of DGSS

Scientific name	Herbal name	Amount (g)
Angelica gigas Nakai	Angelicae gigantis Radix	5.625
Paeonia lactiflora Pall	Paeoniae Radix	3.750
Lindera strychnifolia Fernandez-Villar	Linderae Radix	3.750
Caesalpinia sappan L.	Sappan Lignum	3.750
Cyperus rotundus L.	Cyperi Rhizoma	3.750
Carthamus tinctorius L.	Carthami Flos	3.000
Prunus persica Batsch	Persicae Semen	2.655
Cinnamomum cassia Presl	Cinnamomi Cortex	2.250
Glycyrrhiza uralensis Fisch	Glycyrrhizae Radix et Rhizoma	1.875
Total		30.405

2. Reagents and Antibodies

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sulforaphange, a potent activator of Nrf2, was purchased from Sigma Chemical Co. (St. Louis, MO, USA). TLR4-specific Escherichia coli LPS was purchased from Alexis Biochemical (San Diego, CA, USA). All antibodies used in this study were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

3. Cell Culture

RAW 264.7 cells (American Type Culture Collection, Rockville, MD) were cultured as described previously[19]. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone; Logan, UT, USA) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified incubator at 37℃ and 5 % CO_2 prior to the experiment.

4. MTT assay

The cytotoxicity caused by mDGSS was assessed as described previously[20]. In brief, after Griess reaction, MTT solution (2.0 mg/ml) was added to cells in a 96 well plate. At 4 h after incubation at 37℃ in a CO_2 cell culture incubator, formazan crystals formed in viable cells were measured at 540 nm with a microplate reader. The percentage of living cells was calculated against untreated cells. Each sample was triplicated.

5. Western Blot Analysis

Total proteins were extracted by RIPA buffer and the protocol provided by the manufacturer (Thermo Scientific, IL, USA). Cytoplasmic and nuclear proteins were isolated by using NE-PER nuclear extraction kit and the manufacture's protocol (Thermo Scientific). In brief, cells (5×10^6 cells) were washed with PBS three times and added with buffers in the extraction kit.

The amounts of proteins were measured by Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of proteins were fractionated by SDS-PAGE and then transferred to PVDF membrane (Bio-Rad Laboratories). The PVDF membrane was treated with 5% non-fat dry milk for 1 h at ambient temperature and incubated with polyclonal antibodies for proteins of interest at 4℃ overnight. After incubation with secondary antibodies conjugated with HRP for 1 h at ambient temperature, the bands of interest were revealed by chemiluminescence (SuperSignal® West Femto, Thermo Scientific).

6. ELISA

Supernatant of cultured cells was collected, and TNF-α and IL-1β in the supernatant were measured by ELISA kits and the protocols of the manufacturer (R&D systems, Minneapolis, MN, USA).

7. Reporter Cell Line and Luciferase Assay

To measure NF-κB transcriptional activity, we used RAW 264.7 cells that were stably transfected with an NF-κB reporter construct. The reporter construct harbors four tandem copies of a 36-base enhancer from the 5' HIV-long terminal repeat (containing two NF-κB binding sites, GGGACTTTCC) placed upstream of the HSV minimal thymidine kinase promoter, which was cloned into pEGFPLuc (BD Biosciences Clontech). The resultant vector was stably transfected with lipofectamin LTX (Invitrogen) into RAW264.7 cells under G418 (Invitrogen). Luciferase activity was measured by a luciferase assay kit and the instructions of the manufacturer (Promega, Madison, WI, USA), which was normalized by the amounts of total proteins in the cell extract.

8. Isolation of total RNA from cells and RT-PCR

Total RNA was isolated with the QIAGEN RNeasy® mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Three micrograms of RNA were reverse-transcribed by M-MLV reverse transcriptase (Promega), which was amplified by PCR with specific primers(Table 2).

For PCR amplification, TaqPCRx DNA polymerase, Recombinant (Invitrogen, Carlsbad, CA, USA) and the manufacturer's protocol were used. The reaction conditions were as follows: an initial denaturation at 95 ℃ for 5 min followed by 28 cycles of denaturation for 30 sec at 95 ℃, annealing for 30 sec at 58 ℃ (NQO1 : 55 ℃), and extension for 40 sec at 72 ℃ with a final extension for 7 min at 72 ℃. PCR products were separated in 1.5 % agarose gels in 1× TBE buffer at 100 V for 30 min, stained with ethidium bromide, and visualized under UV light. GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was used as internal controls to evaluate relative expressions GCLC, HO-1, and NQO1.

9. Statistical Analysis

Data is presented as the mean ± SEM (Std. Err.) of at least three separate experiments. For comparison among groups, paired or unpaired T-tests and one-way analysis of variance (ANOVA) tests were used (with the assistance of InStat, Graphpad Software, Inc., San Diego, CA, U.S.A.). P values less than 0.05 was considered statistically significant. All experiment was performed at least three times independently.

Table 2. Oligonucleotide primers used for PCR in this study.

Target gene	Oligonucleotide sequences (5' to 3' direction)	Expected size	Accession number
GCLC	CACTGCCAGAACACAGACCC ATGGTCTGGCTGAGAAGCCT	239 bp	NM010295
HO-1	TGAAGGAGGCCACCAAGGAGG AGAGGTCACCCAGGTAGCGGG	373 bp	NM010442
NQO1	ACTACGCCATGAAGGAGGCT TTCCAGCTTCTTGTGTTCGG	224 bp	NM008706
GAPDH	GGAGCCAAAAGGGTCATCAT GTGATGGCATGGACTGTGGT	203 bp	NM008084
TNF-a	CTACTCCTCAGAGCCCCCAG AGGCAACCTGACCACTCTCC	239 bp	NM013693
IL-1β	GTGTCTTTCCCGTGGACCTT TCGTTGCTTGGTTCTCCTTG	281 bp	NM008361

Results

1. mDGSS activates Nrf2

Prior to studying the anti-inflammatory effect of the methanol extract of DGSS (mDGSS), we determined a cytotoxicity caused by mDGSS. RAW 264.7 cells, a murine macrophage cell line, were treated with various amounts of mDGSS (10 μg/ml to 100 μg/ml), along with the water extract of DGSS (wDGSS) for 20 h. The cytotoxicity caused by mDGSS or wDGSS was measured by MTT assay. As shown in Fig. 1, mDGSS or wDGSS did not show any cytotoxicity to RAW 264.7 cell.

Given no apparent cytotoxicity detectable in RAW 264.7 cells, we determined the possibility that mDGSS activates Nrf2 to exert an anti-inflammatory activity. As in Fig. 1, RAW 264.7 cells were treated with various amounts of mDGSS for 20 h. Since nuclear Nrf2 indicates that Nrf2 gets activated, nuclear proteins were isolated and analyzed by immune- blotting for nuclear Nrf2. As shown in Fig. 2A, nuclear Nrf2 was clearly detectable when the cells were treated with 50 μg/ml of mDGSS. When activated, Nrf2 induces Nrf2-dependent gene expression including heme oxygenase (HO)-1. Therefore, we determined whether mDGSS induces the expression of HO-1. RAW 264.7 cells were similarly treated with mDGSS, and total proteins were isolated and analyzed by immunoblotting for HO-1. As shown in Fig. 2B, the expression of HO-1 was detectable when the cells were treated with 50 μg/ml or 100 μg/ml of mDGSS. To confirm that mDGSS induces the expression of other Nrf2-dependent genes, we analyzed the mRNA of representative Nrf2-dependent genes. Total RNA was isolated from RAW 264.7 cells that were treated with mDGSS

as described in Fig. 2A, and was analyzed by semi-quantitative RT-PCR. As shown in Fig. 2C, the expression of Nrf2-dependent genes was increased by mDGSS. Densitometric analyses show that the expression of those genes was correlated with the amounts of mDGSS treated (Fig. 2D). Together, these results show that mDGSS activated Nrf2 and induced the expression of Nrf2-dependent genes. Given that Nrf2 is an important factor that suppresses inflammation, our results suggest that mDGSS regulates inflammation by activating Nrf2.

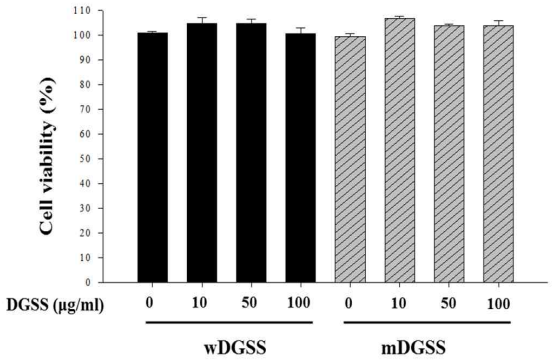

Fig. 1. Effect of wDGSS and mDGSS on cell viability. Cytotoxicity caused by two different extracts was measured by MTT assay. RAW 264.7 cells were treated with indicated amounts of wDGSS or mDGSS for 20 h prior to analyses. Data represent the mean±SEM of triplicated cells. Similar experiment was performed twice more independently, and representative results are shown. No statistical significance was found.

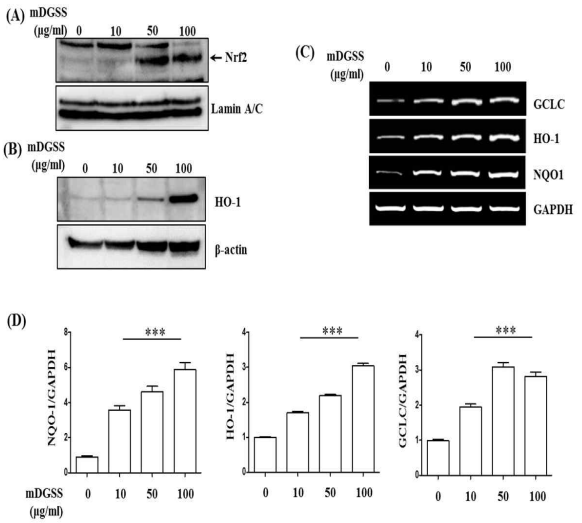

Fig. 2. mDGSS activates Nrf2 and induces Nrf2-dependent genes. RAW 264.7 cells were treated with various concentrations of mDGSS for 20 h. Nuclear proteins or total proteins were isolated and analyzed by immunoblotting for Nrf2 (A) and HO-1 (B), respectively. Blots were stripped and reblotted for internal controls (lamin A/C or β-actin). Total RNA was extracted from RAW 264.7 cells treated as in (A) and analyzed by RT-PCR (C). Specific bands were measured by ImageJ, a densitometric program, and quantitated over GAPDH, a house-keeping gene (D). Data represent the mean±SEM of three measurements of each band. ***P was less than 0.001, compared to untreated controls.

2. mDGSS is more effective in activating Nrf2 than wDGSS

Next, we compared mDGSS and wDGSS in activating Nrf2. Since both mDGSS and wDGSS did not show a significant cytotoxicity in RAW 264.7 cells (Fig. 1), we treated RAW 264.7 cells with mDGSS or wDGSS in parallel and compared the effectiveness of two different extracts of DGSS in activating Nrf2 by performing western blot analysis. RAW 264.7 cells were treated with various amounts of either wDGSS or mDGSS. At 20 h after treatment, nuclear proteins were isolated and analyzed by immunoblotting for Nrf2. As shown in Fig. 3A, both wDGSS and mDGSS similarly activated Nrf2. However, densitometric analyses reveal that mDGSS was more effective in activating Nrf2 because 50 μg/ml of mDGSS robustly activated Nrf2, compare to wDGSS (3rd and 6th columns in Fig. 3B).

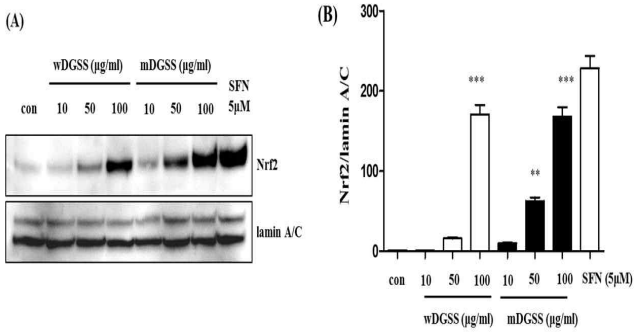

Fig. 3. mDGSS is more effective in activating Nrf2 than wDGSS. RAW 264.7 cells were treated with indicated amounts of mDGSS or wDGSS in parallel, along with sulforaphane (SFN, 5μM for 6h). Nuclear proteins were analyzed by immunoblotting of Nrf2 (A), which was quantitated by ImageJ over lamin A/C (B). Data represent the mean±SEM of three measurements of each band. ***P and **P were less than 0.001 and 0.05, respectively, compared to controls.

3. mDGSS is less effective in suppressing NF-κB activity than wDGSS

Since NF-κB promotes inflammation and wDGSS was reported to suppress NF-κB activity, we tested whether mDGSS suppresses NF-κB. LPS binding to TLR4 translocates NF-κB into the nucleus within 30 min, which is accompanied by rapid degradation of inhibitor κB-α (IκB-α). Therefore, we tested whether mDGSS prevents the nuclear translocation of NF-κB to exert an anti-inflammatory function. RAW 264.7 cells were treated with 100 ng/ml of LPS for 15 or 30 min, with or without mDGSS pre-treated for 3 h. Cytoplasmic and nuclear proteins were isolated and analyzed by immunoblotting for IκB-α and of p65 of NF-κB, respectively, which were quantitated by densitometric analysis. As shown in Fig. 4A and 4B, while not significantly suppressing the degradation of IκB-α at 15 min after LPS treatment, mDGSS slightly but significantly decreased the

degradation at 30 min after LPS treatment. Consistent with this, while not blocking nuclear translocalization of p65 of NF-κB occurred at 15 min after LPS, mDGSS slightly but significantly decreased it at 30 min after LPS (Fig. 4C and 4D), suggesting that mDGSS suppresses, but less effectively, NF-κB activity.

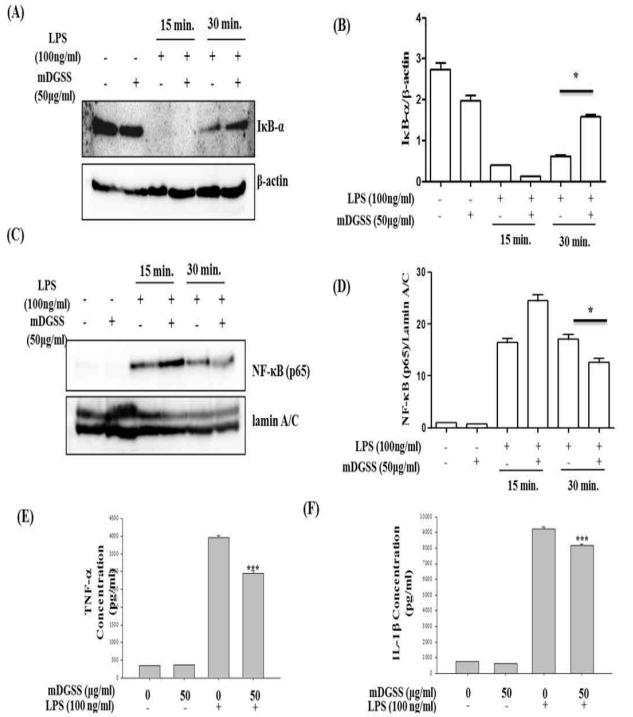

Fig. 4. Effect of mDGSS on NF-κB activity. RAW 264.7 cells were treated with 50 μg/ml of mDGSS for 3 h and subsequently with 100 ng/ml of LPS for 15 min or 30 min. Cytoplasmic (A) and nuclear (C) proteins were analyzed by immunoblotting for IκB-α and p65 of NF-κB, respectively. Membrane was stripped and reprobed for internal controls, α-actin and lamin A/C, respectively. Bands of IκB-α (B) and p65 (D) were analyzed over internal controls by densitometer. Data show the mean±SEM of three measurements of each band. *P was less than 0.05, compared to LPS only. Similarly, RAW 264.7 cells were treated with mDGSS and then with LPS. The supernatant of cell culture was collected and measured by ELISA for TNF-α (E) and IL-1β (F). Data represent the mean±SEM of three measurements. *** P was less than 0.05, compared to LPS alone.

To verify these results, we analyzed the expression of representative pro-inflammatory cytokine genes, TNF-α and IL-1β, whose expressions were largely regulated by NF-κB. RAW 264.7 cells were treated with 50 μg/ml of mDGSS and 3 h later with 100 ng/ml of LPS for 8 h. Total RNA was analyzed by semi-quantitative RT-PCR for TNF-α and IL-1β genes. Consistent with the results in Fig. 4B, the expressions of TNF-α and IL-1β genes were slightly suppressed by mDGSS (Fig. 4E and 4F). Lastly, we compared the effect of wDGSS with mDGSS in suppressing the transcriptional activity of NF-κB. An NF-κB reporter cell line, which was derived from RAW 264.7 cells, was treated similarly with wDGSS or mDGSS and 3 h later with 100 ng/ml of LPS for 8 h. Total cell lysate extracted from variously treated cells was assayed for luciferase activity. As shown in Fig. 5, wDGSS strongly suppressed the NF-κB-driven luciferase activity, suggesting that wDGSS suppresses effectively NF-κB activity. However, mDGSS was less effective in suppressing NF-κB activity, which was consistent with the results in Fig. 4. Taken together, these results suggest that mDGSS suppresses NF-κB activity less effectively than wDGSS.

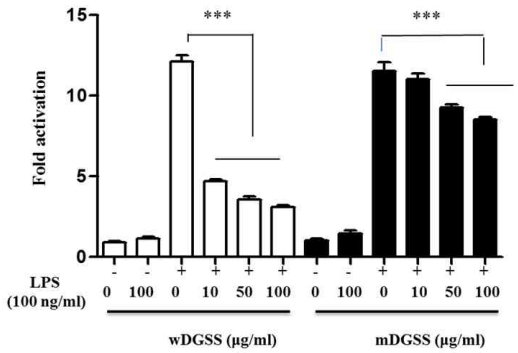

Fig. 5. mDGSS is less effective than wDGSS in suppressing NF-κB. An NF-κB reporter cell line derived from RAW 264.7 were treated with various amounts of wDGSS or mDGSS for 3 h and then with 100 ng/ml of LPS for 8 h. Cytoplasmic proteins were collected for assessing luciferase activity. Data represent the mean±SEM of triplicated samples. *** P was less than 0.05, compared to LPS alone. Similar experiments were performed three more times and representative results are shown.

Discussion

In this study, a possible anti-inflammatory activity of the methanol extract of DGSS (mDGSS) was examined, in parallel with the water extract of DGSS (wDGSS). Since Nrf2 is known as a key anti-inflammatory molecule[11] and NF-κB is a crucial pro-inflammatory factor[21], we addressed whether the anti-inflammatory activity of mDGSS involves regulating the activities of these two factors. Our results show that while more effective in activating Nrf2, mDGSS was less effective in suppressing NF-κB, compared to wDGSS. Consistent with these results, mDGSS induced the expression of Nrf2-dependent genes and suppressed marginally the expression of NF-κB-dependent genes. Therefore, our results suggest that mDGSS has an anti-inflammatory activity by mainly activating Nrf2, while wDGSS uses both activating Nrf2 and suppressing NF-κB.

DGSS is one of the major prescriptions in Korea medicine for patients who suffer from various symptoms associated with blood stagnation[13]. Blood stagnation has rather a broad clinical implication because it has numerous

etiology[13]. For instance, patients who have heart, kidney, or liver diseases have a poor blood circulation, leading to blood stagnation in affected organs. The physical injury also causes blood stagnation; physical damage on tissue elicits inflammatory reactions in the afflicted area. Since inflammation deters normal blood circulation, it would cause blood stagnation. Therefore, we postulated that DGSS relieves the symptoms complicated by blood stagnation, at least in part, by suppressing inflammation. Since DGSS is prepared in a mix of water and alcohol, we prepared mDGSS and wDGSS and compared an anti-inflammatory effect of mDGSS with wDGSS. Interestingly, our results show that mDGSS was more effective in activating Nrf2, a key anti-inflammatory transcription factor, but less effective in suppressing NF-κB, a key pro-inflammatory transcription factor, compared to wDGSS. The differential effects of two different extracts on NF-κB and Nrf2 activities are highly likely due to different chemical constituents found in water and methanol extracts. It is possible that the chemicals responsible for suppressing NF-kB are mainly water soluble and thus mDGSS is less effective than wDGSS in suppressing NF-κB. On the other hand, both mDGSS and wDGSS were effective in activating Nrf2, although mDGSS appeared to be more effective. These results suggest that the chemicals for Nrf2 activation can be found in both water and methanol fractions of DGSS. Although we don't know whether these two fractions contain a similar profile of the chemicals that activate Nrf2, it is more likely that two fractions harbor a different sets of chemicals that account for activating Nrf2. Nevertheless, our results show that DGSS has an efficacy in suppressing inflammation. Inflammation is now recognized as a key underlying pathologic mechanism for various human disorders including cancer, autoimmune diseases, diabetes, and neuronal diseases[22]. Therefore, regulating inflammation could be an important intervention to cure these diseases. Based on our results that DGSS suppressed inflammation, it is possible that DGSS can be applied to treat more diverse symptoms or diseases that involve inflammation. Therefore, it would be interesting whether DGSS can be applicable to treating diseases, including diabetes, autoimmune diseases, and cancer.

The anti-inflammatory function of Nrf2 has been well-documented. Seminal reports show that genetic ablation of Nrf2 in mice exacerbates inflammatory diseases such as acute lung injury[23], smoke-induced emphysema[24], sepsis[25], and asthma[26]. Cumulative experimental evidence has shown that Nrf2 suppresses inflammatory responses[8]. Therefore, our finding that mDGSS activated Nrf2 suggests that mDGSS suppresses inflammation via Nrf2.

We found that mDGSS could not deter the nuclear translocation of NF-κB effectively; mDGSS seemed to suppress it marginally at 30 min after LPS treatment. Consistent with this, mDGSS decreased slightly but statistically significantly the expression of TNF-α and IL-1β (Fig. 4C and 4D). Since the nuclear localization of NF-κB rapidly occurs within 30 min after LPS treatment, which we measured in this study, there was a time gap in measuring the nuclear translocation of NF-κB and the expression of NF-κB-dependent genes. Therefore, it is possible that a slight reduction in the nuclear localization of NF-κB results in a statistically significant reduction of the expression of pro-inflammatory genes such as TNF-α and IL-1β. Concordant with this notion, mDGSS similarly suppressed the NF-κB-driven transcriptional activity (Fig. 5). Alternatively, this minor reduction of pro-inflammatory cytokine expression could be due to the activation of Nrf2. As shown in this study, NF-κB activation occurs right after LPS treatment. On the other hand, Nrf2 activation usually happens in a late phase of inflammation. Because of the time difference between NF-κB and Nrf2 activation, Nrf2 may not effectively suppress a full-blown expression of pro-inflammatory cytokines. Although we did not examine, it is possible that measuring the expression of pro-inflammatory cytokine at much later time points could show more reduction of the cytokine expression. Since NF-κB reporter assay showed that wDGSS effectively suppressed the NF-κB transcriptional activity, as opposed to mDGSS, these results suggest that mDGSS has an anti-inflammatory activity, which is mainly mediated by activating Nrf2 and in a lesser degree by suppressing NF-κB.

Conclusion

In this study, we hypothesized that DGSS suppresses inflammation, alleviating the symptoms associated with blood stagnation. We show that mDGSS was more effective in activating Nrf2 but less effective in suppressing NF-κB, compared with wDGSS, we propose that DGSS needs to be prepared in a traditional way, a mix of alcohol and water, to achieve a high efficacy in suppressing inflammation.

Acknowledges

This study was supported by a 2-year Research Grant of Pusan National University (to MJ).

References

1. Maity B, Yadav SK, Patro BS, Tyagi M, Bandyopadhyay SK, Chattopadhyay S. Molecular mechanism of the anti-inflammatory activity of a natural diarylnonanoid, malabaricone C. Free Radic Biol Med. 2012;52(9):1680-91.
2. Saxena M, Yeretssian G. NOD-Like Receptors: Master Regulators of Inflammation and Cancer. Front Immunol. 2014;5:327.
3. Hamidzadeh K, Christensen SM, Dalby E, Chandrasekaran P, Mosser DM. Macrophages and the Recovery From Acute and Chronic Inflammation. Annu Rev Physiol. 2017;79:567-92.
4. Beutler B, Rietschel ET. Innate immune sensing and its roots: the story of endotoxin. Nat Rev Immunol. 2003;3:169-76.
5. Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. Cell. 2008;132(3):344-62.
6. Serhan CN. The resolution of inflammation: the devil in the flask and in the details. FASEB J. 2011; 25:1441-8.
7. Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. Proc Natl Acad Sci USA. 1994;91:9926-30.
8. Brune B, Dehne N, Grossmann N, Jung M, Namgaladze D, Schmid T, et al. Redox control of inflammation in macrophages. Antioxid Redox Signal. 2013;19:595-637.
9. Niture SK, Khatri R, Jaiswal AK. Regulation of Nrf2-an update. Free Radic Biol Med. 2014;66:36-44.
10. Lee JM, Calkins MJ, Chan K, Kan YW, Johnson JA. Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. J Biol Chem. 2003;278:12029-38.
11. Boutten A, Goven D, Artaud-Macari E, Boczkowski J, Bonay M. NRF2 targeting: a promising therapeutic strategy in chronic obstructive pulmonary disease. Trends Mol Med. 2011;17:363-71.
12. van Muiswinkel, FL Kuiperij, HB. The Nrf2-ARE Signalling Pathway: Promising Drug Target to Combat Oxidative Stress in Neurodegenerative Disorders. Curr Drug Targets CNS Neurol Disord. 2005;4:267-81.
13. Park B, Yun KJ, Jung J, You S, Lee JA, Choi J, et al. Conceptualization and utilization of blood stasis syndrome among doctors of Korean medicine: results of a web-based survey. Am J Transl Res. 2014;6:857-68.
14. Furie, B. Furie, BC. Thrombus formation in vivo. J Clin Invest. 2005;115:3355-62.
15. Shen BQ, Situ Y, Huang JL, Su XM, He WT, Zhang MW, et al. A clinical study on the treatment of chronic pelvic inflammation of Qi-stagnation with blood stasis syndrome by Penyanqing capsule. Chin J Integr Med. 2005;11:249-54.
16. Kobayashi N, Hirano K, Nakano M, Ito Y, Sakai T, Ishimori H, et al. Measuring Procedure and Maximal Hyperemia in the Assessment of Fractional Flow Reserve for Superficial Femoral Artery Disease. J Atheroscler Thromb. 2016;23:56-66.
17. Lyu JH, Kim KH, Kim HW, Cho SI, Ha KT, Choi JY, et al. Dangkwisoo-san, an herbal medicinal formula, ameliorates acute lung inflammation via activation of Nrf2 and suppression of NF-kappaB. J Ethnopharmacol. 2012;140:107-16.
18. Hur J. Donguibogam, Seoul:Namsandang; 2007. 578 p.
19. Kim KH, Kwun MJ, Han CW, Ha KT, Choi JY, Joo M. Suppression of lung inflammation in an LPS-induced acute lung injury model by the fruit hull of Gleditsia sinensis. BMC Complement Altern Med. 2014;14:402.
20. Lyu JH, Lee GS, Kim KH, Kim HW, Cho SI, Jeong SI, et al..ent-kaur-16-en-19-oic Acid, isolated from the roots of Aralia continentalis, induces activation of Nrf2. J Ethnopharmacol. 2011;137:1442-49.
21. Jawa RS, Anillo S, Huntoon K, Baumann H, Kulaylat M. Analytic review: Interleukin-6 in surgery, trauma, and critical care: part I: basic science 2. J Intensive Care Med. 2011;26:3-12.
22. Nathan C, Ding A. Nonresolving inflammation. Cell. 2010;140:871-82.
23. Chan K, Kan YW. Nrf2 is essential for protection against acute pulmonary injury in mice. Proc Natl Acad Sci USA. 1999;96:12731-36.
24. Rangasamy T, Cho CY, Thimmulappa RK, Zhen L, Srisuma SS, Kensler TW, et al. Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. J Clin Invest. 2004;114:1248-59.
25. Thimmulappa RK, Lee H, Rangasamy T, Reddy SP, Yamamoto M, Kensler TW, et al. Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. J Clin Invest. 2006;116:984-95.
26. Rangasamy T, Guo J, Mitzner WA, Roman J, Singh A, Fryer AD, et al. Disruption of Nrf2 enhances susceptibility to severe airway inflammation and asthma in mice. J Exp Med. 2005;202:47-59.