Protective effect of *Salvia miltiorrhiza* Bunge on 5-fluorouracil-induced oral mucositis

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Abstract. Oral mucositis is a common side-effect caused by chemotherapy or radiotherapy occurring in the majority of cancer patients and is characterized by inflammation and ulcers in the oral mucosa. In the present study, we examined the protective effects of *Salvia miltiorrhiza* Bunge (SM) on oral mucositis induced by 5-fluorouracil (5-FU) in human pharyngeal cells and golden Syrian hamsters. We investigated the proliferation and antioxidant abilities of SM using MTT, 2-diphenyl-1-picrylhydrazyl (DPPH) and reactive oxygen species (ROS) assays in vitro. Additionally, TUNEL assay was performed, and the expression levels of nuclear factor-κB (NF-κB), caspase-3 and proinflammatory cytokines were assessed by immunoblotting. The results showed that SM increased the cell proliferation rate in human pharyngeal cells up to 128.97±9.7% compared with this rate in the untreated cells and exerted protective effects on mucosal injury caused by 5-FU treatment. In addition, all concentrations of SM increased DPPH scavenging ability and blocked ROS generation in the treated cells. Taken together, following SM treatment, expression of NF-κB and cleaved caspase-3 were significantly decreased followed by inhibition of cell death. These data suggest that SM could be used for the prevention and treatment of oral mucositis caused by cancer therapies.

Introduction

Oral mucositis is inflammation of the oral and oropharyngeal mucosa and usually occurs as a side-effect of cancer chemotherapy or radiotherapy in the head and neck region (1). This condition affects 10-40% of patients receiving the standard dose of chemotherapy for tumors and 60-100% of patients undergoing myeloablative chemotherapy for hematopoietic stem cell transplant and high-dose radiation therapy (2,3). Symptoms include pain, vomiting, dry mouth and diarrhea, and sequentially secondary infection of the oral mucosa leading to decreased quality of life (3,4). Oral mucositis can reduce the effectiveness of chemotherapy as a result of the alteration or discontinuation of the chemotherapy (5). Therefore, the development of therapeutic agents that can be combined with chemotherapy to prevent oral mucositis as a side-effect of chemotherapy is crucial.

The incidence of oral mucositis is divided into five biological steps: initiation, upregulation and generation of messenger signals, signal amplification, ulceration and healing (6). Chemotherapy-induced damage to cells or tissues is caused by reactive oxygen species (ROS) (7), thus ROS play a main role in the initiation phase of oral mucositis and activate several transcription factors such as nuclear factor-κB (NF-κB), a key transcription factor involved in the development of mucositis. NF-κB increases the production of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and induces apoptosis (8,9). Upregulation of proinflammatory cytokines amplifies the primary damage (10). Recently, mechanistically based oral mucositis drugs were evaluated and developed such as keratinocyte growth factor (KGF), benzylamine HCl and COX-2 inhibitors (10-12).

The dried root of *Salvia miltiorrhiza* Bunge (SM) (Lamiaceae), has been used in Korea, China and Japan for the treatment of various diseases, including coronary heart disease (13), cerebrovascular disease (14), Alzheimer’s disease (15), Parkinson’s disease (16), renal deficiency (17), hepatocirrhosis (18), cancer (19) and bone loss (20). The antioxidant (21), antidiabetic (22) and hepatoprotective (23) effects of SM on apoptosis and inflammation were investigated in a rat model of stroke (24). However, knowledge regarding the effects of SM extract on oral mucositis is limited. The effects of SM on molecular mechanisms in vitro and in vivo need to be elucidated. In the present study, we examined whether SM could be used in the development of a novel therapeutic agent for the treatment of mucositis.
induced by 5-fluouracil (5-FU). To identify the effects of SM on human pharyngeal cells and hamster, we performed scavenging of free radical activities against 2,2-diphenyl-1-picrylhydrazyl (DPPH), cell viability assay, ROS level measurements, TUNEL assay and immunoblotting.

Materials and methods

Preparation of SM extract. SM was purchased from Kyung Hee University Medical Center (Seoul, Korea). A 300 g sample of dried medicinal herb was boiled in 3 l water for 2 h at 100°C, and the suspension was filtered and evaporated under reduced pressure. The filtrate was lyophilized and yielded 69.1 g powder. The dried extract was dissolved in distilled deionized water (Millipore, Billerica, MA, USA) and vortexed for 2 min at room temperature.

In vitro studies

Human pharyngeal cell culture. Human pharyngeal cell line (Detroit 562, ATCC CCL-138) was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The Detroit 562 cell line was cultured in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS; both from Gibco-BRL, Grand Island, NY, USA), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂.

Cell viability and proliferation assays. Cell proliferation was determined using the MTT assay. The Detroit 562 cells were starved for 24 h and simultaneously treated with several concentrations of SM (1, 5, 10, 50 or 100 µg/ml) only or with several concentrations of SM plus 10 µM 5-FU. After 48 h, the medium was removed, and the cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to measure metabolic activity. Spectrophotometric analysis at 450 nm to measure metabolic activity was performed using a microtiter plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

ROS assay. ROS production was performed according to the protocol of the intracellular ROS assay kit (Cell Biolabs, Inc., San Diego, CA, USA). Cells were cultured in a 96-well cell culture plate and treated with several concentrations of SM (1, 5, 10, 50 or 100 µg/ml) with 10 µM 5-FU for 48 h. Next, the cells pretreated with 1 mM 2',7'-dichlorofluorescein diacetate (DCFH-DA) were incubated for 60 min at 37°C. After a brief incubation, the cell fluorescence was read on a fluorometric plate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 480/530 nm. ROS production was determined by comparison with the predetermined 2',7'-dichlorofluorescein (DCF) standard curve.

DPPH assay. Mixtures of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution with methanol and SM (1, 5, 10, 50 or 100 µg/ml) were incubated in the dark for 30 min at room temperature. After 30 min, the absorbance at 517 nm was read for each sample on a microtiter plate reader (Molecular Devices, LLC). Radical scavenging activity was calculated using the following formula: DPPH radical scavenging activity (%) = [(AB-AT)/AB] x 100; where AB is the absorbance of the blank sample and AT is the absorbance of the tested extract solution.

In vivo studies

Animals and experimental protocol. Seven-week-old male golden Syrian hamsters (SLC, Inc., Hamamatsu, Japan) weighing 100-110 g each were used. The animals were housed in a specific pathogen-free environment with a 12 h light/dark cycle at the Center for Laboratory Animal Care and Use at Kyung Hee University. Animal care and experimental procedures conformed to the 'Guide for the Care and Use of Laboratory Animals'. The protocol for the induction of oral mucositis was modified on the basis of a previously published protocol (25). The protocols for the use of hamsters in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Kyung Hee University. Briefly, all the animals received intraperitoneal (i.p.) administration of 80 mg/kg of the chemotherapy drug 5-FU on day 0, followed by i.p. administration of 60 mg/kg 5-FU on day 2. The cheek pouch of the animals was everted and the mucosa was irritated by superficial scratching with the tip of an 18-gauge needle by the same operator on days 3 and 4.

Experimental groups. The hamsters were randomly divided into six groups: normal group (vehicle-treated, n=6), control (5-FU 80 mg/kg, i.p. n=6), positive control (0.15% benzylidine HCl, o.p. n=6) and three groups treated with different concentrations of SM with 80 mg/kg of 5-FU (5-FU + SM: 100, 500 and 1,000 mg/kg of SM and 5-FU, o.p. n=6 Each group). 5-FU was administered on days 1 and 2 from the beginning of the study and SM and benzylidine HCl were administered for 5 days a week for 2 weeks. The animals were weighed weekly in order to adjust the gavage volume and to monitor their general health.

Histological evaluation of oral mucositis. For histological studies, the cheek pouches were fixed overnight in Bouin's solution, dehydrated in 70, 80, 95 and 100% ethanol, xylene and embedded in paraffin. Tissue sections (5 µm) were prepared in order to perform hematoxylin and eosin (H&E) staining. The sections were deparaffinized and rehydrated in xylene, 100, 95, 80 and 70% ethanol. The sections were over-stained with hematoxylin, usually 3-5 min and excess stain was rinsed off in deionized water. Then the sections were destained for a few seconds in acid alcohol until the sections appeared red in color (usually 4-5 dips) and then rinsed briefly in deionized water to remove the acid. Hematoxylin-stained slides from the last tap water were rinsed and placed in 70% ethanol for 3 min. Slides were placed in eosin for 2 min and then slides were put through 95 and 100% ethanol and xylene. After H&E staining, the slides were mounted with Canada balsam.

TUNEL assay. 5-FU-induced cell death was investigated on the day 14 using the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labelling (TUNEL) method (ApopTag®, no. S7101; Merck Millipore, Darmstadt, Germany). Briefly, after deparaffinizing, the samples were rehydrated and incubated with 20 mg/ml proteinase K for 15 min at room temperature. Endogenous peroxidases were
blocked by treatment with 3% (v/v) hydrogen peroxide in phosphate-buffered saline (PBS) for 5 min at room temperature. After washing, the sections were then incubated in a humidified chamber at 37˚C for 1 h with TdT buffer containing TdT enzyme and reaction buffer. Specimens were incubated for 10 min at room temperature with a stop/wash buffer and then incubated in a humidified chamber for 30 min with anti-digoxigenin peroxidase conjugate at room temperature. After a series of PBS washes, the slides were covered with peroxidase substrate for color development and then washed in three changes of dH₂O and counterstained in 0.5% (w/v) methyl green for 10 min at room temperature. The TUNEL-positive cells were counted (10 fields/slide; magnification, x1,000) for statistical comparisons.

Western blotting. Proteins from homogenized cheek pouches of hamsters were separated using a nuclear extraction kit following a modification of the manufacturer's instructions (Active Motif, Carlsbad, CA, USA). SDS-PAGE and western blotting were performed as previously described (26). Samples for protein extraction were half of the same cheek pouches of hamsters used for RNA extractions. Equivalent amount (50 µg) of protein extracts was separated on 10% Tris-glycine gels by SDS-PAGE and transferred to nitrocellulose membranes using 25 mM Tris and 250 mM glycine buffer containing 20% methanol, pH 8.3. Transfer was performed at a constant voltage of 120 mA for 1 h. After transfer, the membranes were blocked in PBS containing 0.05% Tween-20 (PBS-T) with 5% skim milk for 2 h at room temperature and incubated with the primary antibodies (1:1,000) for IL-1β (sc-12742), TNF-α (sc-1350), NF-κB (sc-109) and IL-4 (sc-32242) (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in PBS-T overnight at 4˚C. Following overnight incubation, the membranes were rinsed with 1X PBS three times and incubated with conjugated goat anti-rabbit IgG for 1 h at room temperature, followed by three additional washes with 1X PBS.

Statistical analysis. Statistical analysis was performed using GraphPrism 4.0.3 software (GraphPad Software, Inc., San Diego, CA, USA). Data are presented as means with standard deviation (SD) and analyzed using the statistical software SPSS, version 12.0 (SPSS, Inc., Chicago, IL, USA).

Results

SM enhances proliferation of the human pharyngeal cells. The effects of SM on proliferation of the Detroit 562 human pharyngeal cells were determined from cell growth kinetics using the MTT assay, which measures the metabolic activity of viable cells. Growth-arrested Detroit 562 cells were cultured in starvation medium for 24 h prior to the experiment and incubated for 48 h with SM and 10 µM 5-FU. SM activated cell proliferation; specifically, the number of cells treated with 100 µg/ml SM increased by 128.97±9.7% compared with that noted in the untreated cells (p<0.01; Fig. 1A). The cell viability in the 5-FU and SM-treated groups (1, 5, 10, 50 or 100 µg/ml) was significantly increased compared with the cell viability of the control (10 µM 5-FU-only treated) group (75.19±7.01, 78.43±7.0, 81.33±2.41, 102.1±6.8 and 95.97±8.54 vs. 56.01±3.48%, respectively; p<0.01, Fig. 1A), indicating that
SM enhanced the proliferation rate of Detroit 562 cells and exerted protective effects against 5-FU-induced cytotoxicity.

SM decreased intracellular ROS production and increases the DPPH-scavenging activity. As shown in Fig. 1B, the production of superoxide anions in the 10 µM 5-FU-treated cells was decreased markedly by co-treatment of 1, 10 and 100 µg/ml of SM compared with the (10 µM 5-FU-only treated) control. Next, the effects of SM on DPPH-scavenging activity were assessed. The DPPH-scavenging activity increased to 20.30, 24.21, 24.95, 58.65 and 61.96% at 1, 5, 10, 50 and 100 µg/ml of SM treatment, respectively. The IC₅₀ value of SM was 42.6 µg/ml. As a positive control, we measured the effects of ascorbic acid (AC) on DPPH-scavenging activity, which increased to 20.35, 54.08, 53.39, 59.95 and 64.23% at 1, 5, 10, 50 and 100 µg/ml SM concentrations, respectively. The IC₅₀ value of AC was 4.6 µg/ml (Fig. 1C).

SM reduces apoptosis in the human pharyngeal cell line. Nuclear DNA breaks in human pharyngeal cells were detected using TUNEL staining to estimate the extent of apoptosis and assessed under magnification, x400. The results of the TUNEL staining showed positive staining for DNA fragmentation in the 5-FU-treated compared with the untreated cells (Fig. 2). The number of TUNEL-positive cells was reduced by 1, 10 and 100 µg/ml following SM treatment.

SM regulates apoptosis-related signaling pathways. We investigated whether SM is associated with NF-κB and caspase-dependent apoptosis. The NF-κB and caspase-3 signaling pathways were activated by 5-FU treatment but were decreased by all concentrations of SM (Fig. 3).

Histological effects of SM on cheek pouches of hamsters. H&E staining was carried out to observe the histological changes in the cheek pouches. The normal group is the vehicle-treated group and the control is the 5-FU (80 mg/kg, i.p.) only treated group. Positive control is the benzydilimine HCl-treated group. In addition, there were three co-treatment groups with SM (100, 500 and 1,000 mg/kg) and 5-FU (80 mg/kg, i.p.). Histological examination demonstrated a normal arrangement of cellular components in the cheek pouches of the hamsters (Fig. 4A). Stratum corneum exfoliation, degradation of the epithelial layer and ulcers were observed in the control (Fig. 4B). The observed damages were recovered in the positive control (Fig. 4C) and the 500, 1,000 mg/kg of SM-treated groups (Fig. 4E and F); epithelial layers were recovered.

Detection of apoptosis in cheek pouches of hamsters. To examine the apoptosis in cheek pouches of the hamsters, TUNEL staining was carried out. The cheek pouches of hamsters showed a significant increase in apoptotic changes in the group treated with 5-FU (Fig. 5B, arrow) when compared with the normal group (Fig. 5A), while the positive control group (Fig. 5C) and SM and 5-FU-treated groups showed epithelial layer and granular layer (Fig. 5D-F).

Effect of SM on the expression of IL-1β, TNF-α, NF-κB and caspase-3 in cheek pouches of hamsters. Western blotting was performed to determine the effect of SM on proinflammatory cytokines (IL-1β and TNF-α), NF-κB and caspase-3 expression level in cheek pouches of hamsters. As shown in Fig. 6, NF-κB, TNF-α and IL-1β protein expression levels in the control group were increased compared to the normal group (136.01%, p<0.05; 141.5%, p<0.05; 147.0%, p<0.01, respectively). NF-κB, TNF-α and IL-1β protein levels in the positive control (benzydilime HCl-treated) group were decreased compared to the control group (94.7 vs. 136.0%, p<0.01; 110.1 vs. 141.5%, p<0.01 and 129.9 vs. 147.0%, p<0.01, respectively). In contrast, in the 1,000 mg/kg SM and 5-FU-treated...
Figure 3. Immunoblot analysis of nuclear factor-κB (NF-κB) and caspase-3 proteins from 5-fluorouracil (5-FU)-induced apoptotic human pharyngeal cells after SM treatment. β-tubulin was used as the internal control. Western blot results of (A) NF-κB and cleaved caspase-3 and (B) the relative expression levels.

Figure 4. Examination of histological changes in cheek pouches of hamsters using hematoxylin and eosin (H&E) staining. (A) Normal (vehicle-treated) group, (B) control [5-FU (80 mg/kg, i.p.) only], (C) positive control (benzydamine HCl-treated group), (D) 100 mg/kg SM + 5-fluorouracil (5-FU)-treated group, (E) 500 mg/kg SM + 5-FU-treated group and (F) 1,000 mg/kg SM + 5-FU-treated group. Arrows indicate the histological changes in the epithelial layer, granular layer and muscle layer on the cheek pouches of the hamsters. Images were obtained at an objective magnification, x200. Scale bar, 200 µm.
Figure 5. TUNEL assay in the cheek pouches of hamsters subjected to 5-fluorouracil (5-FU)-induced oral mucositis. (A) Normal (vehicle-treated) group, (B) control [5-FU (80 mg/kg, i.p.) only], (C) positive control (benzyldine HCl-treated group), (D) 100 mg/kg SM + 5-FU-treated group, (E) 500 mg/kg SM + 5-FU-treated group and (F) 1,000 mg/kg SM + 5-FU-treated group. Arrows indicate TUNEL-positive nuclei in the epithelial layer and granular layer on the cheek pouches of the hamsters. Images were obtained at an objective magnification, x200. Scale bar, 200 µm.

Figure 6. Effect of Salvia miltiorrhiza Bunge (SM) on the expression of nuclear factor-κB (NF-κB), interleukin (IL)-1β, tumor necrosis factor (TNF)-α and caspase-3 proteins in cheek pouches of hamsters. (A) Western blot showing the protein expression levels. (B) The western blot bands were quantified by normalization to those from the vehicle-treated group and individuals. Each column represents the mean ± SD (n=3). Significantly different from the normal value (*p<0.05, **p<0.01). Significantly different from the control value (#p<0.05, ##p<0.01).
group, NF-κB, TNF-α and IL-1β protein expression levels were significantly decreased when compared to these levels in the control group (108.7 vs. 136.0%; 118.19 vs. 141.5%; 115.3 vs. 147.0%, p<0.05, respectively; Fig. 6).

Caspase-3 cleaved protein level in the control group was increased compared to that noted in the normal group (136.62, p<0.05, respectively). This level was decreased in the positive control (benzyldimine HCl) group compared to the control group (108.3 vs. 136.6%, p<0.05, respectively). In addition, in the SM and 5-FU-treated groups, caspase-3 protein expression was significantly decreased when compared with that noted in the control group (103.7%, p<0.05 and 96.1%, p<0.01 vs. 136.6%, respectively; Fig. 6).

**Discussion**

Cancer chemotherapy targets rapidly dividing cancer cells but also interferes with DNA, RNA and protein synthesis (27). Therefore, normal tissue cells as well as cancer cells are damaged. Oral mucositis is an adverse effect of cancer chemo-therapeutic drugs (28). Ulceration of the oral mucosa and oropharynx can lead to decreased quality of life and modification with highly expensive cancer treatments (29). The use of current medications for mucositis such as palifermin and benzylamine is limited due to their high cost (30). Therefore, the development of new oral mucositis therapeutic drugs that can be universally used to promote effectiveness is necessary. *Salvia miltiorrhiza* Bunge (SM) is known for its antioxidative and anti-inflammatory effects (22,23). However, the molecular mechanisms of SM are poorly understood, and its effects on oral mucositis have yet to be determined. In the present study, we investigated the effects of SM on 5-FU-induced oral mucositis.

5-FU is an anticancer drug which is used most frequently for carcinomas of the breast, colon, and skin (31). 5-FU inhibits thymidylate synthase or incorporation of nucleic acid into RNA and DNA and causes cell death. But it can induce oral mucositis. Therefore, inhibition or the prevention of the cytotoxic effects of 5-FU on the mucosa is a reasonable strategy (32).

In this study, we examined cell viability using an MTT assay, DPPH and ROS reproduction to determine the antioxidant activities of SM. Generation of ROS linked to increased oxidative stress causes oxidative damage and the pathogenesis of several diseases (33). ROS are an important mediator of downstream biological of oral mucositis (34). The antioxidant ability of natural products is crucial in oral mucositis treatment. SM promoted proliferation of human pharyngeal cells without cytotoxic effects. Additionally, SM showed protective effects against 5-FU-induced cytotoxicity and, as expected, stimulated cell growth.

The DPPH assay is widely used to assess free radical-scavenging abilities of natural products reflecting their antioxidant properties (35). SM reduced the stable radical DPPH to yellow-colored diphenylpicryl hydrazine dose-dependently. Based on this result, SM showed antioxidant activities against scavenging DPPH free radicals. Additionally, ROS production was effectively suppressed at all concentrations in the SM-treated cells. To further understand the antioxidant effects of SM on the human pharyngeal cell line, we examined ROS production. SM-treated cells showed significantly lowered ROS production implying that SM may protect mucosal injury initiated by ROS generation.

DNA strand breaks caused by ROS activate transcription factors such as NF-κB (36). Activation of the NF-κB pathway leads to the expression of antiapoptotic genes and induces proinflammatory cytokines (37). Additionally, these reactions cause oral mucositis. The biological steps of oral mucositis involve various pathways including those associated with mitogen-activated protein kinase (MAPK), ceramide and matrix metalloproteinases (MMPs) (38). Thus, therapeutic drugs for oral mucositis will help reduce apoptosis and recover epithelial cells in the oral mucositis. To examine the regulation of apoptosis by SM in our study, the NF-κB and caspase-independent apoptotic pathways were examined by immuno blotting using 5-FU-treated human pharyngeal cells and hamster cheek pouches. In the TUNEL assay, the number of apoptosis-positive cells in the SM-treated groups was decreased compared with the control, and SM treatment also reduced the expression of NF-κB and cleaved caspase-3 both in vitro and in vivo. Several transcription factors and proinflammatory cytokines such as IL-1β and TNF-α are known to be involved in the development of mucositis (39). SM treatment for 14 days led to a decrease in the expression level of proinflammatory cytokines (IL-1β and TNF-α) and NF-κB in the 5-FU-induced oral mucositis.

In conclusion, the present study demonstrated that SM promoted cell proliferation and had protective effects against oxidative stress. Additionally, SM inhibited apoptotic cell death mediated by the NF-κB-caspase-3 signaling pathways. Moreover, changes in NF-κB and proinflammatory cytokine expression following SM treatment suggest that SM reduces inflammation during the development of mucositis. Although other molecular mechanisms remain to be elucidated in further studies, this study showed the possible use of SM as a therapeutic agent for oral mucositis.

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