Deoxyschisandrin inhibits H_2O_2-induced apoptotic cell death in intestinal epithelial cells through nuclear factor-κB

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Abstract. Oxidative stress is a pathogenesis for a typical inflammatory intestinal disease known as ulcerative colitis (UC) characterized by erosion and mucosal ulceration. For the treatment of UC, many kinds of traditional Asian medical plants have been used. *Schisandra chinensis* fruits (SC) are known to possess anti-ulcer, anti-hepatotoxic and anti-neurotoxic activity. However, its mechanism is still unknown. In the present study, we investigated the cytoprotective effect of deoxyschisandrin, a lignan compound comprised of SC fruits, on H_2O_2-induced apoptotic cell death in human intestinal epithelial cells (HCT116). In flow cytometry assay using Annexin V and propidium iodide, deoxyschisandrin inhibited H_2O_2-induced apoptotic cell death. To further evaluate the apoptotic signaling by H_2O_2, we detected caspase-3 activation using cleavage of pro-caspase-3. Deoxyschisandrin inhibited H_2O_2-induced caspase-3 activation by blocking cleavage of pro-caspase-3. Furthermore, it has been reported that oxidative stress by H_2O_2 induces an activation of nuclear factor-κB (NF-κB). In our results, H_2O_2 stimulated the degradation of IκBα, inhibitor of NF-κB, in a concentration-dependent manner. On the contrary, deoxyschisandrin inhibited H_2O_2-stimulated degradation of IκBα and activation of NF-κB by blocking translocation of NF-κB to the nucleus. Therefore, we suggest that deoxyschisandrin inhibits H_2O_2-induced apoptotic cell death.

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Abbreviations: IBD, inflammatory bowel disease; IECs, intestinal epithelial cells; NF-κB, nuclear factor-κB; ROS, reactive oxygen species; SC, *Schisandra chinensis* fruits; UC, ulcerative colitis

Key words: apoptosis, deoxyschisandrin, intestinal epithelial cells, nuclear factor-κB, oxidative stress

Introduction

Intestinal epithelial cells (IECs) constitute a single layer of the intestine and act as a critical physical barrier against pathogen invasion. It is considered to be an important component associated with initiation and regulation of adaptive and innate defense mechanisms in the mucosal immune system (1). In IECs, the activation of the pro-inflammatory gene is related with chronic and acute intestinal inflammation (2-4).

Ulcerative colitis (UC) is a type of inflammatory bowel disease (IBD) characterized by mucosal ulceration, up-regulation of pro-inflammatory cytokines, and infiltration of inflammatory cells into the colonic mucosa (5,6). Although the etiology of UC is not clearly understood, it has been reported that exaggerated immune response by immune cells such as white blood cells (mainly neutrophils) is a considerable pathogenesis factor (7). Responsibility of neutrophils for tissue damage in the colonic mucosal surface has been well described but an explanation of the mechanism has not been proposed (8). However, the possible mechanism has been reported that reactive oxygen species (ROS)-mediated oxidative stress is a major pathogenic mechanism in IBD. ROS, reactive molecules containing the oxygen atom, can initiate inflammatory cascades and cause subsequent tissue damage (9). ROS-mediated oxidative stress provokes oxidative damage, results in neutrophil infiltration into the colonic mucosa. H_2O_2 is a major contributor to ROS-mediated oxidative stress (10-12).

H_2O_2 is produced as a toxic by-product in living organisms through the mitochondrial electron transport chain (ETC) respiratory process (13). When H_2O_2 is produced excessively by aberrant cellular metabolism in intestinal epithelial cells, H_2O_2 can have an effect on damage of colonic barrier function structures to induce UC (10). Furthermore, H_2O_2-induced oxidative stress regulates the intracellular signaling pathway. Representatively, nuclear factor-κB (NF-κB), a heteromeric transcription factor, is activated by H_2O_2 (14). NF-κB mediates cell survival and inflammation via regulation of apoptotic genes such as caspase-3 and anti-apoptotic genes such as Bcl-2 (15).

Schisandra Chinensis fruits (SC) is widely used as a traditional Asian medical plant for diarrhea. It has been reported that SC has anti-ulcer, anti-hepatotoxic and anti-neurotoxic activity (16-18). We also previously demonstrated

inhibitory effects of aqueous extract from SC in dextransulfate sodium (DSS)-induced colitis mouse model, but the molecular mechanism has still not been identified (19). SC contains a variety of pharmacologically active lignan components (20).

Deoxyschisandrin is one of the lignan components of SC. It consists of dibenzocyclooctadiene which possesses hepatoprotective, neuroprotective, and antioxidant activities (21-24). It was reported that deoxyschisandrin has neuroprotective activity in glutamate-induced neurotoxicity. Deoxyschisandrin attenuated the content of cellular peroxide and enhanced the glutathione antioxidative defense system in rat cortical cells (24).

In the present study, we investigated the effect of deoxyschisandrin on apoptotic cell death of intestinal epithelial cells (HCT116) induced by H_2O_2. We attempted to identify the molecular mechanisms. The study demonstrated that deoxyschisandrin suppresses H_2O_2-induced apoptotic cell death through inhibition of the activities of NF-κB.

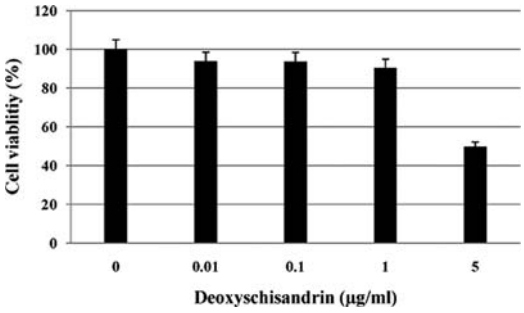

Figure 1. Effects of deoxyschisandrin on the viability of HCT116 cells. Cells were treated with deoxyschisandrin in various concentrations from 0.01 to 5 μg/ml for 24 h. Cell viability was determined using CCK-8 assay. Values are presented as percent of control and results are the mean ± SEM of three independent experiments.

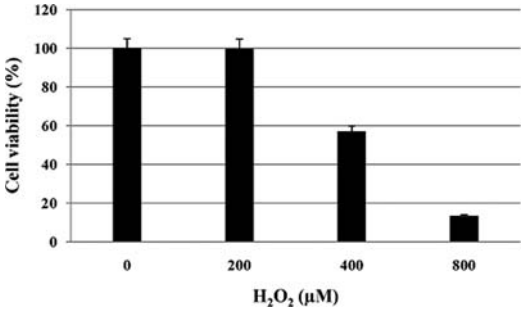

Figure 2. Suppression of H_2O_2 on the viability in HCT116 cells. Cells were treated with H_2O_2 in different concentrations (0-800 μM) for 24 h. Cell viability was determined using CCK-8 assay. Values are represented as percent of control and the results are the mean ± SEM of three independent experiments.

Materials and methods

Materials and cell culture. Deoxyschisandrin was purchased from Chromadex (CA, USA). HCT116 cell line was incubated in Dulbecco's modified Eagle's medium (DMEM) (Thermo Scientific Hyclone, UT, USA) with penicillin (100 units/ml) - streptomycin (100 μg/ml) (Welgene, Korea), and 10% fetal bovine serum (Thermo Scientific Hyclone, UT, USA) at 37˚C under 5% CO_2 humidified air.

CCK-8 assay. To evaluate cell viability, we used CCK-8 cell counting kit (Dojindo Molecular Technologies, MD, USA). Cells were prepared in 96-well plates ($5x10^3$ cells/plate) to treat H_2O_2 or deoxyschisandrin. Absorbance was measured at 450 nm.

Determining early apoptosis and necrosis by flow cytometry. For apoptosis and necrosis analysis of HCT116 cells, flow cytometry assay was performed using FITC-labeled Annexin V (BD Biosciences Parmingen, CA, USA) and propidium iodide (PI) (Sigma Aldrich, Germany). Cells were seeded into 12-well plates ($4x10^4$ cells/plate), and 24 h later, were treated with H_2O_2 and deoxyschisandrin in each concentration. After treatment, cells were gently detached with Detachin (Genlantis, CA, USA) and washed with PBS containing 0.1% sodium azide. Cells were incubated with Annexin V (final concentration, 1 μg/ml) for 15 min in the dark, washed one more time, and suspended in PI (final concentration 5 μg/ml). Stained cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences, CA, USA). A total $5x10^3$ cells were analyzed per sample.

Western blot analysis. To detect protein expression levels, cells were detached using cell scrapers and washed with PBS. Lysis buffer (Intron Biotech, Korea) was used to prepare total cell lysates. Lysates were electrophoresed using 10-15% SDS-PAGE and transferred to PVDF membranes (Amersham Pharmacia Biotech, NJ, USA). Membranes were incubated with blocking solution, containing a 1:1,000 dilution of anti-IκBα (Santa Cruz Biotechnology, CA, USA), or anti-caspase-3 (Santa Cruz Biotechnology, CA, USA) antibodies. Antibody of anti-GAPDH (Epitomics, CA, USA) diluted 1:2,000 with blocking solution was used as a loading control. Then, the membranes were incubated with blocking solution containing a 1:10,000 dilution of horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (Amersham Pharmacia Biotech, NJ, USA). An enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, NJ, USA) was used to detect the epitope on the proteins recognized by the specific antibody.

Immunofluorescent staining and nuclear staining. HCT116 cells were grown in culture slides for 24 h, and then treated either with or without H_2O_2 and deoxyschisandrin. After treatment, cells were treated with 4% paraformaldehyde (PFA) for 15 min. After washing 3 times, cells were blocked with 1% bovine serum albumin (BSA)-PBS for 3 h and incubated with rabbit monoclonal NF-κB p65 antibody (Epitomics, CA, USA) diluted 1:100 with 1% BSA-PBS overnight in 4˚C. Incubated cells were washed 3 times, and treated with FITC-conjugated anti-rabbit secondary antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) diluted 1:200 with 1% BSA-PBS for 20 min. Then, cells were stained with DAPI solution for the nuclei staining. The fluorescence and translocation of NF-κB were evaluated by confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss Co., Germany).

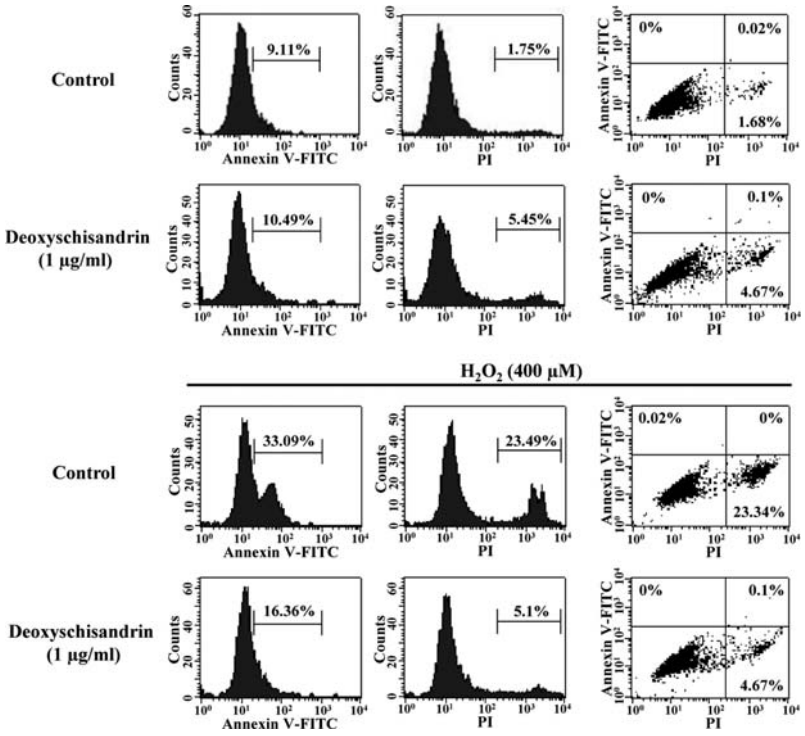

Figure 3. Inhibitory effect of deoxyschisandrin on H_2O_2-induced apoptotic cell death in HCT116 cells. Cells were pretreated with deoxyschisandrin (1 μg/ml) for 30 min and stimulated with H_2O_2 (400 μM) for 4 h. Flow cytometry assay was performed with Annexin V and PI staining.

Results

Deoxyschisandrin has a cytotoxic effect in HCT116 cells. We performed CCK-8 assay in human intestinal epithelial cell line (HCT116) with deoxyschisandrin (Fig. 1). Deoxyschisandrin was treated with various concentrations for 24 h. As a result, the cell viability was not affected up to 1 μg/ml of deoxyschisandrin, whereas 5 μg/ml of deoxyschisandrin decreased cell viability.

H_2O_2 induces apoptotic cell death. We examined the cytotoxic effect of H_2O_2 to investigate whether H_2O_2 induces apoptosis in HCT116 cells using CCK-8 assay (Fig. 2). Cell viability was measured after treatment with various concentrations of H_2O_2 for 24 h. In an H_2O_2 concentration-dependent condition, cell viability decreased in over 400 μM of H_2O_2. When cells were treated with 400 μM of H_2O_2 for 24 h, the viability of cells was reduced to ~57% of control, whereas the viability of cells treated with 800 μM of H_2O_2 was significantly reduced to 13% of control. Next, we confirmed H_2O_2-induced apoptotic cell death in flow cytometry assay using co-staining with Annexin V and PI (Fig. 3). Annexin V-FITC and PI fluorescent intensity were increased in H_2O_2-treated cells more than H_2O_2-non-treated cells. The percentage of apoptotic/necrotic cells increased from 1.68% in H_2O_2 non-treated control to 23.34% in H_2O_2 treated control.

Activation of caspase-3 has been shown to play a role in oxidative stress-induced apoptotic-cell death (15). Therefore, the examination of the effect of H_2O_2-induced apoptosis focuses on activation of caspase-3, we measured the cleavage level of pro-caspase-3. We exposed cells to 400 μM of H_2O_2 at various times (Fig. 4A). Expression of pro-caspase-3 was time-dependently decreased. In the treatment of cells for 4 h, expression level of pro-caspase-3 was highly decreased. It reveals that caspase-3 was activated by stimulation of H_2O_2. These findings support that H_2O_2 induces apoptotic cell death in the intestinal epithelial cell line.

Deoxyschisandrin inhibits H_2O_2-induced apoptotic cell death. To determine whether deoxyschisandrin has an inhibitory effect on H_2O_2-induced apoptotic cell death, we first analyzed cell apoptosis using flow cytometry assay. FACS was used to quantitatively determine and distinguish the percentage of viable, apoptotic, and necrotic cells after pretreatment of deoxyschisandrin (1 μg/ml) for 30 min and H_2O_2 (400 μM) treatment for 4 h (Fig. 3). The percentage of apoptotic/necrotic cells pre-treated with deoxyschisandrin was significantly attenuated up to 4.67% compared to H_2O_2-treated control. It was similar with H_2O_2 non-treated cells. This shows that deoxyschisandrin can protect cells from H_2O_2-induced apoptotic cell death. Furthermore, we detected activation of caspase-3 in Western blot analysis (Fig. 4B). In cells treated with deoxyschisandrin and H_2O_2, expression of pro-caspase-3 was higher than in just H_2O_2-treated cells. Therefore, activation of caspase-3 was inhibited by deoxyschisandrin.

Deoxyschisandrin inhibits activation of NF-κB in stimulation by H_2O_2. Oxidative stress by H_2O_2 induces apoptotic cell death. H_2O_2 is known as a stimulant of NF-κB-translocation from the cytoplasm to the nucleus. We examined whether stimulation by H_2O_2 activates NF-κB through detection of the presence level for IκBα in cells (Fig. 5A). H_2O_2 (400 μM) was

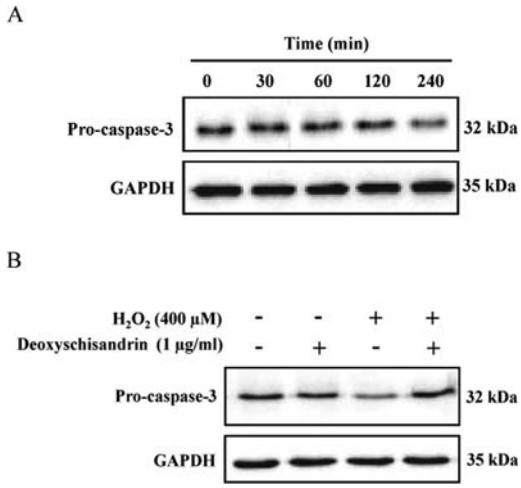

Figure 4. Effects of H_2O_2 (A) and deoxyschisandrin (B) on caspase-3 activation. Cells were treated with H_2O_2 (400 μM) in a time-dependent manner (A). Cells were pretreated with deoxyschisandrin (1 μg/ml) for 30 min and stimulated with H_2O_2 (400 μM) for 4 h (B). Blots were examined with anti-caspase-3 antibody.

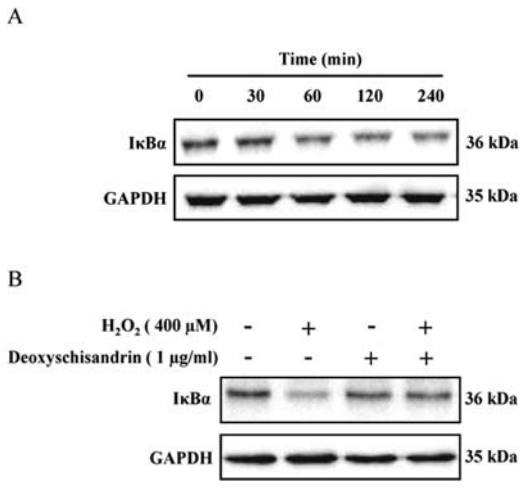

Figure 5. Effects of deoxyschisandrin on IκB degradation by H_2O_2 exposure. Cells were treated with H_2O_2 (400 μM) in a time-dependent manner (A). Cells were pretreated with deoxyschisandrin (1 μg/ml) for 30 min and stimulated with H_2O_2 (400 μM) for 4 h (B). Blots were examined with anti-IκBα antibody.

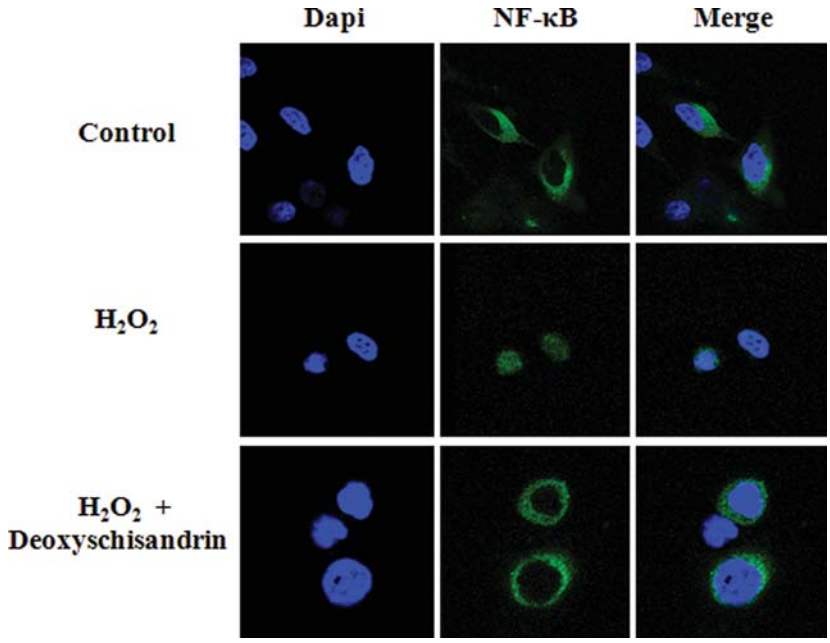

Figure 6. Effects of deoxyschisandrin on NF-κB translocation by H_2O_2 exposure. Cells were treated with H_2O_2 (400 μM) with or without deoxyschisandrin (1 μg/ml) for 4 h. In order to perform NF-κB translocation analysis, cells were stained with FITC-conjugated anti-NF-κB antibody (green) and DAPI solution (blue) for nuclei staining. Fluorescence was visualized using confocal laser scanning microscope.

treated in a time-dependent manner, and degradation level of IκBα was detected using Western blot analysis. Degradation of IκBα by phosphorylation was decreased from 60 min, and significantly decreased after 4 h treatment with H_2O_2. This result supports that H_2O_2 induced the activation of NF-κB through degradation of phosphorylated IκBα, inhibitor of NF-κB. To determine the effect of deoxyschisandrin on H_2O_2-induced NF-κB, cells were treated with deoxyschisandrin (1 μg/ml) prior to H_2O_2 (400 μM) stimulation (Fig. 5B). Interestingly, cells treated with deoxyschisandrin before H_2O_2 treatment showed a similar expression level of IκBα with non-stimulated cells. It showed that deoxyschisandrin inhibited degradation of IκBα to prevent activation of NF-κB.

To confirm the nuclear translocation of the p65 subunit of NF-κB, we performed an immunofluorescence assay (Fig. 6). Subsequent to H_2O_2 (400 μM) exposure for 4 h, nuclear translocation of the NF-κB p65 subunit was confirmed. DAPI staining was used to inspect the nucleus. In contrast, treatment with deoxyschisandrin (1 μg/ml), which was identified to have an inhibitory effect for degradation of IκBα, inhibited translocation of the NF-κB p65 subunit from the cytoplasm to the nucleus in HCT116 cells. These data suggest

that deoxyschisandrin suppresses H_2O_2-induced apoptotic cell death by inhibition of NF-κB.

Discussion

IECs are considered an important physical barrier in response to many inflammatory stimuli in intestinal inflammation, because most pro-inflammatory genes are expressed in the activated IECs (3,4). In IECs, it has been demonstrated that ROS-induced oxidative stress is involved in the development of IBD (8). Excess oxidative stress by H_2O_2, a major contributor, induces infiltration of immune cells, and then, immune cells activate NF-κB to produce pro-inflammatory mediators such as tumor necrosis factor α (TNF-α) and pro-apoptosis factors in IECs. Subsequently, the colonic tissue is destroyed leading to UC with diarrhea and rectal bleeding.

In the present study, we identified whether deoxyschisandrin has an inhibitory effect on H_2O_2-induced apoptotic cell death by stimulation in IECs. We exposed exogenous H_2O_2 (400 μM) to HCT116. Apoptotic cell death was induced time-dependently by H_2O_2. Caspase-3 is known as a central component of the apoptosis machinery (25). We verified that H_2O_2-induced apoptotic cell death was related to activation of caspase-3. Interestingly, the percentage of apoptotic/necrotic cells was significantly decreased with deoxyschisandrin treatment in flow cytometry assay using Annexin V and PI. Deoxyschisandrin attenuated activation of caspase-3 by H_2O_2 in IECs. In addition, NF-κB plays an essential role as a transcriptional factor involved in inflammatory processes and cell apoptosis. In the resting state, NF-κB is sequestered in the cytoplasm in an inactive form by inhibitory protein IκB. In response to oxidative stress by H_2O_2, IκB is phosphorylated by IκB kinases (IKKs) and degraded by proteasomes (26-28). NF-κB translocates subsequently to the nucleus in an active form (26). We tried to clarify whether H_2O_2-induced apoptotic cell death is related to NF-κB in IECs. In stimulation by H_2O_2, NF-κB was activated and translocated into the nucleus. On the contrary, pretreatment with deoxyschisandrin inhibited the activation and translocation of NF-κB by H_2O_2 through blocking degradation of IκBα. It is likely that deoxyschisandrin prevents degradation of IκBα by the ubiquitin-proteasome pathway to inhibit activation of NF-κB. Furthermore, these results support our previous *in vivo* data that SC significantly inhibited the development of UC in the mouse model (19).

When the balance between pro-inflammatory and anti-inflammatory mediators is impaired, IBD breaks out. NF-κB is a key factor in IBD including UC, because it regulates transcriptional activity for various pro-inflammatory mediators like TNF-α, interleukin-6 (IL-6), and interleukin-1 (IL-1) as well as pro-apoptotic factors. Although exact mechanisms were not identified, it has been reported that factors such as TNF act to stimulate NF-κB for regulation of cell survival and pro-apoptotic signaling in UC (29). In fact, the expression level of NF-κB subunit p65 was increased in epithelial cells of UC (30). Activation of NF-κB was significantly augmented in severe intestinal inflammation (31). Furthermore, some therapeutic drugs for IBD like sulfasalazine, methotrexate, corticosteroids and sucralfate were established to improve the anti-inflammatory effect by inhibition of NF-κB activation (32-36).

In conclusion, we focused on oxidative stress as a pathogenesis of UC. This study suggests that deoxyschisandrin may be a useful therapeutic agent for oxidative stress-induced UC through inhibiting activation of NF-κB. NF-κB is involved in transcription of diverse genes. Therefore, further studies are necessary to identify what kind of factors are mediated by inhibitory effect of deoxyschisandrin for NF-κB activation in ROS-induced oxidative stress manner in *in vivo* model.

Acknowledgements

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