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Dietary squalene supplementation improves DSS-induced acute colitis by downregulating p38 MAPK and NFkB signalling pathways

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Abbreviations: ***COX***, cyclooxygenase; ***DAI,*** disease activity index; ***FOXP3***, forkhead box P3; ***IBD***, inflammatory bowel disease; ***iNOS***, inducible nitric oxide synthase; ***JAK-STAT***, Janus kinase-signal transducer and activator of transcription; ***MAPKs***, mitogen activate protein kinases; ***NFkB***, nuclear factor-kappa B; ***Nrf2***, nuclear factor (erythroid-derived 2)-like 2; ***ROS***, reactive oxygen species; ***TNF-α*** , tumor necrosis factor-α; ***UC***, ulcerative colitis.

Key words: dextran sulfate sodium, MAPK, NFkB, squalene, ulcerative colitis

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Abstract

SCOPE: Squalene is a polyunsaturated triterpene which has exhibited anti-cancer and anti-oxidant activities among others. We investigated dietary squalene supplementation effect on an acute colitis model induced by dextran sulfate sodium (DSS) in C57BL/6 mice.

METHODS AND RESULTS: Mice were fed from weaning with squalene at 0.02% and 0.1%. After 4 weeks, mice were exposed to 3% DSS for 5 days developing acute colitis. After DSS removal (5 days), colons were histological and biochemically processed. Our results showed that dietary squalene treatment exerts anti-inflammatory action in DSS-induced acute colitis. Western blot revealed that squalene downregulated COX-2 and iNOS system by inhibition of MAPK p38 and of the NFkB signalling pathways, preventing an increase in the cytokines levels. Under our experimental conditions, STAT3 and FOXP3 were not modified and the transcriptional regulation of anti-oxidant and/or detoxifying enzymes, Nrf2, was reduced in DSS-induced colitis. However, any change could be observed after squalene supplementation. CONCLUSION: squalene was able to improve the oxidative events and returned pro-inflammatory proteins expression to basal levels probably through p38 MAPK and NFkB signalling pathways. However, supplementary studies are needed in order to provide a basis for developing a new dietary supplementation strategy.

1. Introduction

Ulcerative colitis (UC) is one of the two major forms of inflammatory bowel disease, which is a chronic relapsing inflammatory disorder of the bowel involving mainly the colonic mucosa and submucosa. Although the exact mechanism remains unclear, it has been commonly accepted that upregulation of certain proteins i.e. cyclooxygenase (COX)-2 or inducible nitric oxide synthase (iNOS), play an important role in immune dysregulation of UC [1,2]. On the other hand, signalling pathways such as mitogen activate protein kinases (MAPKs) are also implicated by leading to the activation of nuclear transcriptors factors. Among them, nuclear factor-kappa B (NFkB) participates controlling the activation of various pro-inflammatory cytokine genes such as IL-1β, IL-6 and tumor necrosis factor-α (TNF-α) supporting a critical role in the pathogenesis of UC [3-5]. In addition, the Janus kinase-signal transducer and activator of transcription (JAK-STAT) is another relevant inflammatory pathway activated in response to cytokines [6-8]. Among STAT family member, the role of STAT3 in the pathogenesis of UC has been most well documented by studies obtained from experimental and clinical data [6-8]. Moreover, nuclear factor (erythroid-derived 2)-like 2 (Nrf2) has also shown a central role in the transcriptional regulation of anti-oxidant and/or detoxifying enzymes, as well as suppression of pro-inflammatory signalling pathways [9]. Additionally, natural Tregs, which are characterized by the expression of forkhead box P3 (FOXP3) participate in the control of pathogenic autoreactivity and the maintenance of immuno homeostasis [10-12]. In fact, maintenance of Tregs functions may ameliorate inflammatory bowel disease (IBD) [13, 14]

Squalene is a triterpene polyunsaturated comprising six isoprene units, structurally similar to β-carotene, which acts as precursor of cholesterol and other steroids. It is widely distributed in nature, where we can find it in the shark liver oil in wheat germ and rice bran

[15]. It is also the most abundant compound in the unsaponifiable fraction of the olive oil [16].

Up to date, anti-cancer, anti-oxidant, drug carrier, detoxifier, skin hydrating, and emollient activities of squalene have been reported both in animal models and *in vitro* environments [17, 18]. Moreover, squalene is a highly effective anti-oxidant by direct reactive oxygen species (ROS) scavenging agent reducing intracellular oxidant stress and also protects human skin surfaces from lipid peroxidation as a quencher of singlet oxygen. Additionally ROS scavenging activity in the striatum of a Parkinson's disease mouse model has been reported [19]. Moreover, a cardioprotective action has also been shown in several experimental models mainly by blocking lipid peroxidation [20, 21]. On the other hand, squalene is considered to be an important component in the diet due to its chemopreventive potential against cancer [22].

Taken this background into account and inasmuch as no studies to evaluate its role in inflammatory disorders, the present study was designed to assess the effects of dietary squalene supplementation on an acute colitis model induced by dextran sulfate sodium (DSS) in C57BL/6 mice by macroscopic and histological methods. The inflammatory mediators TNF-α and IL-1β were determined by ELISA. Protein expression of COX-2 and iNOS was evaluated by Western blot. Moreover, we studied the role of MAPKs, NFkB, JAK-STAT, Nrf2 and FOXP3 signalling pathways in the potential beneficial effects of dietary squalene treatment in colonic mucosa under early acute inflammatory conditions.

2. Material and Methods

2.1.Animals and diets

A total of 45 6-week-old female C57BL/6 mice were provided by Charles River (Tokyo, Japan) and maintained in our Animal Laboratory Center under standard conditions (temperature 24-25ºC, humidity 70-75%, lighting regimen of 12L/12D). They were fed pellet

diets and water at libitum. Mice were randomized into four groups during all experimental period: sham group and control group received standard diet (AIN76A) and a third and fourth groups were fed with squalene (Sigma-Aldrich Company Ltd, Spain) at 0.02% and 0.1% of diet, respectively. All of them were made adding a solution 0,02 g or 0,1 g of squalene to 100 g of crushed AIN76A (0,02 and 0,1 %), which is mixed, divided in pellets and air drying under yellow light. Control group received the diet elaborated at the same manner without squalene addition (standard diet). Fresh diet was provided daily. Animals from dietary groups consumed an average of 3 g/day of diet, resulting in a dose of 25 or 125 mg/kg body weight of squalene ingested. The administered dose was chosen in based of previous study described in the literature [23]. Body weight, food and water intake were evaluated weekly. The protocol for animal handling and experimentation was in accordance with the European Union European Community guidelines for the ethical treatment of animals (2010/63/UE) and was approved by the Ethical Committee for Animal Research of the University of Seville.

2.2. Induction of colitis

Colitis was induced according to the procedure described by Melgar et al. (2005) [24]. Four weeks after weaning and fed with the diets in study, all animals groups, except sham, received 3% DSS (DSS group; MW: 40000, ICN Pharmaceuticals, Costa Mesa, CA) in drinking water for 5 days, developing acute colitis. After of these 5 days, DSS is removed and animals followed feeding for 5 days more and then they were sacrificed by i.p. chloral hydrate at 12% (v/v).

2.3. Evaluation of the severity of clinical colitis

The clinical activity of colitis was evaluated by an independent observer who was blinded to the treatment in order to determine the disease activity index (DAI) as described Melgar et al. (2005) with slight modifications [25] (Supporting Information Table 1). The presence of

diarrhea, rectal bleeding and weight loss were registered during DSS treatment as well as during the follow five days when the animals were without DSS. The average of the three values constituted the DAI.

2.4. Macroscopic and histopathological evaluation

At the end of the experimental period, the colons were removed, slightly cleaned in physiological saline to remove fecal residues, weighed and measured in order to evaluate variations in the weight/length as an inflammation index.

Samples of three regions (proximal, middle and rectum) were excised out of every segment, fixed in 4% buffered formaldehyde, dehydrated by increasing concentrations of ethanol and embedded in paraffin. 4 μm thick slices from paraffin sections were stained with haematoxylin and eosin in accordance with the standard procedures for histological evaluation of colonic damage. Rest of pieces of the colon were collected and frozen in liquid nitrogen to measure biochemical parameters.

For DSS colitis, 3 colonic sections of each animal were scored by a pathologist who was unaware of the experimental protocol using a colitis score as previously was described [26]. In brief, for each category of the score (inflammation, extent, crypt damage), points were multiplied by a factor of involvement of the visible epithelium (Supporting Information Table 2). The sum of the 3 category scores ads up to the total score of each section.

2.5.Cytokine levels

Colon samples were weighed and homogenized, after thawing, in phosphate buffer saline solution (PBS pH 7.2) containing a proteases cocktail at 4 ºC and centrifuged at 12,000g for 10 min. Mucosal cytokine levels were assayed with quantitative DuoSet® ELISA kits (R&D Systems, USA). TNF-α and IL-1β values were measured as pg/mg tissue.

2.6. Isolation of cytoplasmic and nuclear proteins and immunoblotting detection

Frozen colonic tissues were processed as described by Sánchez-Hidalgo et al. (2005) [27] in order to isolate cytoplasmatic or nuclear proteins. Protein concentration of the homogenates were determined following Bradford's colorimetric method. Aliquots of supernatants containing equal amounts of protein (50 µg) were separated on 10% acrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In the next step, the proteins were electrophoretically transferred onto a nitrocellulose membrane and incubated with specific primary antibodies: rabbit anti-COX-2 and rabbit anti-iNOS (Cayman, Ann Arbor, MI, USA; 1:2500 and 1:1000, respectively), rabbit anti-IκBα (Cell Signalling, Danvers, MA, USA; 1:1000), mouse anti-pJNK, rabbit-JNK, mouse anti-pp38, rabbit-p38, rabbit anti-p65, mouse anti-pSTAT3, rabbit anti-Nrf2, rabbit anti-FOXP3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000),

overnight at 4°C. After rinsing, the membranes were incubated with a horseradish peroxidase- labeled secondary antibody anti-rabbit (Cayman Chemical, Ann Arbor, MI, USA; 1:50.000) or anti-mouse (Dako, Atlanta, GA, USA; 1:2000) containing blocking solution for 1–2 h at room temperature. To prove equal loading, the blots were analyzed for β-actin expression using an anti-β-actin antibody (Sigma–Aldrich, MO, USA). Immunodetection was performed using enhanced chemiluminiscence light-detecting kit (SuperSignal1 West Femto Chemiluminescent Substrate, Pierce, IL, USA). Densitometric data were studied following normalization to the control (housekeeping gene). The signals were analyzed and quantified by an Image Processing and Analysis in Java (Image J, Softonic, USA).

2.7.Statistical analysis

All values in the figures and text are expressed as arithmetic means ± standard error (S.E.M.). Data were evaluated with GraphPad Prism® Version 5.01 software. The statistical

significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA), using Tukey–Kramer multiple comparisons test as post hoc test. P values of <0.05 were considered statistically significant. In the experiment involving histology and Western blot, the figures shown are representative of at least 4-5 experiments performed on different days.

3. Results

3.1. Squalene supplemented diets improve DSS-induced acute colitis in mice

Loss of body weight, not formed stool and rectal bleeding are symptoms present in all animals DSS treated. Significant loss of body weight was observed from 5th day of DSS treatment and after DSS removal; although mice fed with squalene supplemented diets didn't show significant differences versus DSS control group (Fig 1A). On the other hand, the DAI in animals from DSS control group showed a marked increase from 5th day of DSS treatment, which was increasing until to sacrificed of the animals versus sham group. Moreover, squalene groups improved significantly the DAI index versus DSS control, since it was significantly diminished from 6th day of DSS-treatment until the final (Fig 1B).

After animals were sacrificed a significant decrease of length of the mice colon was observed in DSS-treated group versus sham control group and squalene supplemented diets didn't present significant difference respect to DSS control group (Data no show).

3.2.Histopathological analysis of DSS-induced acute colitis after squalene supplementation

The histological evaluation of colonic tissue from healthy animals revealed a normal structure without histological changes. By contrast, mice with DSS-induced colitis exhibited disruption of the epithelial barrier, a pronounced decrease in the number of crypts, and marked infiltration of inflammatory cells into the mucosa and submucosa, overcoat in middle and rectum sections. In contrast, slides from the group of animals treated with DSS but fed with

squalene supplemented diets revealed a significant reduction of signs of inflammation as well as a minor extent of affected mucosa and loss of epithelial cells, resulting in decreased microscopic damage score, compared with colon of DSS-control group mice in proximal and rectum sections (Supporting Information Figure 1 and Fig. 1).

3.3. IL-1β and TNF-α were decreased after squalene-supplemented diets in acute DSS-induced colitis in mice

As shown in Fig 2, a marked increase in the proinflammatory cytokines IL-1β and TNF-α was characterized in the colitis caused by DSS ($P<0.001$ vs sham group). In contrast, the levels of both were significantly ameliorated in squalene dietary groups (squalene 0.02%: $P<0.05$ and $P< 0.01$; squalene 0.1%: $P< 0.01$ and $P<0.01$ vs DSS-control group, respectively).

3.4. iNOS and COX-2 protein expression inhibition in colon tissue after squalene supplemented diets in DSS-induced acute colitis

The levels of inflammatory proteins expression were measured by Western blotting of cytosolic extracts from colonic mucosa. As shown in Figure 3, iNOS and COX-2 were significantly expressed in colonic samples from DSS-control group ($P<0.01$ and $P<0.05$, respectively). Nevertheless, preventive treatment with squalene -supplemented diets significantly blocked the upregulation of both iNOS and COX-2 immunosignals in colonic tissue (squalene 0.02%: $P<0.05$ and $P<0.01$ and squalene 0.1%: $P<0.05$ and $P<0.05$ vs DSS-control group respectively).

3.5. Effects of dietary squalene supplementation on activation of mitogen-activated protein kinase signalling in DSS-induced acute colitis

In order to determinate the potential implication of MAPKs in the anti-inflammatory effects observed after squalene supplementation on the DSS-induced colitis, p38 and JNK activation were studied by Western blot analysis using phosphospecific MAPKs antibodies. To

standardize protein loading in each line, blots were stripped and reproved with the corresponding antibodies against non-activate MAPKs proteins. As expected, DSS was found significantly to activate p38 and JNK proteins ($P<0.001$ and $P<0.05$ vs. sham group respectively), indicating that both MAPKs protein activation are induced at the acute stage of colonic lesion. However, after preventive dietary squalene treatment, our results demonstrated that only p38 activation was significantly reduced (squalene 0.02% and 0.1%: $P<0.001$ vs. DSS-control group) (Fig. 4).

3.6.Effect of squalene supplementation on NFkB -mediated transcriptional activation and induced IKBα degradation in DSS-induced acute colitis

We tested the potential effect of squalene supplementation on the IκBα degradation and NFkB activation in colonic tissue of mice from DSS-induced colitis. According to the results obtained DSS produced an IκBα degradation which is consistent with an up-regulation of the nuclear translocation levels of p65 protein ($P<0.05$ vs sham group). However, dietary treatment with squalene (0.02 and 0.1% diets) prevented IκBα degradation and significantly reduced the nuclear translocation level of p65 in colonic mucosa ($P<0.05$ vs DSS-control group) (Fig 5). Thus, our results demonstrate that dietary squalene exerts an inhibitory effect on the DSS-induced activation of NFkB.

3.7.Effect of dietary squalene supplementation on STAT3 activation in DSS-induced acute colitis

STAT3 is also known to be involved in colonic inflammation and activated by variety of cytokines and growth factors. As shown in Supporting Information Figure 2, the expression of p-STAT3 in DSS group, which was considered a marker of STAT3 activation, was maintained at the same levels that sham control. After dietary squalene supplementation was not observed any significant modification.

3.8. Effect of dietary squalene supplementation on Nfr2-mediated transcriptional activation in DSS-induced acute colitis

Nrf2 is a key transcription factor that regulates the cellular anti-oxidant response. Upon cell stimulation, Nrf2 is translocated from the cytosol to the nucleus, and sequentially binds to a promoter sequence called the anti-oxidant response, resulting in a cytoprotective response characterized by upregulation of anti-oxidant enzymes and decreased sensitivity to oxidative stress damage. Several studies have demonstrated that activation of Nrf2 signalling and the induction of its target genes could exert anti-inflammation and anti-oxidative effects [28]. To identify whether squalene is effective on regulation of Nrf2, we investigated its protein expression by Western blot. As shown in Supporting Information Figure 2, DSS treatment downregulated Nrf2 expression ($P<0.05$ vs sham group); however dietary squalene supplementation was not able to modify its expression after 5 days of DSS removal.

3.9. Effect of dietary squalene supplementation on FOXP3 in DSS-induced acute colitis

Next, we postulated that squalene supplementation could modulate the Tregs, which are characterized by the expression of FOXP3. The results showed that the expression of FOXP3 was not modified in DSS group neither after dietary squalene supplementation in the colonic tissue (Supporting Information Figure 2).

4. Discussion

Our results show that the dietary administration of squalene was able to improve the clinical signs of the disease ameliorating the first stage in this model of acute UC. Moreover, dietary squalene supplementation was able to block TNF-α and IL-1β activation and mediate COX-2 and iNOS downregulation ameliorating the UC acute stage.

TNF-α and IL-1 play a key role in the mucosal inflammation amplification in IBD. It has been confirmed that selective blockade of both inflammatory mediators decrease

neutrophil/macrophage migration improving colitis progression [29, 30]. On the other hand, it is well known that inducible enzymes COX-2 and iNOS are inflammatory proteins predominantly expressed at sites of inflammation. Their activation produces an excessive inflammatory response which may affect to colon mucosa integrity and contributes to the development of intestinal damage [31, 32]. Therefore, regulation of these pro-inflammatory biomarkers by squalene could contribute to the improvement of the intestinal damage observed after DSS administration

A signalling pathway that control the regulation of those key inflammatory molecules are the MAPKs [33, 34], which are kinases serine/threonine protein families that mediate fundamental biological processes and cellular responses to external stress signals. In fact, preclinical studies with MAPKs inhibitors have demonstrated significant efficacy repeatedly in experimental colitis models making them potential targets for anti-inflammatory therapeutics [35]. It has been recognized that MAPKs are implicated in regulate cytokines production [36] and COX-2 and iNOS upregulation in intestinal epithelial IBD patients cells [37]. In this way, squalene supplementation reduced significantly p38 activation in the colon of the mice with UC although no changes in JNK phosphorylation were observed. One of the well-studied transcription factors downstream of MAPKs signalling is the nuclear factor NFkB. The NFkB family of transcription factors consists of five mammalian members (p50, p52, p65, cRel and RelB), which can form either homodimers or heterodimers. NFkB pathway is activated in mucosal cells of IBD patients [38], as well as in experimental colitis models [36, 39-41]. Phosphorylation of IkBα generally leads to the degradation of IkBα, with the concomitant release of p65NFkB and its nuclear translocation, which binds to the promoter of many genes that are important for the activation of immune responses as iNOS, COX- 2, and cytokines among others [42, 43]. Dietary squalene supplementation significantly inhibited the IκBα degradation and blocked the translocation of p65 into the

nuclei, providing further evidence of the role of NFkB pathway in the beneficial effects observed of squalene in UC.

Different studies showed that mice lacking Nrf2 are more susceptible to DSS induced colitis, suggesting that Nrf2 is essential in protecting intestinal integrity through the regulation of pro-inflammatory cytokines and induction of phase II detoxifying enzymes [9, 44]. We found that DSS-induced colitis was associated with decreased expression of Nrf2 in colon, which is consistent with the mentioned above. However, under our experimental conditions, dietary squalene administration was not able to modify its expression.

The release of inflammatory mediators, in addition to MAPKs activation, modulate other intracellular signalling such as STATs, which can be also activated and translocate from the cytoplasm into the nucleus [45]. There, they recognize and bind to specific promoter regions of their respective target genes and induce expression of STAT-regulated cytokine gene expression, regulating immune and inflammatory responses [45]. Among STATs, the role of STAT3 in IBD has been documented by recent studies obtained from human IBD [8, 46, 47] and experimental IBD models [48-51]. Recently, activation of STAT3 has been shown in different models of DSS colitis model [52, 53]. Although these studies suggest an important role of this transcription factor in UC, our results showed that activation of STAT3 was not modified in DSS-induced acute colitis. Moreover, any change could be observed after dietary squalene supplementation. These results could be explained on the basis of our experimental conditions and colitis experimental model selected.

Interestingly Treg cells, characterized by the expression of FOXP3, are essential to gastrointestinal immuno homeostasis, and a genetic defect in Treg development or dysfunction of Treg suppressive mechanisms can lead to a severe autoimmune colitis [54, 55]. Clinical and experimental studies have shown induction and maintenance of Tregs functions downregulating the established inflammatory response could ameliorate IBD [13,

14]. In a recent paper by Kim et al., 2014 [56], the frequency of FoxP3+ cells was not significantly modified, in parallel with the inflammatory response, in WT mice under DSS-induced colitis. In agreement with above data our results showed that expression of FOXP3 was unchanged significant in DSS group and dietary squalene supplementation groups.

In conclusion, we describe for the first time, the protective effects of squalene in colonic inflammation under early acute conditions. Squalene was able to improve the oxidative events and returned pro-inflammatory proteins expression to basal levels probably through p38 MAPK and NFkB signalling pathways. However supplementary studies are needed in order to provide a basis for developing a new dietary supplementation strategy.

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Figure 1. Squalene ameliorates DSS-induced acute colitis. Preventive dietary treatment with squalene improved the DAI score (A) although no changes were observed in body weight loss (B) compared with mice from the DSS group. Histological analysis (C), the colonic sections of each animal were scored in a blinded fashion by a pathologist who was unaware of the experimental protocol using a colitis score as previously described by Dieleman et al [26]. The resulting scores showed a significant reduction in histological changes in animals fed with squalene in proximal and rectum sections. Data are reported as means±S.E.M. *P< 0.05 and **P<0.01 vs. squalene 0.02% group; +P< 0.05 and ++ P<0.01 vs. squalene 0.1% group; & P<0.05 and && P<0.01 vs DSS control group.

Figure 1

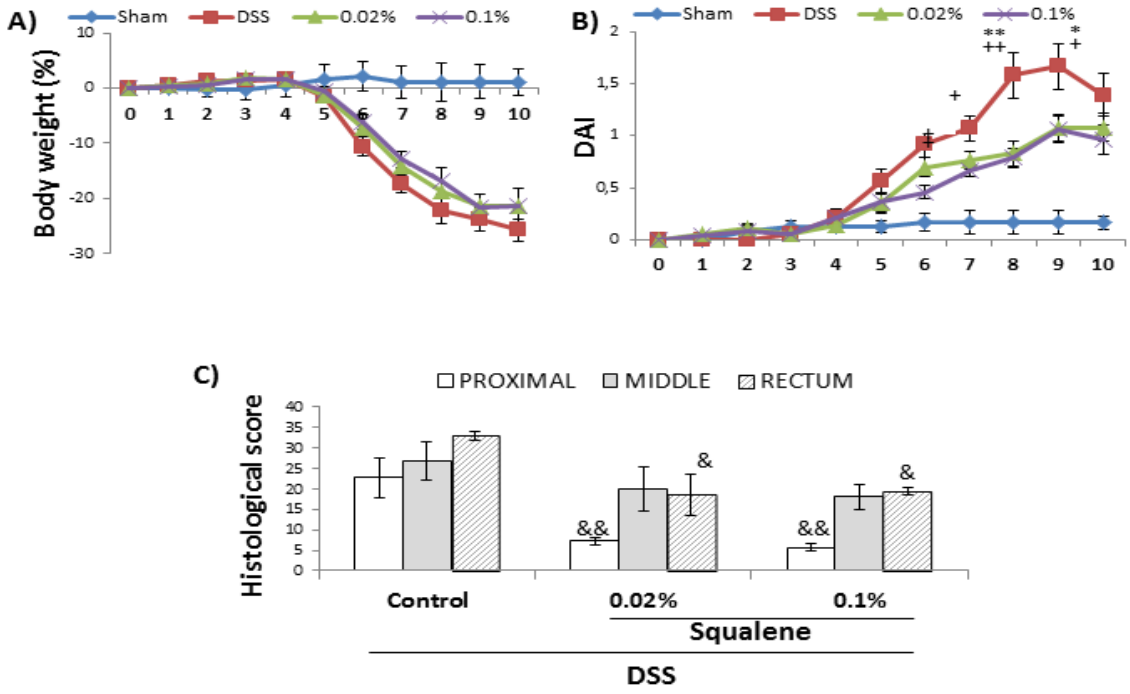

Figure 2. Effect of squalene-supplemented diets on IL-1β and TNF-α mRNA in acute DSS-induced colitis in mice. IL-1β (A) and TNF-α (B) were evaluated by means ELISA kits. Values of IL-1β and TNF-α in colon are shown as means±S.E.M. ***P<0.001 vs. control sham group; +P<0.05 and ++P<0.01 vs. DSS control group.

Figure 2

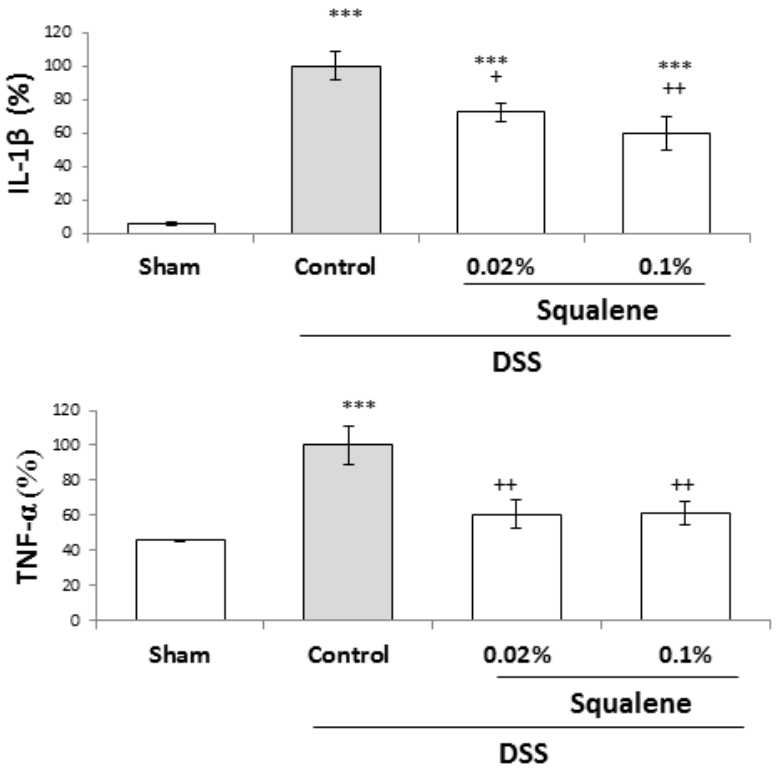

Figure 3. Squalene supplemented diet reduces iNOS and COX-2 in colonic tissue of DSS-induced mice. Western blot gel and quantification of the ratio between cytosolic iNOS and COX-2 protein bands and β-actin protein band in colonic tissue of DSS-induced mice. Each column represents the mean ± SEM. *P< 0.05 and **P<0.01 vs sham group; + P< 0.05 and ++P<0.01 vs DSS control group

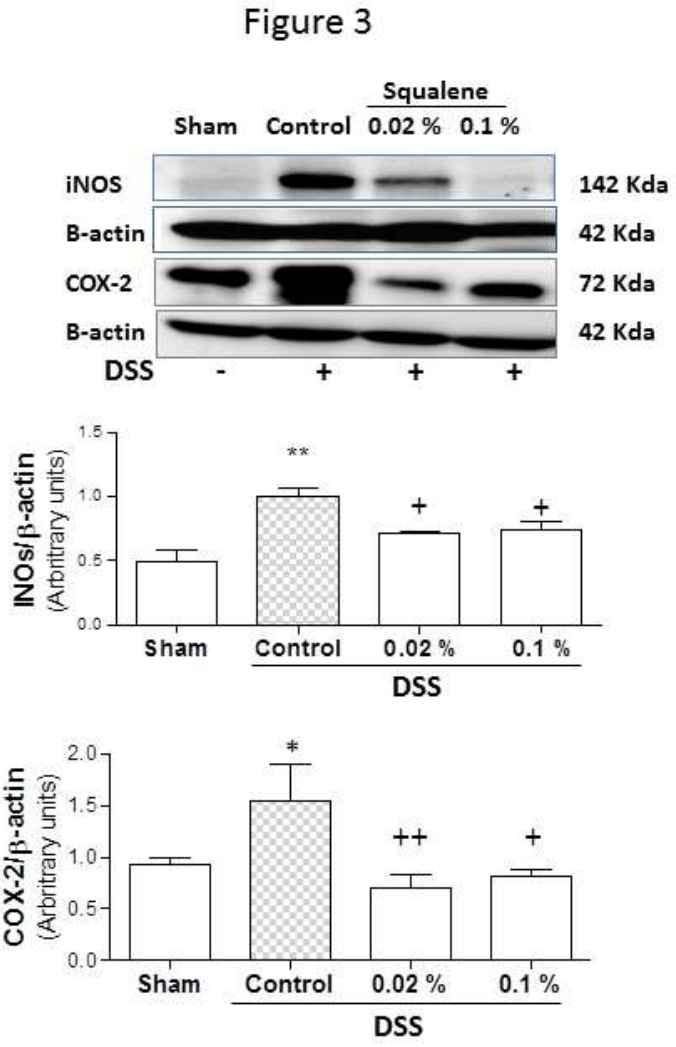

Figure 4. Effect of dietary squalene supplementation on activation of cytosolic p38 and JNK in the colon tissue after 3% of DSS for 5 days followed by 5 days of water. Densitometry was performed following normalization to the control (p38 and JNK housekeeping genes, respectively). Data are expressed as the means ± S.E.M. *P<0.05 and ***P<0.001 vs. control sham group; +++P<0.001 vs. DSS control group.

Figure 4

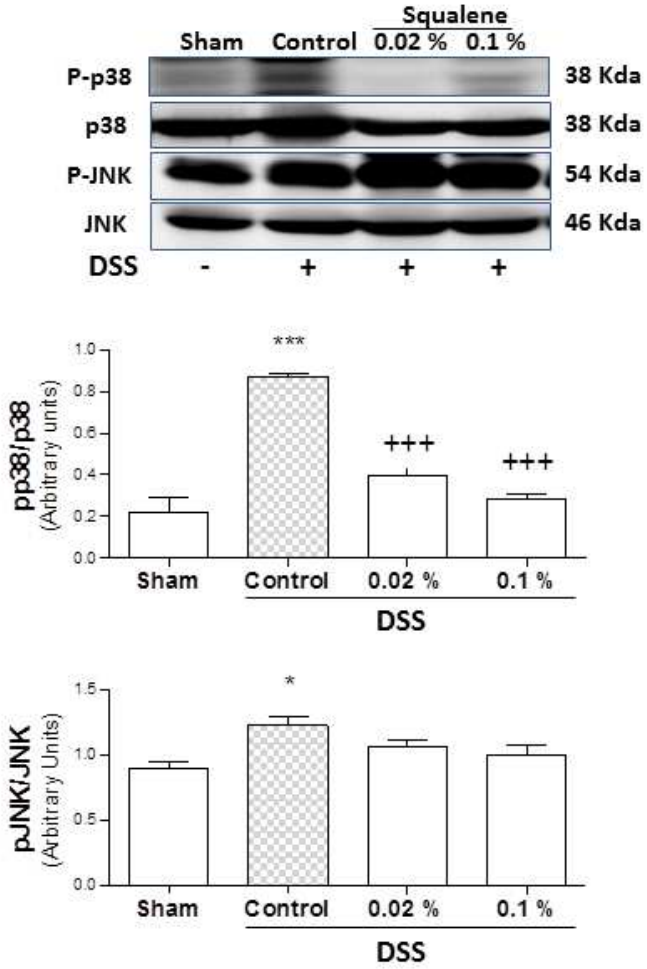

Figure 5. Effect of dietary squalene supplementation on NF-κB signaling pathway in colonic mucosa after 3% of DSS for 5 days followed by 5 days of water. Western blot gel and quantification of the ratio between cytosolic IKBα and nuclear p65 protein bands and β-actin protein band in colonic tissue of DSS-induced mice. Data are expressed as the means ± S.E.M. *P<0.05 vs. control sham group; +P<0.05 vs. DSS control group.