Cellular Physiology and Biochemistry

Cell Physiol Biochem 2022;56(S1):24-35
DOI: 10.33594/000000504
Published online: 8 March 2022
Accepted: 2 February 2022

Original Paper

Sodium Selenite Modulates IDO1/ Kynurenine, TLR4, NF-κB and Bcl2/Bax Pathway and Mitigates Acetic Acid-Induced Colitis in Rat

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Key Words
Colitis • Sodium selenite • IDO1 • Kynurenine • NF-κB • TLR4 • Rat

Abstract
Background/Aims: Colitis is a main presentation of inflammatory bowel disease (IBD) and yet, has no definitive cure. Currently, corticosteroids, anti-tumor necrosis factor (anti-TNF) agents and 5-aminosalicylic acid derivatives are prescribed for management of colitis. Except their failure rate, they are not always tolerated because of their severe adverse effects. Additive formulas with fewer adverse effects may improve the treatment of colitis. ***Methods:*** In this study, colitis was induced with intra-rectal injection of three concentrations of acetic acid (4, 6 and 8 v/v). Each group received sodium selenite (0.5 mg/kg) or saline, gavaged on days 0 and 1 for treatment. Two days after induction of colitis, rats were sacrificed and the end part of their colons were resected for macroscopic and microscopic evaluation and molecular measurement. ***Results:*** Sodium selenite improved macroscopic and microscopic view of the colon, decreased cryptitis, crypt abscess and inflammatory cells infiltration and partly maintained mucosal structure. Sodium selenite markedly reduced tissue levels of malondialdehyde (MDA), TNF-α and interferon γ (INF-γ) and decreased myeloperoxidase (MPO) activity. Treatment with sodium selenite also significantly downregulated IL17, IL22, indoleamine 2,3-dioxygenase (IDO1), and kynurenine levels. Western blotting revealed that sodium selenite prevented apoptosis by increasing bcl2/Bax ratio. Furthermore, our findings showed that sodium selenite significantly downregulated the upstream inflammatory molecules such as nuclear factor kappa B (NF-κB) and toll-like receptor 4 (TLR4) in colitis. ***Conclusion:*** These findings show that sodium selenite alleviates inflammatory response and oxidative stress and protects against colitis.

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Introduction

Colitis is defined as inflammation of the colon and is a major component of IBD. It varies in depth (transmural or non-transmural) and length (pan colitis or segmental), so the treatment will vary, as well. A sick colon imposes many limitations in eating habits, affects bowel movement, produces abdominal discomfort, and disturbs the normal living and function [1, 2]. Contrary to consuming different immunosuppressive drugs such as corticosteroids, anti-TNF agents, 5-aminosalicylic acid derivatives and cytotoxic drugs, a large number of colitis cases particularly patients with IBD remain untreated [3, 4]. Except the failure rate, these drugs bring severe adverse effects, which is not always tolerated and some of them impose high economic burden.

Selenium is a micronutrient with antioxidant function. Selenium modulates inflammation by altering the expression of pro- and anti-inflammatory mediators [5, 6]. It is relatively safe but consuming very high doses of selenium leads to selenium toxicity that presents with diarrhea, nausea, fatigue, hair loss, arthralgia, and nail discoloration [7]. It was reported that serum selenium concentration of 400–30,000 μg/L and 500–1400 μg/L were associated with acute and chronic selenium toxicity, respectively [8]. These concentrations of selenium are much higher than normal serum concentrations of selenium (1.14 μmol/L or 90 μg/L) [9]. Selenium deficiency was associated with decreased selenoproteins expression, increased oxidative stress and inflammatory cytokines production in the animal models and selenium supplementation attenuated these inflammatory changes [10, 11]. Studies revealed that selenium and selenoproteins alter the expression of TLR4, NF-κB, bcl2 and bax to suppress inflammation and enhance cellular longevity [12-14].

Several clinical studies have reported that selenium deficiency is observed in a notable percentage of patients with IBD and may be a contributory factor for colitis [15-17]. Consistently, selenium deficiency expanded the extent of tissue destruction in animal models of colitis [18]. Similarly, it was found that selenium alone or in combination with other substances such as vitamin E mitigates the intensity of damage due to its anti-oxidant properties [19-21]. Furthermore, selenium supports gut commensal microbiota and enhances their protective effects on intestinal lining [21].

The aim of this study was to evaluate the effect of sodium selenite on mild, moderate and severe colitis at the gross and microscopic level. In addition, we investigated the effect of sodium selenite on the expression of inflammatory mediators and apoptosis regulators.

Materials and Methods

Animals

This experiment was performed with seventy-two male Wistar rats, aged 10 to 12 weeks and weighing 200 to 250 g. Animals were obtained from the Animal house of Faculty of medicine, Tehran University of Medical Sciences, Tehran, Iran. All animals were housed in a controlled ambient temperature (22±2 °C) on a 12:12h light/dark cycle. Rats were fed a standard diet and had free access to water. After acclimatization to their environment, the study began. Anesthesia was induced by ketamine (87 mg/kg) and xylazine (13 mg/kg) [22] and rats were followed until 1 hour after awaking. All efforts were spared to reduce animal suffering and the number of animals used. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tehran University of Medical Sciences (IR.TUMS.VCR.REC.1397.833). The study was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication, 8th Ed.).

Acute Colitis Model

Experimental rats fasted for at least 24 h before the induction of colitis with free access to water. Colonic inflammation was induced under light anesthesia by administering 2 ml of acetic acid in three increasing concentrations (4% - 6% - 8% v/v) dissolved in 0.9% physiologic saline. A polypropylene tube with an outer diameter of 3 mm was used for intra-rectal acid instillation. The tube was inserted intrarectally into

the colon to a distance of 8 cm from the anus. Rats were hold vertically (with their head downward) for 2 minutes to prevent rapid outflow of acetic acid.

Treatment Schedule

After induction of acute colitis by acetic acid, treatment was performed with two consecutive gavages of 0.5 mg/kg sodium selenite on days 0 and 1. Control group received an equal amount of normal saline. Two days after the induction of colitis, the animals underwent colectomy surgery, extracting their terminal colon. This part was used for microscopic, macroscopic and molecular assessments (Fig. 1).

Animals Grouping

Experimental rats were randomly divided into 6 groups (n=6) as shown in Table 1.

Because of the high mortality rate, the sample size in groups 5 and 6 did not reach to the aim of this study. Therefore, the investigation was ended in these groups because of the ethics. Specimens from group 3 and 4 were used for molecular assessments.

Macroscopic and Microscopic Measurement

The terminal part of colon was opened longitudinally to expose the internal luminal surface. After washing mucosal surface and removing the feces, the photos were taken from the gross view. Then samples were collected for molecular and microscopic measurements.

For histopathological examination, specimens were fixed in 4% buffered paraformaldehyde solution (PFA 4%). Thereafter, sections with 5-µm thickness were cut and stained with hematoxylin and eosin (H&E). An experienced pathologist blinded to the identity of specimens, performed all histopathological processing and interpreted the results.

Measurement of Pro-Inflammatory Cytokines by ELISA

For molecular assessments specimens were snap frozen in -80 °C and kept in -20 °C before processing. The colon tissue samples were sonicated (10 w, 2×5s) in a mixture containing protease inhibitors and then centrifuged at 50,000×g for 20 min at 4 °C. TNF-α (DuoSet®, DY510-05), INF-γ (DuoSet®, Rat IFN-γ DY585) and Myeloperoxidase (MPO Colorimetric Activity Assay Kit MAK068), MDA (Teb Pazhouhan Razi (TRP), Tehran, Iran), IL22 (ERA27RB), IL17 (DY4437) and kynurenine (Rat Kynurenine (KYNU) Elisa kit MBS745507) were measured in the supernatant by ELISA as explained by the manufacturer.

Western Blot

Colon tissue was sonicated and homogenized in lysis buffer (included: Tris HCl, SDS, DTT, Glycerol, NP40). The homogenate was centrifuged at 10000 g for 10 min in 4 °C. The supernatants were extracted for immunoblotting protein expression. After the samples were boiled at 100 °C for 5 minutes, loading samples on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and using running buffer, transferred to Polyvinylidene difluoride (PVDF) membranes. The nonspecific binding sites on membranes were blocked 90 min with 5% non-fat skim milk at room temperature and incubated with the following primary antibodies overnight: IDO1 (Santa Cruz Biotechnology, sc-137012), p-NF-κB p65 (Santa Cruz Biotechnology,

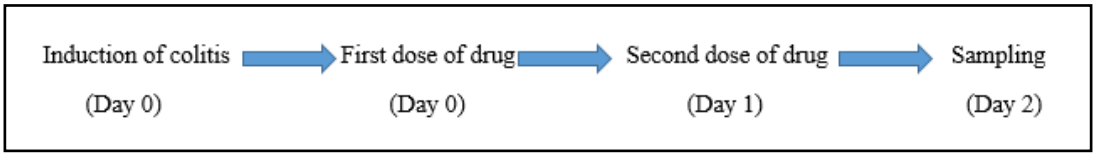

Fig. 1. Study timeline.

Table 1. Experimental groups

Treatment	Acetic acid 4%	Acetic acid 6%	Acetic acid 8%
Normal saline (control group)	Group 1	Group 3	Group 5
Sodium selenite 0.5 mg/kg	Group 2	Group 4	Group 6

sc-136548), Anti-NF-κB p65 antibody (ab16502, abcam), Bax (Santa Cruz Biotechnology, sc-7480), TLR4 (Santa Cruz Biotechnology, sc-293072), Bcl2 (Santa Cruz Biotechnology, sc-492), and β-actin (Santa Cruz Biotechnology, sc-47778). Membranes were then washed with TBST (TBS+ tween 80) and incubated with secondary antibodies for 60 min at room temperature. BM chemiluminescence western blot kit used for detection immunostained protein bands by gel doc. An open source image processing program, Image J, was applied to quantify the optical density of each band. β-actin was used as an internal control. The relative activity of all proteins was calculated by Prism 7 (GraphPad Inc.) and compared with the control group [23].

Statistical Analysis

Data are presented as Mean ± SEM and analyzed using GraphPad Prism7 software (version 6.07). Tests of homogeneity of variance were used to ensure normal distribution of data. One-way analysis of variance (ANOVA) and T test were used to analyze the data. Probability (p) value less than 0.05 was considered significant.

Results

Macroscopic Finding

Acetic acid enema led to colitis in all rats. Gross colonic injuries characterized by diffuse mucosal edema and granularity (blue arrow), erythema (yellow arrow), ulceration and even necrosis (red arrow) were observed in saline-treated group. The intensity and extent of destruction increased proportional to the increase of acid concentration. Oral administration of sodium selenite after induction of colitis alleviated the macroscopic signs of destruction. Treatment with sodium selenite reduced the extent of the colonic injury and mitigated the degree of edema, granulation and erythema. In addition, differences were more perceptible in lower concentrations of acetic acid (Fig. 2).

Histopathological Findings

In saline-treated groups, a significant infiltration of inflammatory cells was observed. Inflammation of crypts (cryptitis), collection of neutrophils within crypts lumen (crypt abscess) and mucosal ulcers were prominent in the saline-treated groups compared to sodium selenite-treated groups. Histopathological analysis of the colon revealed that acetic acid-induced changes of colon tissue such as loss of tissue architecture, cellular infiltration into the mucosa and submucosa, abscess formation, hemorrhage in the crypts were alleviated in animals treated with sodium selenite (Fig. 3 & Fig. 4).

Sodium Selenite Exhibits Anti-Oxidant Effects in the Treatment of Acetic Acid-Induced Colitis

MDA assay kit (Teb Pazhouhan Razi, Iran, Code No. TPR-MDA-96T) was used for measurement of MDA as a marker of oxidative stress. As shown in Fig. 4, MDA levels were markedly decreased in acetic acid 4% (**p<0.01) and acetic acid 6% (*p<0.05), compared with saline-treated groups. MPO activity was significantly (*p<0.05) lower in sodium selenite-treated group (Fig. 5).

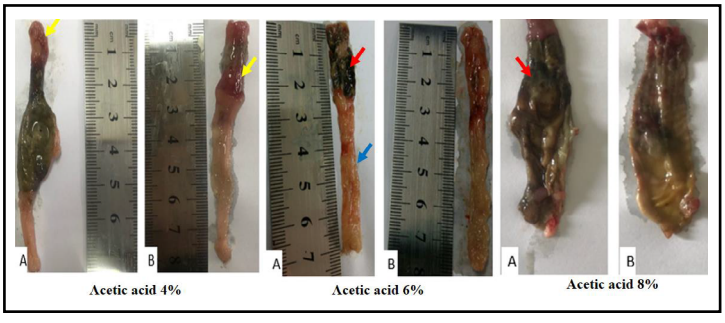

Fig. 2. Gross examination of end part of colon showed that sodium selenite (B) could partly confine the necrosis (red arrow), edema and granularity (blue arrow), compared to saline-treated group (A).

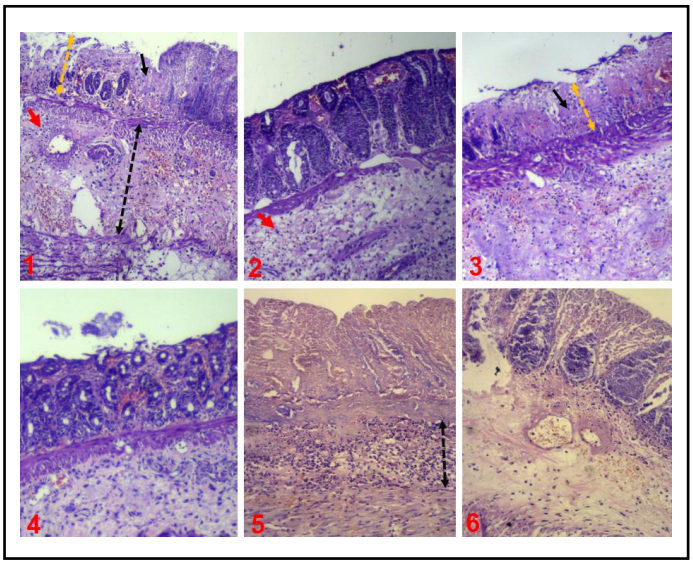

Fig. 3. Microscopic comparison of saline-treated and sodium selenite-treated groups showed that sodium selenite-treated rats have lower abundance of inflammatory cells (red arrows) in their colon. In addition, mucosal (yellow dashed lines) and submucosal (black dashed lines) derangement, cryptitis and crypt abscess (black arrows) were more prominent in control groups, compared to sodium selenite-treated group. Photos were magnified for 100 times. (1) acetic acid 4% control group, (2) sodium selenite-treated (0.5 mg/kg) + acetic acid 4%, (3) acetic acid 6% control group, (4) selenium-treated (0.5 mg/kg) + acetic acid 6%, (5) acetic acid 8% control group, (6) selenium-treated (0.5 mg/kg) + acetic acid 8%.

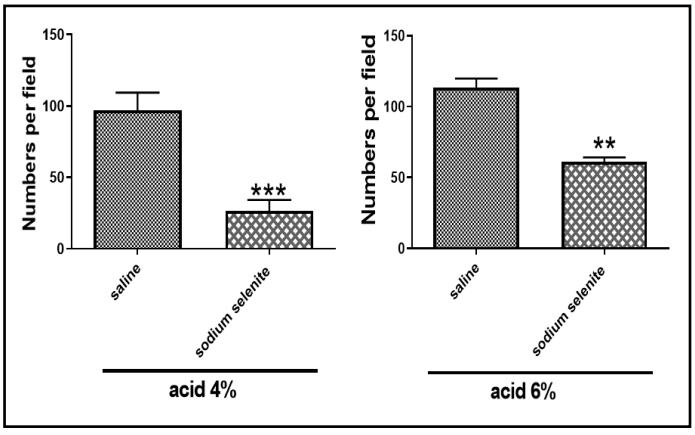

Fig. 4. Treatment with sodium selenite significantly (***P<0.001) decreased the number of inflammatory cells in the submucosal layer in colitis induced with acetic acid 4%. Similarly, treatment with sodium selenite significantly (**P<0.01) decreased the abundance of inflammatory cells in the submucosal layer in colitis induced with acetic acid 6%.

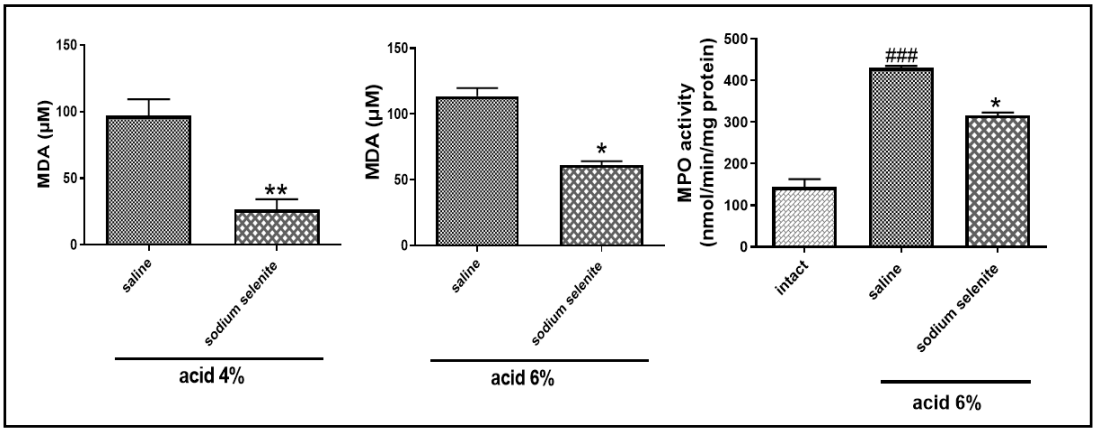

Fig. 5. Treatment with sodium selenite significantly decreased MDA levels in both concentrations of acetic acid (**p<0.01 for acid 4%, p<0.05 for acid 6%). MPO activity was significantly (*p<0.05) lower in sodium selenite-treated group.

Sodium Selenite Exhibits Anti-inflammatory Effects in the Treatment of Acetic Acid-Induced Colitis

As shown in Fig. 5, treatment with sodium selenite significantly decreased TNF-α (***p<0.001) and INF-γ (**p<0.01) levels. In addition, sodium selenite could significantly decrease the increased levels of IL22 (***p<0.001), IL17 (*p<0.05) and kynurenine (***p<0.001) (Fig. 6).

Sodium Selenite Modulate TLR4, NF-κB, IDO1 and Apoptosis Signaling Pathways to Protect against Acetic Acid-Induced Colitis in Rats

Western blotting was used to measure major inflammatory signaling pathways and apoptosis markers after induction and treatment of colitis. Acid enema caused a significant increase in TLR4/β-actin (###p<.0.001), p-NF-κB/NF-κB (###p<0.001) and indoleamine 2,3-dioxygenase IDO1/β-actin (####p<0.0001). In addition, acid enema significantly decreased bcl2/Bax (####p<0.0001). Treatment with sodium selenite 0.5 mg/kg meaningfully decreased TLR4 (***p<0.001), p-NF-κB/NF-κB (*p<0.05) and IDO1/β-actin (**p<0.01) and increased bcl2//Bax ratio (****p<0.0001) (Fig. 7).

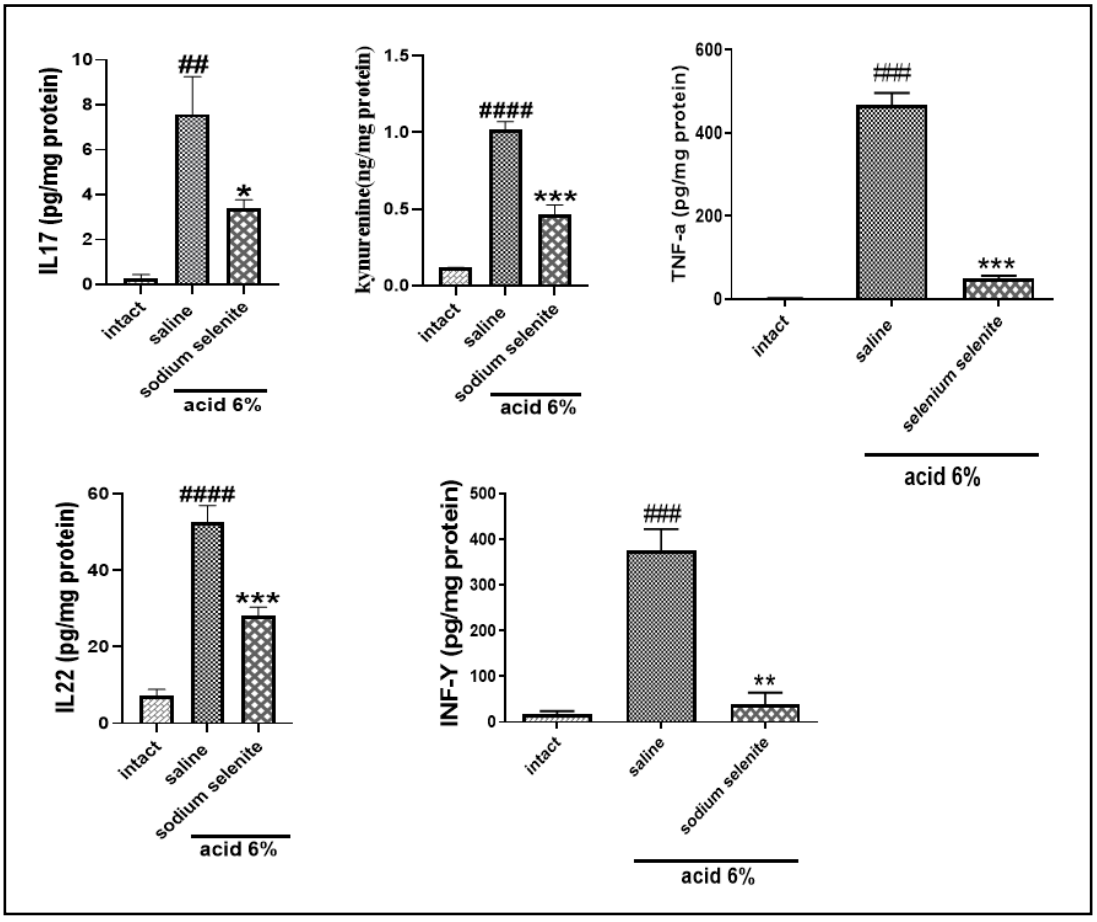

Fig. 6. Acetic acid instillation significantly increased TNF-α (###p<0.001) and INF-γ (###p<0.001). After treating rats with sodium selenite TNF-α (***p<0.001) and INF-γ (**p<0.01) markedly decreased. Enema of acetic acid significantly increased tissue levels of IL22 (####p<0.0001), IL17 (##p<0.01) and kynurenine (####p<0.0001). Treatment with sodium selenite caused a significant decrease in IL22 (***p<0.001), IL17 (*p<0.05) and kynurenine (***p<0.001) levels.

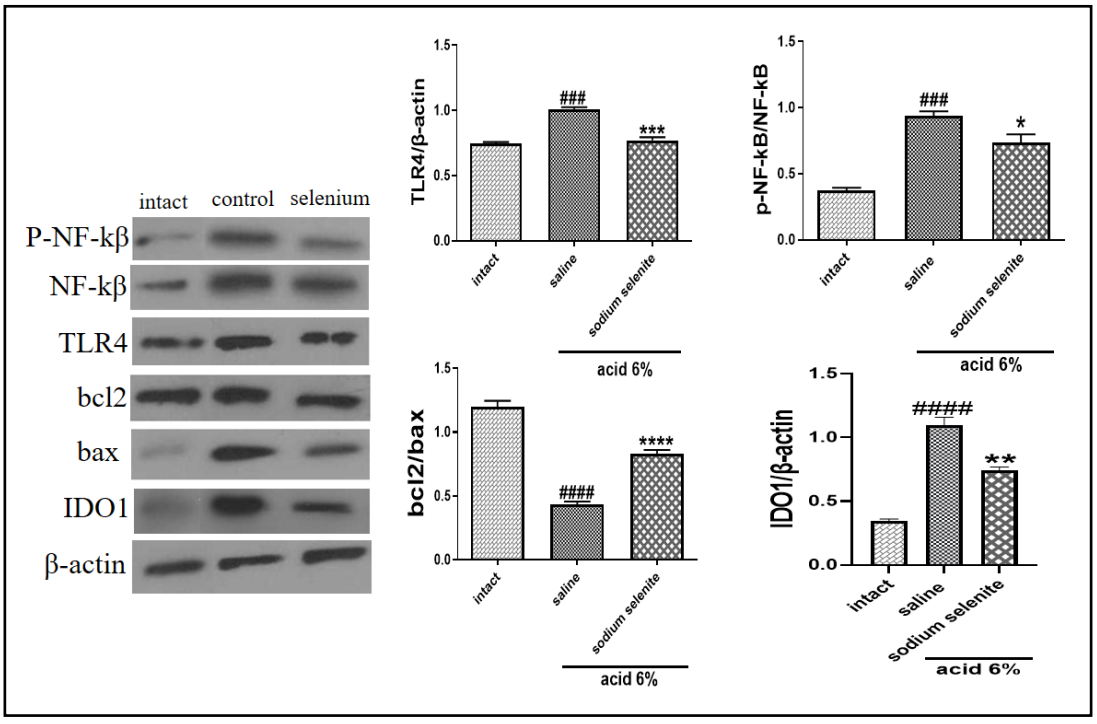

Fig. 7. Acid instillation significantly increased TLR4/β-actin (###p<0.001), p-NF-κB/NF-κB (###p<0.001) and IDO1/β-actin (####p<0.0001), whereas decreased bcl2//Bax (####p<0.0001). Treatment with sodium selenite decreased TLR4 (***p<0.001), p-NF-κB/NF-κB (*p<0.05) and IDO1/β-actin (**p<0.01) and increased bcl2/Bax (****p<0.0001).

Discussion

The current study demonstrated that, sodium selenite significantly improved the macroscopic and microscopic structure of the colon, confined inflammatory cells infiltration, partly attenuated oxidative stress, and decreased inflammatory cytokines release. In addition, sodium selenite downregulated thelevels of IDO1, TLR4, and NF-κB in the colon and protected against apoptosis and suppressed oxidative stress related to colon injury.

Selenium increases the expression of selenoproteins, thereby attenuating NF-κB signaling pathway [24, 25]. NF-κB activates innate immunity and T cells, stimulates their migration to inflammatory foci, and augments inflammatory cytokines production [26]. Overactivation of mutant NF-κB has been implicated in the pathophysiology of IBD [27, 28]. Furthermore, particular polymorphisms and mutations of NF-κB are associated with increased risk of ulcerative colitis [29, 30]. Selenium deficient animals had a higher level of inflammatory cytokines in their gastrointestinal tract [31]. This study showed that sodium selenite downregulates NF-κB and decreases inflammatory cytokines release.

Previously, it was found that activation of oxidative stress increases the severity of ulcerative colitis and attenuation of oxidative stress can vigorously alleviate colitis [32, 33]. Modulation of NF-κB could suppress oxidative stress in experimental model of colitis [34]. MPO is a cytotoxic enzyme which is abundantly expressed in neutrophils, produces hypochlorous acid from hydrogen peroxide and chloride and mediates the destructive effect of neutrophils in colitis [32, 35]. MPO activity during acute colitis is positively associated with disease severity [36]. Sodium selenite downregulated NF-κB and decreased MDA and MPO levels in this study.

INF-γ undermines epithelial integrity and increases mucosal permeability in the intestinal lining. Higher levels of INF-γ and increased mucosal permeability helps bacterial penetration through the epithelial layer [37, 38]. Similarly, it was shown that TNF-α has the same effect and anti-TNF agents preserve mucosal structure [39, 40]. Anti-TNF agents play an important role in the induction phase of ulcerative colitis treatment [41]. Sodium selenite decreased TNF-α and INF-γ to mitigate colonic inflammation (Fig. 8).

IDO1 is an enzyme involved in degradation of tryptophan into its metabolites such as kynurenine. Inflammatory cytokines such as INF-γ instigate the expression of IDO1 [42, 43]. IDO1 is overexpressed in IBD and is associated with disease activity [44]. Mucosal IDO1 expression is also associated with endoscopic inflammation degree in patients with IBD [45, 46]. IDO1 promotes the production of kynurenine, thereby stimulating aryl hydrocarbon receptor (AHR). Activation of AHR leads to production of IL22 or differentiation of T regulatory cells [47, 48]. As a compensatory mechanism, IL22 accelerates tissue healing and shows the intensity of pre-existing damage [49]. It was shown that patients with ulcerative colitis who were in remission had higher IL22 levels, compared to healthy individuals. In addition, IL22 was significantly higher in patients with active ulcerative colitis, compared to IL22 in those with quiescent ulcerative colitis [50]. IL22 levels were also positively associated with disease severity in patients with Crohn's disease and in animal model of IBD [51, 52]. Kynurenine pathway appears to be an endogenous anti-inflammatory mechanism that simultaneously indicates the presence and severity of gut inflammation. The expression of IDO1 and kynurenine levels are positively associated with the severity of inflammation and possess prognostic value in IBD [44, 46, 53]. In this study, sodium selenite markedly decreased IDO expression and tissue levels of kynurenine and IL22, consistent with its protective effect on histopathology (Fig. 8).

IL17 is produced by Th17 and is responsible for induction of severe colitis [54]. Th17 induces Th1 response through IL17 [54]. Th17 profoundly relies on IL17 to induce severe colitis and inhibition of IL17 with anti-IL17 monoclonal antibody markedly attenuated Th17-mediated colitis [54]. Sodium selenite decreased tissue level of IL17 in this study that was associated with alleviation of colitis and decreased infiltration of inflammatory cells (Fig. 8).

TLR4 is a member of PRRs (pathogen recognition receptors) and acts on the first line of recognition of external stimulus [55]. Previous studies illuminated that stimulation of TLR4 activates NF-κB in experimental models of colitis [56, 57]. Likewise, attenuation of TLR4 signaling alleviates colitis [58-60]. However, TLR4 is considered as a member of innate immunity, it also activates T cells and interacts with adaptive immunity [61]. Consistently,

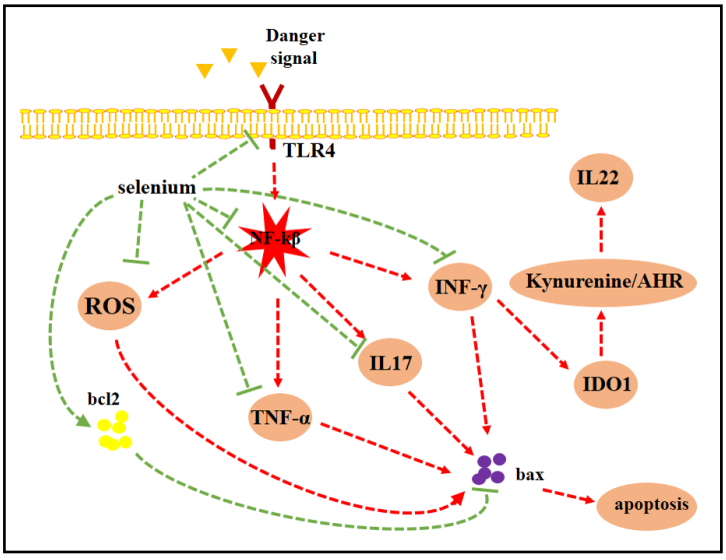

Fig. 8. This figure depicts the pathological pathway discussed in this article. TLR4 stimulates NF-κB to provoke inflammation. Subsequently, inflammatory cells infiltration, inflammatory cytokines release and apoptosis distort the microarchitecture of the colon. Meanwhile, inflammatory mediators, particularly INF-γ activate IDO1/kynurenine pathway to counteract the inflammatory response and alleviate the immune response. Sodium selenite breaks this chain from various points and preserves the normal structure of the colon.

attenuation of TLR4 signaling in sodium selenite-treated rats was associated with alleviation of colitis and downregulation of downstream inflammatory molecules in this study (Fig. 8).

Effective treatment of colitis increases bcl2/bax ratio [62]. Enhancing bcl2/bax ratio prevents cellular death and ameliorates colitis [63]. Selenium can change the expression of apoptosis-related proteins such as bcl2 and bax, in favor of anti-apoptotic proteins [64, 65]. In this study, administration of sodium selenite augmented the bcl2/bax ratio to protect against apoptosis in colitis (Fig. 8).

Conclusion

All in all, the present study revealed that sodium selenite can ameliorate acetic acid-induced colitis in rats and its effect was not limited to its anti-oxidant effects. Sodium selenite downregulated TLR4 and NF-κB, decreased MPO activity and MDA levels and reduced the release of inflammatory cytokines such as TNF-α, INF-γ and IL17. Eventually, sodium selenite improved cell survival, protected against apoptosis, preserved colonic microstructure and decreased colitis severity markers such as IDO1, kynurenine and IL22. Based on these findings, supplementation of selenium is warranted in patients with colitis, particularly in those with lower serum levels of selenium.

Acknowledgements

Author Contributions

In this study every member respectively performed these responsibilities:

Moein Ala (surgery and following animals, writing the article), Razieh Mohammad Jafari (Western blot performance and editing assay, co-corresponding), Hossein Nematian (surgery), Amir Shadboorestan (Western blot performance), Ahmad Reza Dehpour (conceived and designed research, principal investigator).

Funding

This work was supported by a grant from Experimental Medicine Research Center, Tehran University of Medical Sciences, Tehran, Iran (Grant No. 97-03-158-40561) and Iran National Science Foundation (INSF) (Grant No. 96002757).

Disclosure Statement

The authors declare that no conflicts of interest exist.

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