

Protective effect of naringenin on acetic acid-induced ulcerative colitis in rats

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Abstract

AIM: To evaluate the ameliorative effect of naringenin (NG) during ulcerative colitis (UC) in rats.

METHODS: Rats were treated with three different doses (25, 50 and 100 mg/kg per day) of NG and a single dose of mesalazine (MES, 300 mg/kg per day) for seven days prior to ulcerative colitis induction by 4% acetic acid (AA). Twenty four hours after AA rectal administration, animals were scarified and the colonic tissues were dissected. Colonic mucus content was estimated using Alcian blue dye binding technique. In colon tissues, levels of total glutathione sulphadryls (T-

GSH), non-protein sulphadryls (NP-SH) and thiobarbituric acid reactive substances (TBARS) were evaluated. The activities of the antioxidant enzymes, catalase (CAT) and superoxide dismutase (SOD) were measured. Concentrations of nucleic acids (DNA and RNA) and total protein were also estimated in colon tissues. Colonic levels of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), prostaglandin E₂ (PGE₂) and nitric oxide (NO) were estimated. In cross section of colitis tissue the histopathological changes were observed.

RESULTS: Colonic mucus content was decreased in AA compared to controls (587.09 ± 65.59 mg/kg vs 941.78 ± 68.41 mg/kg, $P < 0.001$). AA administration markedly reduced T-GSH (5.25 ± 0.37 nmol/L vs 3.04 ± 0.24 nmol/L, $P < 0.01$), NP-SH (3.16 ± 0.04 nmol/L vs 2.16 ± 0.30 nmol/L, $P < 0.01$), CAT (6.77 ± 0.40 U/mg vs 3.04 ± 0.2 U/mg, $P < 0.01$) and SOD (3.10 ± 0.11 U/mg vs 1.77 ± 0.18 U/mg, $P < 0.01$) while TBARS, TNF- α , IL-1 β , IL-6, PGE₂ and NO levels (15.09 ± 3.84 nmol/L vs 59.90 ± 16.34 nmol/L, $P < 0.01$; 113.56 ± 1.91 pg/mg vs 134.24 ± 4.77 pg/mg, $P < 0.01$; 209.20 ± 36.38 pg/mg vs 422.19 ± 31.47 pg/mg, $P < 0.01$; 250.83 ± 25.09 pg/mg vs 638.58 ± 115.9 pg/mg, $P < 0.01$; 248.19 ± 36.98 pg/mg vs 541.74 ± 58.34 pg/mg, $P < 0.01$ and 81.26 ± 2.98 mmol/g vs 101.90 ± 10.73 mmol/g, $P < 0.001$) were increased in colon of rats with UC compared controls respectively. Naringenin supplementation, significantly and dose dependently increased the colonic mucus content. The elevated TBARS levels were significantly decreased (39.35 ± 5.86 nmol/L, $P < 0.05$; 26.74 ± 3.17 nmol/L, $P < 0.01$ nmol/L and 17.74 ± 2.69 nmol/L, $P < 0.01$) compared to AA (59.90 ± 16.34 nmol/L) group while the decreased levels of T-GSH and NP-SH and activities of CAT and SOD found increased by NG treatments in dose dependent manner. The decreased values of nucleic acids and total protein in AA group were also significantly ($P < 0.01$) increased in all three NG supplemented groups

respectively. NG pretreatment inhibited the TNF- α levels (123.76 ± 3.76 pg/mg, 122.62 ± 3.41 pg/mg and 121.51 ± 2.61 pg/mg *vs* 134.24 ± 4.78 pg/mg, $P < 0.05$) compared to AA group, respectively. Interleukins, IL-1 β and IL-6 levels were also decreased in NG50 + AA (314.37 ± 16.31 pg/mg and 292.58 ± 23.68 pg/mg, $P < 0.05$) and NG100 + AA (416.72 ± 49.62 pg/mg and 407.96 ± 43.87 pg/mg, $P < 0.05$) when compared to AA (352.46 ± 8.58 pg/mg and 638.58 ± 115.98 pg/mg) group. Similar decrease ($P < 0.05$) was seen in PGE₂ and NO values when compared to AA group. The group pretreated with MES, as a reference drug, showed significant ($P < 0.01$) protection against the changes induced in colon tissue by AA administration respectively.

CONCLUSION: In present study, NG produced antioxidant and anti-inflammatory effects demonstrating protective effect in inflammatory bowel disease.

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Key words: Naringenin; Ulcerative colitis; Inflammatory bowel disease; Oxidative stress

Core tip: Inflammatory bowel disease (IBD), consisting of Crohn's disease (CD) and ulcerative colitis (UC), results in substantial morbidity and is difficult to treat. New strategies for adjunct therapies are needed. Systemic corticosteroids are highly effective at inducing clinical remission in cases of acute exacerbation of CD and UC; however, their use is limited by their frequent and sometimes severe side effects. Results of present study revealed that, naringenin has protective effects against acetic acid-induced UC by inhibiting inflammatory and oxidative bio-markers. Thus, it may pose promising outcomes for future clinical usage as a natural non-toxic effective supplement in IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a common chronic inflammatory disease of the gastrointestinal tract. Several etiological factors, such as genetic, immunological, and environmental have been linked with the pathophysiology of the disease^[1]. There are two main subtypes of IBD; Crohn's disease (CD) and ulcerative colitis (UC) having a combined prevalence of 150-250/100000 population^[2,3]. Moreover, the prevalence of hospitalization due to CD and UC is estimated to be 50.1 and 50.6 per 100000 population, respectively^[4]. UC involves only the colon and rectum. Al-

though the etiology of UC is not completely understood, it has been commonly associated with reduced antioxidant capacity as well as increased free radical production such as reactive oxygen species (ROS)^[5]. Over production of ROS leads to lipid peroxidation (LPO), which can inhibit cellular antioxidant capability finally resulting in prominent colonic inflammation^[6]. Clinically, colitis patients were found to overproduce ROS and nitrogen species leading to LPO of membranes and attack on tissue proteins and DNA^[7,8]. Endogenous antioxidant defenses against ROS production even in low concentrations influence on two main types: (1) enzymatic such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT); and (2) non-enzymatic such as glutathione (GSH) and ascorbic acid (vitamin C). It is suggested that inflammatory response amplification can induce inflammatory cells chemotaxis resulting in release of ROS and inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-6 and IL-1 β , which triggers the pathological responses and symptoms during IBD^[9]. Elevated levels of pro-inflammatory cytokines in both the IBD forms reported to have a vital role of such mediators, which also play in determining the severity of the disease^[10]. Medications that have ability to inhibit the production of these inflammatory mediators are shown to be clinically effective, which indicate their contribution to IBD and other chronic inflammatory conditions aggravation^[11].

Some natural products, such as flavonoids, are getting more attention as novel agents for therapeutically usage. Flavonoids are one of the most abundant natural antioxidants present in plants and the human diets. Naringenin (4,5,7-trihydroxy flavanone) a flavonoid, widely distributed in citrus fruits, tomatoes, cherries, and cocoa^[12]. Several pharmacological studies revealed its effects including antidiabetic^[13], antiatherogenic^[14], antidepressant^[15], immunomodulatory^[14], antitumor^[16], DNA protective^[17], hypolipidemic^[18] and peroxisome proliferator-activated receptors activator^[19]. It has also been shown to have prominent antioxidant^[20] and anti-inflammatory^[21] potentials. InêsAmaro *et al.*^[22] reported that, naringenin (NG) has reducing effect on intestinal edema-induced by dextran sodium sulphate in mice.

In several studies, pathogenesis of UC disease has demonstrated that excessive inflammation and oxidative stress play a significant role^[23,24]. Amelioration of LPO as well as free radicals scavenging would provide a useful, protective and therapeutic treatment for UC. With respect to the high antioxidant capacity and anti-inflammatory activity, NG would be expected to reduce injury and/or improve tissue healing following injury from ulcerative colitis. In the present study we had evaluated the protective effect of NG during experimental ulcerative colitis and the possible mechanism of action.

MATERIALS AND METHODS

Animals and ethical approval

Eight weeks old male Wister albino rats weighting 250-280 g were received from Experimental Animal

Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. Animals were housed under controlled environmental conditions (25 °C and a 12 h light/dark cycle). Animals had free access to Purina rat chow (Manufactured by Grain Silos and Flour Mills Organization, Riyadh, Saudi Arabia) and tap water. All experimental procedures and protocols in this study including euthanasia were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (NIH Publications No. 80-23; 1996) as well as the Ethical Guidelines of the Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

Induction of ulcerative colitis

Experimental ulceration in colon tissue was done according to the method described by Mousavizadeh *et al.*^[25] with slight modification. In brief, under light ether anesthesia rats were administered 2 mL of 4% acetic acid solution (v/v; Merck, Darmstadt, Germany) by transrectally using a (2.7 mm) soft pediatric catheter. After AA administration, rats were holed horizontally for 2 min to prevent AA leakage. Control animals underwent the same procedure using equal volume of normal saline instead of AA solution.

Experimental design

Forty two rats were divided into seven groups (six animals in each) as follows: (1) Control (Cont); (2) NG 100 mg/kg per day (NG100); (3) AA treated rats (AA); (4) NG 25 mg/kg per day + acetic acid (NG25 + AA); (5) NG 50 mg/kg per day + AA (NG50 + AA); (6) NG 100 mg/kg per day + AA (NG100 + AA); and (7) MES 300 mg/kg per day + AA (MES + AA). Naringenin (Sigma Aldrich, United States) and MES treatments were continued for 7 consecutive days by gavage^[26]. At end of the treatment, ulcerative colitis was induced in all AA groups. Twenty four hours after the colitis induction, animals were sacrificed under deep anesthesia^[27]. The colon (5-6 cm) specimens were dissected, washed with saline solution, imaged, weighted and small cross section was fixed in 10% formaldehyde solution for histopathological evaluation. The remaining tissues were stored at -75 °C (Ultra-low freezer, Environmental Equipment, Cincinnati, Ohio, United States) till analysis.

Evaluation of the adherent colonic mucus

The modified procedure of Popov *et al.*^[28] was used to determine adherent colonic mucus concentration. Briefly, a small portion of colon tissue was excised, weighted then transferred immediately to 1% Alcian blue solution (in 0.16 mol/L sucrose solution buffered with sodium acetate, pH 5) for 24 h. The excess dye was removed by rinsing with sucrose solution. The dye complexed with the gastric wall mucus was extracted using 10 mL of 0.5 mol/L MgCl₂ solution. A 4 mL aliquot of blue extract was then shaken with an equal volume of diethyl ether. The resulting emulsion was centrifuged at 4000 rpm and the absorbance of the aqueous layer was recorded at 580

nm by using spectrophotometer (LKB-Pharmacia, Mark II, Ireland). The quantity of Alcian blue extracted (µg) per grams of wet colonic tissue was then calculated.

Histopathological investigations

Colon sections were fixed 10% neutral buffered formalin then put for 24 h in decal. Samples were then cut into several sections and embedded into paraffin wax blocks. Tissues were stained with haematoxylin and eosin and were mounted and observed microscopically for histopathological changes by a pathologist in blinded fashion.

Estimations of total glutathione sulphadryls and non-protein sulphadryls concentrations in colon

In colon tissues, total glutathione sulphadryls (T-GSH) and non-protein sulphadryls (NP-SH) levels were estimated according to the method described by Sedlak *et al.*^[29]. Tissues were homogenated in ice-cold 0.02 mol/L ethylenediamine tetra-acetic acid. An aliquots of 0.5 mL of tissue homogenate was mixed with 0.2 mol/L Tris buffer, pH 8.2 and 0.1 mL of 0.01 mol/L Ellman's reagent, [5,5'-dithiobis-(2-nitr-benzoic acid)] (DTNB). Each sample tube was centrifuged at 3000 rpm at room temperature for 15 min the absorbance of the clear supernatant was measured using spectrophotometer (LKB-Pharmacia, Mark II, Ireland) at 412 nm. For NP-SH estimation, homogenate was diluted with distilled H₂O and mixed with 1 mL of 50% trichloroacetic acid (TCA). The tubes were shaken intermittently for 10-15 min and centrifuged for 15 min at approximately 3000 g. Two milliliter of supernatant was then added to 4 mL of 0.4 mol/L Tris buffer (pH 8.9) then 0.1 mL DTNB added. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank.

Estimation of thiobarbituric acid reactive substances levels in colon

A thiobarbituric acid reactive substances (TBARS) assay kit (ZeptoMetrix, United States) was used to measure the LPO products, malondialdehyde (MDA) equivalents. Briefly, one hundred microliters of colon homogenate was added to 2.5 mL reaction buffer (provided by the kit) and heated at 95 °C for 60 min. After the mixture cooling, supernatant absorbance was measured at 532 nm using a spectrophotometer (LKB-Pharmacia, Mark II, Ireland). The LPO products are expressed in terms of nmoles MDA/mg protein.

Estimation of CAT and SOD activities in colon

Catalase activity in colon tissues was estimated by the method described by Aebi^[30]. In brief, aliquot of 0.5 mL post-mitochondrial supernatant was mixed with 2.5 mL of 50 mmol/L phosphate buffer (pH 7.0) and 20 mmol/L H₂O₂. CAT activity was estimated spectrophotometrically following the decrease in absorbance at 240 nm and expressed in terms of units/mg protein as compared to a standard curve.

The SOD activity in colon tissue was measured by using the method described by Kono^[31]. The principle of this method was that superoxide anions generated by the

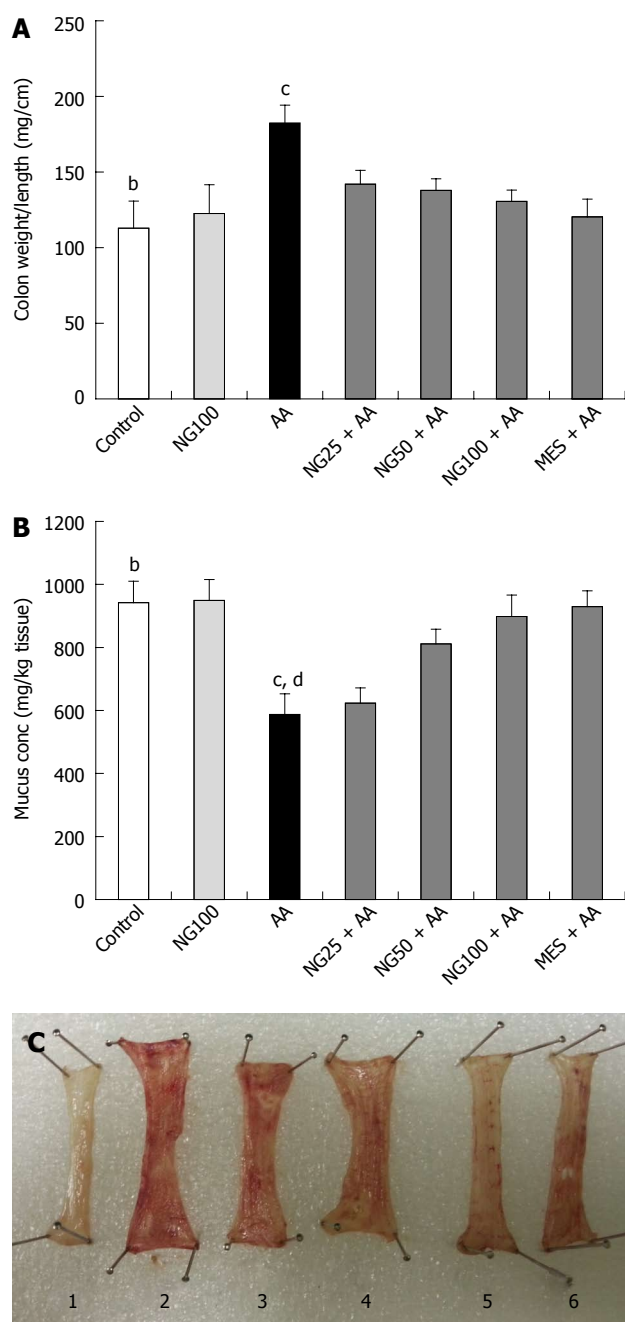


Figure 1 Effect of naringenin on colon weight/length (A), mucus concentration (B) and induction of ulceration and its protection by treatments in colonic tissue of rats with acetic acid-induced ulcerative colitis (C) ($n = 6$). Values in (A) and (B) are expressed as mean \pm SE and analyzed using one way analysis of variance followed by Newman-Keuls *post hoc* test. ^b $P < 0.01$ control vs acetic acid (AA); ^c $P < 0.05$, ^d $P < 0.01$ AA vs naringenin (NG) 25 + AA, NG50 + AA, NG100 + AA or mesalazine (MES) + AA groups. Groups in (C) are arranged as follows: control (1), AA (2), NG25 + AA (3), NG50 + AA (4), NG100 + AA (5) and MES + AA (6).

oxidation of hydroxylamine hydrochloride can mediate the reduction of nitrobluetetrazolium to blue formazon. The color was then measured at 560 nm under aerobic conditions. Addition of superoxide dismutase inhibited nitrobluetetrazolium reduction and the extent of this inhibition was taken as a measure of enzymatic activity. The SOD activity was expressed as units/mg protein.

Determination of nucleic acids and total protein levels in colon

The method described by Bregman^[32] was used to determine the levels of nucleic acids (DNA and RNA) in colon. In brief, colon tissues were homogenized in 4 mL ice-cold distilled water and 2 mL homogenate was suspended in 5 mL of 10% ice-cold trichloroacetic acid (TCA). After centrifugation, the pellet was extracted twice with 95% ethanol. Finally, the nucleic acids were extracted in 5% TCA. DNA was determined by treating the nucleic acid extract with diphenylamine reagent and measuring the intensity of blue color at 600 nm. For quantification of RNA, the nucleic acid extract was treated with orcinol reagent and the green color was read at 660 nm. Standard curves were used to determine the amounts of nucleic acids present. Total protein in colon was estimated by Lowry *et al*^[33] method using Bovine plasma albumin as a standard.

Determination of inflammatory cytokines, PGE₂ and NO levels in colon

In colon, TNF- α , IL-1 β , IL-6 and PGE₂ levels were assessed and quantified according to the method of Mousavizadeh *et al*^[25] using enzyme-linked immunosorbent assay ELISA (R and D systems, United States). The results were expressed as pg/mg tissue. Levels of colonic nitric oxide were assayed by Griess reaction method using commercial kit (R and D systems, United States).

Statistical analysis

Data were expressed as mean \pm SE. Statistical analysis was carried out using one-way ANOVA followed by Newman-Keuls *post hoc* test. P value of ≤ 0.05 was considered statistically significant. All statistics tests were conducted using Graph Pad Prism (version 5) software.

RESULTS

Acetic acid significantly ($P < 0.01$) increased the colonic weight as compared to control group. Pretreatment with NG following three doses and MES for 7 d, showed significant ($P < 0.05$) inhibition in weight increase while compared to AA group (Figure 1A). Mucus concentration was significantly ($P < 0.01$) reduced in AA administered group when compared to control animals. Pretreatment with higher doses (50 and 100 mg/kg) of NG and MES significantly elevated the reduced colonic mucus concentration ($P < 0.05$, $P < 0.01$ and $P < 0.05$, respectively) as compared to AA group (Figure 1B). The colon images were clearly showed the induction of ulceration and its protection by the treatments (Figure 1C).

Histopathological changes with their intensity are presented in Figure 2. Slide from control group, showing benign mucosal epithelium of tall columnar epithelial cells with goblet cells (Table 1 and Figure 2A). In the AA group, the slide revealed diffused active colitis with widely eroded mucosa with ulcerations and necrosis associated with edema, goblet cell hyperplasia, lymphoid follicular

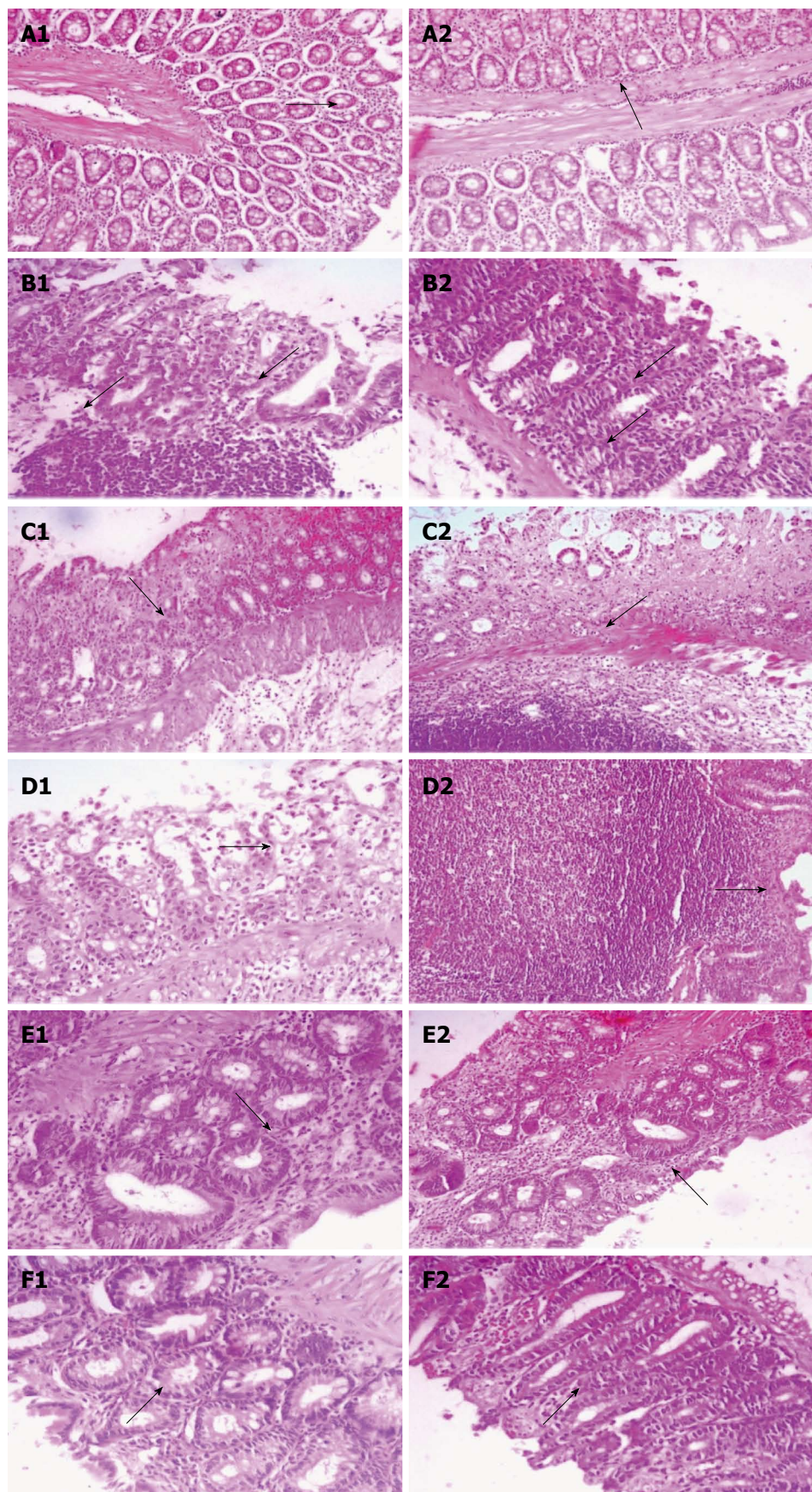


Figure 2 Histopathological changes with their intensity are presented. A-1 and 2: Histopathological colonic sections showing normal benign looking mucosa; B-1 and 2: Diffused active colitis with superficial erosions, stromal edema, dense acute and chronic inflammatory cells infiltrate with widely ulcerating mucosa; C-1 and 2: Reparative epithelial changes with little ulcer healing and inflammatory cells infiltrate; D-1 and 2: Reparative epithelial changes and healing ulcer with lymphoid follicle form; E-1 and 2: Healing ulcer and reparative epithelial changes; F-1 and 2: Attenuated cell damage with complete ulcer healing. A1-F1 ($\times 400$), A2-F2 ($\times 200$).

Table 1 Effect of naringenin on microscopic scoring of histopathological sections of colonic tissue of rats with acetic acid-induced ulcerative colitis

Groups	Ulceration	Hyperemia	Necrosis	Edema	Cellular infiltrate	Goblet cell hyperplasia
Control	0	0	0	0	0	0
AA	+++	+++	++++	+++	++++	++
NG25 + AA	++	++	+++	++	+++	++
NG50 + AA	++	++	++	++	+++	+
NG100 + AA	+	+	+	+	++	+
MES + AA	+	+	+	+	+	+

MES: Mesalazine; AA: Acetic acid; NG: Naringenin; NG25: NG 25 mg/kg per day; NG50: 50 mg/kg per day; NG100: 100 mg/kg per day; 0: Normal; +: Mild; ++: Moderate; +++: Severe; ++++: Very severe.

hyperplasia and transmural lymphoplasmacytic infiltrate with few intraepithelial neutrophilic cells within stromal (Table 1 and Figure 2B). In NG25 + AA group, slight healing epithelial cells with scattered superficial ulcers lined by colonic glands with reparative epithelial changes with hyperchromatic nuclei and infrequent mitosis and less goblet cells surrounded by transmucosal fewer lymphoplasmacytic infiltrate within stromal edemas was seen (Table 1 and Figure 2C). Slide from NG50 + AA group showed intestinal rat lined by healing epithelial cells with tall columnar epithelium, with superficial shredded epithelial cells, less eroded surface surrounded by few inflammatory edema and less necrosis with colonic gland showed reparative epithelial changes (Table 1 and Figure 2D). In higher dose of NG treatment (NG100 + AA) group, superficial tiny eroded mucosa with mucosal, hemorrhage, edema and scattered acute and chronic inflammatory cells infiltrate surrounding colonic glands with reparative epithelial changes and few goblet cells were seen (Table 1 and Figure 2E). Slide from MES + AA group revealed intestinal section with more better healed and improvement of intestinal mucosa compared to positive controlled sections with few mucosal lymphoplasmacytic infiltrate within stromal edema (Table 1 and Figure 2F).

Acetic acid administration resulted in a significant ($P < 0.01$) decrease in colon levels of both T-GSH and NP-SH when compared to control animals. Pretreatment with NG with higher doses (50 mg/kg and 100 mg/kg) significantly ($P < 0.01$) attenuated T-GSH and NP-SH ($P < 0.05$) the reduced levels as compared to AA group. Pretreatment with MES significantly inhibited the decreased levels of T-GSH and NP-SH ($P < 0.001$ and $P < 0.05$, respectively) (Figure 3A and B). Concentration of TBARS in the colons of AA treated rats were significantly ($P < 0.01$) increased compared to control animals. A significantly lower concentrations of TBARS values were found in NG25 + AA ($P < 0.05$), NG50 + AA ($P < 0.01$), NG100 + AA ($P < 0.01$) and MES + AA ($P < 0.01$) groups as compared to AA group (Figure 3C). CAT activity was significantly ($P < 0.01$) decreased in colon tissues of AA administered rats compared to control animals. Pretreatment with 50 mg/kg per day and 100 mg/kg per day of NG, significantly ($P < 0.05$) inhibited the decrease CAT activity in colon as compared to AA group (Figure 3D). SOD activity was significantly ($P < 0.01$) reduced in the colons of AA treated animals as compared to control rats. Group

of rats pretreated with 100 mg/kg per day of NG for 7 d showed a significant ($P < 0.05$) increase in colon SOD activity while compared to AA treated animals (Figure 3E). Pretreatment with MES also markedly ($P < 0.05$ and $P < 0.01$, respectively) enhanced the CAT and SOD activities as compared to AA group (Figure 3D and E).

There was a significant ($P < 0.001$) decrease in colon levels of DNA, RNA and total protein in AA administered group as compared to control animals. Pretreatment with NG (100 mg/kg per day) or MES (300 mg/kg per day) significantly ($P < 0.01$) increased the DNA content in colon tissue compared to AA group. The RNA levels in NG higher doses and MES groups found significant ($P < 0.01$) elevation compared to AA group. Total protein levels were also significantly ($P < 0.05$) increased in NG50 + AA, NG100 + AA and MES + AA groups compared to AA group (Table 2).

Pro-inflammatory cytokines including TNF- α , IL-1 β and IL-6 levels produced significant ($P < 0.01$) increase in AA-induced ulcerative colitis and levels found significantly ($P < 0.05$ and $P < 0.01$) diminished in NG higher doses and MES pretreated groups as compared to AA group, respectively (Figure 4A-C). Similar changes in PGE₂ levels were seen in colon tissue of rats (Figure 4D). In colon tissue, NO levels were significantly ($P < 0.01$) increased AA group compared to controls. The elevated NO levels were markedly ($P < 0.05$) reduced in NG and MES treated group compared AA group respectively (Figure 4E).

DISCUSSION

Present investigation outlines the anti-ulcerogenic effect of NG against experimentally induced UC in rats as a model for IBD. The preventative effect of NG was confirmed by histological evaluation and also using MES as a standard drug. Seven days pretreatment with NG significantly reduced the AA-induced colonic mucus content and prevented oxidative and inflammatory response in dose dependent manner.

Ulcerative colitis is characterized by mucosal inflammation and ulcerations with a variable extent and severity^[34]. Rectal administration of 4% AA to experimental rodents to induce UC is a well-established animal model, which phenotypically resembles human colon inflammation^[35]. It also causes colonic epithelial lesions and

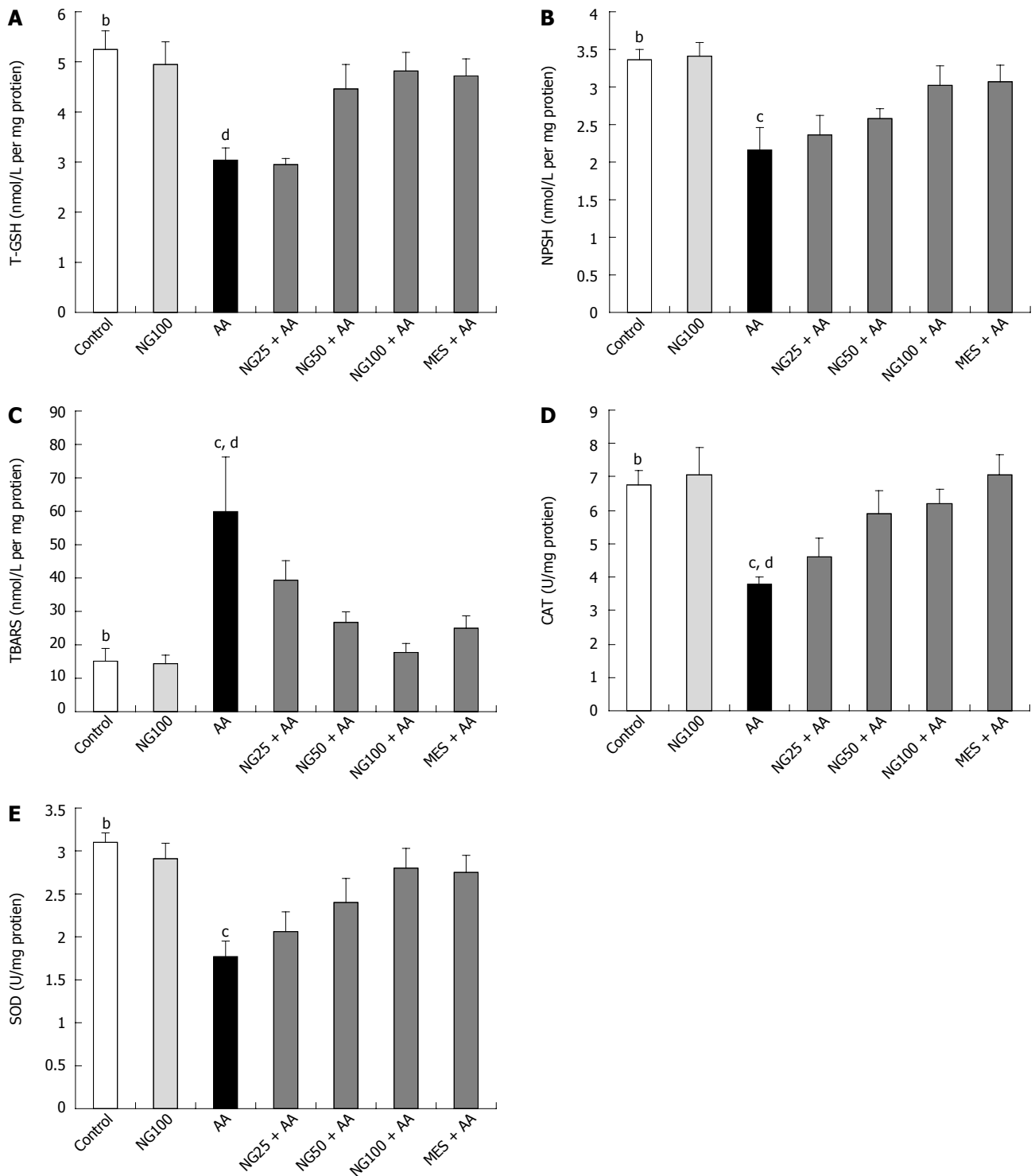


Figure 3 Effect of naringenin on total glutathione sulphadryls (A), non-protein sulphadryls (B) and thiobarbituric acid reactive substances levels (C) as well as catalase (D) and superoxide dismutase activities (E) in colonic tissue of rats with acetic acid-induced ulcerative colitis ($n = 6$). Values are expressed as mean \pm SE and analyzed using one way analysis of variance followed by Newman-Keuls *post hoc* test. ^b $P < 0.01$ control vs AA; ^c $P < 0.05$, ^d $P < 0.01$ AA vs NG25 + AA, NG50 + AA, NG100 + AA or MES + AA groups. T-GSH: Total glutathione sulphadryls; NP-SH: Non-protein sulphadryls; TBARS: Thiobarbituric acid reactive substances; CAT: Catalase; SOD: Superoxide dismutase; AA: Acetic acid; UC: Ulcerative colitis; MES: Mesalazine; NG: Naringenin.

necrosis associated with neutrophils and macrophages infiltration to the damaged colon indicating inflammatory conditions^[28,35]. In present study, the 4% AA administration resulted a significant increase in colonic weight and induced sever ulceration and tissue necrosis associated with inflammatory infiltrate and goblet cell hyperplasia

as indicated in the results of the histopathological estimations. Similar pathological impairments were reported in earlier studies using the same animal model^[26,36]. Application of AA in the present study disturbed the colonic mucus, which is in agreement with Popov *et al*^[28]. Colonic mucus plays an important protective role against

Table 2 Effect of naringenin on DNA, RNA and total protein levels in colonic tissue of rats with acetic acid-induced ulcerative colitis

Groups	DNA ($\mu\text{g}/100$ mg wet tissue)	RNA ($\mu\text{g}/100$ mg wet tissue)	Total protein (mg/100 mg wet tissue)
Control	652.05 \pm 17.12 ^b	378.51 \pm 38.44 ^b	3.0 \pm 0.19 ^b
NG100	688.84 \pm 47.69	380.24 \pm 48.96	2.43 \pm 0.31
AA	222.69 \pm 18.78 ^d	167.35 \pm 15.16 ^d	0.95 \pm 0.08 ^e
NG25 + AA	293.93 \pm 33.49	208.68 \pm 25.05	1.32 \pm 0.13
NG50 + AA	338.54 \pm 21.44	250.06 \pm 10.11	2.02 \pm 0.46
NG100 + AA	415.50 \pm 41.51	298.34 \pm 12.92	2.15 \pm 0.27
MES + AA	425.04 \pm 38.23	307.44 \pm 18.31	2.14 \pm 0.26

Values are expressed as mean \pm SE ($n = 6$) and analyzed using one way ANOVA followed by Newman-Keuls *post hoc* test. ^b $P < 0.01$ control *vs* acetic acid (AA); ^c $P < 0.05$, ^d $P < 0.01$ AA *vs* Naringenin (NG) 25 + AA, NG50 + AA, NG100 + AA or mesalazine (MES) + AA groups.

chemically induced ulceration which may also facilitate the repair of the damaged epithelium^[37]. Although, numerous pharmacotherapies have been suggested for UC treatment, the side effects or toxicity of these medications are a major clinical problem^[38]. That is why naturally occurring products such as flavonoids are now suggested as an alternative option beside the conventional therapies^[39]. Indeed, earlier experimental studies demonstrated flavonoids such as quercitrin, kushenin, kaempferol and baicalin to promote UC healing^[40-43].

Previous studies demonstrated that NG administration effectively protected the experimentally induced gastric lesions and ulcers^[44,45]. Protection against experimental UC induced by NG was accompanied by restoration of the increased colon thickening in AA group, which is an indirect assessment of colon inflammation. Microscopic scoring of the histopathological sections confirmed the protective action of NG as it decreased colonic tissue ulceration, necrosis and inflammation in dose dependent manner. Motilva *et al.*^[45] reported that NG treatment increased the gastric mucus levels in rats induced gastric lesions by absolute ethanol. In the present study, NG was also found to inhibit the depletion of colonic mucus caused by AA treatment. This protective activity could be attributed to its antioxidant and anti-inflammatory properties.

Oxidative stress is known to play an important role in IBD initiation and progression^[46]. Experimentally induced colitis in animals is characterized by oxidative damage and an imbalance between oxidant and antioxidant substances^[47]. The AA-induced colitis model is known to cause vascular dilatation and white blood cells accumulation, as well as an increase in blood flow, leading to increased production of oxygen and hence the excessive generation of free radical and ROS^[35,48]. Several studies have indicated the vital role that free radicals play in the pathogenesis of mucosal injuries^[49,50]. Moreover, free radicals and ROS were reported in colorectal specimens of ulcerative colitis^[51,52]. The first line of oxidative defense system against free radicals is the sulphadryls groups in peptide namely GSH or NP-SH. It is widely distributed in all biological

tissues and work as a non-enzymatic antioxidant. GSH inhibits ROS oxidative injuries directly *via* its sulfhydryl group and indirectly as a cofactor or a coenzyme in ROS enzymatic detoxification process^[53,54]. Another line in oxidative defense system is the enzymatic antioxidants. Examples for important antioxidant enzymes are SOD, CAT, and GPx^[55]. In present study, levels and activities of non-enzymatic and enzymatic defense systems were severely decreased in the colon of AA administered animals indicating oxidative cellular injury. Furthermore, free radicals are known to attack lipid contents of cellular membranes leading to activation of LPO process and cellular damage. Therefore, the concentrations of LPO specific products such as TBARS were elevated, while the critical cellular macro- and micro-molecules such as nucleic acids and total proteins levels were decreased in the present work indicating cellular oxidative injury and cytotoxicity. These results, which are in agreement with previous findings, suggest the harmful effects of AA on cellular macromolecules and its ability to impair the epithelial cell integrity and hinder mucosal recovery^[23,36].

In present study, NG was able to attenuate AA induced oxidative damage and injury of colon tissues confirming its strong antioxidant and anti-inflammatory properties. It has seen in earlier studies that NG markedly increased the antioxidant markers such as GSH, NP-SH levels and SOD, and CAT activities^[12,56,57]. Han *et al.*^[56] found that NG pretreatment can increase the activity of antioxidant enzyme GPx, which suggest the ability of NG to attenuate oxidative stress by decreasing the lipid peroxide level and to inhibit accumulation of free radicals generation during LPO process^[57]. In the current study, NG treatment significantly corrected the impaired levels of nucleic acids and total protein in colon tissue suggesting the cytoprotective properties of the naturally occurring flavonoids. The antioxidant activity of NG depends mainly on the presence of B-ring catechol group, which can stabilize a radical species by donating hydrogen (H^+)^[12].

Inflammatory cytokines are known to play a crucial role in modulating mucosal immune system where the neutrophils and macrophages are responsible for disrupting epithelial integrity and causing colon injury^[58]. The pathogenesis of UC is characterized by migration of granulocytes and other leukocytes to the inflamed mucosa and superficial ulcers leading to increased levels of pro-inflammatory cytokines such as TNF- α , IL-6 and IL-1 β ^[59,60]. In present study, the elevated colon level of TNF- α , IL-6 and IL-1 β in AA administered group is an evidence for epithelial cell necrosis, edema, and neutrophil infiltration, which is also supported by the histopathological results. These results are in accordance with earlier experimental and clinical studies^[6,28,36,61]. The reported increased levels of colonic PGE₂ in AA group of animals is in agreement with Otani *et al.*^[62], where the enhanced level of PGE₂ was attributed to its overproduction rather than decreased metabolism, both of which are mediated by pro-inflammatory cytokines. Naringenin was found in the current

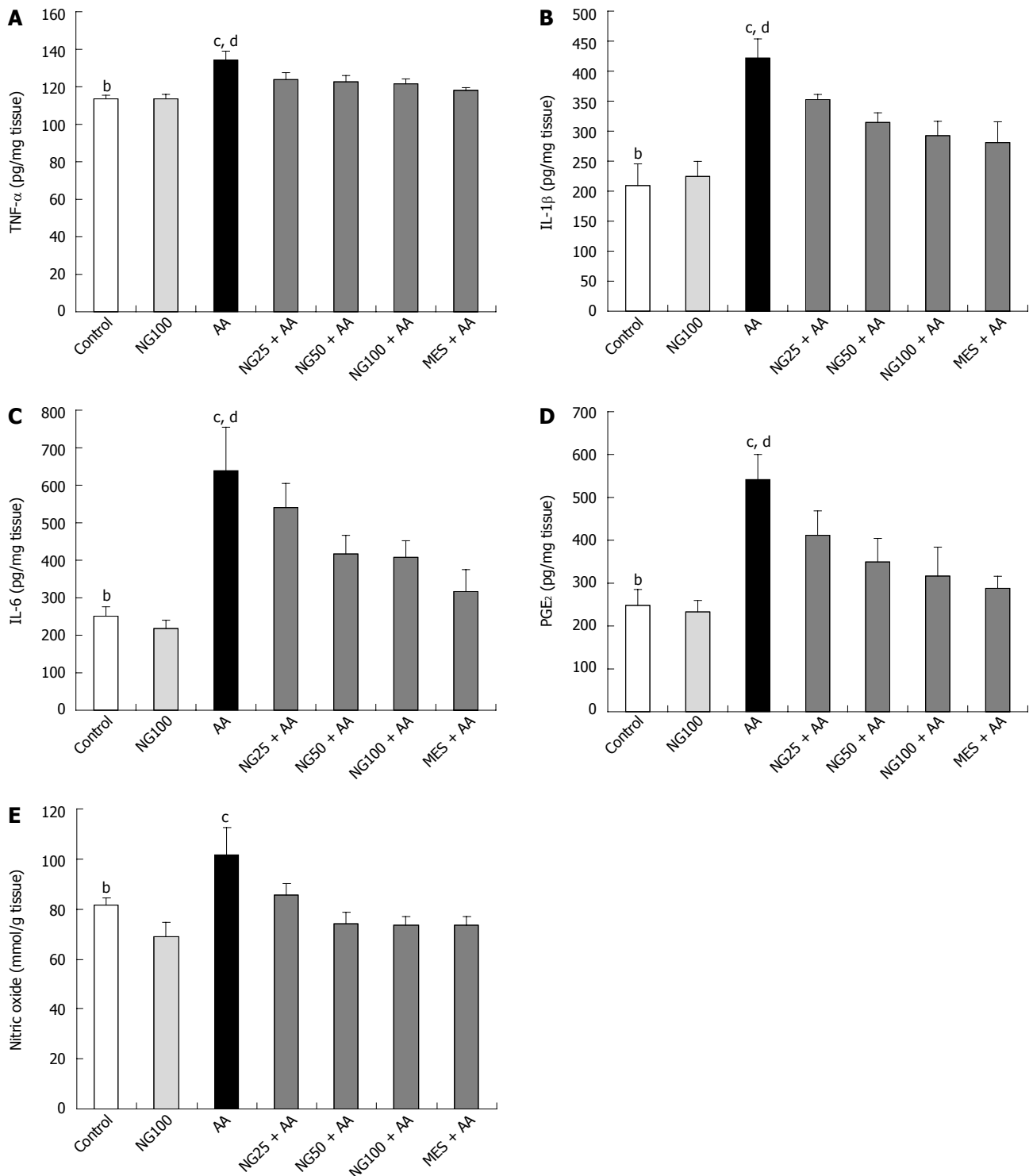


Figure 4 Effect of naringenin on tumor necrosis factor- α (A), interleukin-1 β (B), interleukin-6 (C), prostaglandin E $_2$ (D) and nitric oxide (E) levels in colonic tissue of rats with acetic acid-induced ulcerative colitis ($n = 6$). Values are expressed as mean \pm SE and analyzed using one way analysis of variance followed by Newman-Keuls *post hoc* test. ^b $P < 0.01$ control vs AA; ^c $P < 0.05$, ^d $P < 0.01$ AA vs NG25 + AA, NG50 + AA, NG100 + AA or MES + AA groups. TNF- α : Tumor necrosis factor- α ; IL-1 β : Interleukin-1 β ; IL-6: Interleukin-6; PGE $_2$: Prostaglandin E $_2$; AA: Acetic acid; NG: Naringenin; MES: Mesalazine.

and earlier studies to inhibit the level of inflammatory cytokines including TNF- α , IL-6 and IL-1 β ^[63]. The anti-inflammatory properties of naringenin were suggested to be through several mechanisms including increased phosphorylation of ERK 5 and P38 MAPK and inhibition of NF- κ B and activator protein-1 signaling^[64,65]. Additionally, naringenin, which present in high concentrations in cit-

rus fruits, was found to block NF- κ B activation resulting in down regulation of the downstream target genes of NF- κ B such as iNOS and COX-2 expression^[66]. These enzymes catalyze oxidative stress-induced production of NO and prostaglandins respectively, which are known as an important inflammatory mediators in the pathogenesis of colitis^[63,67]. These findings are in agreement with our

results where pretreatment with naringenin significantly ameliorated AA induced elevation of the level of PGE₂ and NO in rats' colon.

In conclusion, the present study revealed that NG-protects the AA-induced ulcerative colitis by inhibiting inflammatory and oxidative bio-markers. Finally, our results may pose promising outcomes for future clinical usage of NG as a natural non-toxic effective supplement in IBD.

COMMENTS

Background

The pathogenesis of inflammatory bowel disease (IBD) such as ulcerative colitis (UC) is usually associated with reduced antioxidant capacity. Generation of free radicals like reactive oxygen species (ROS) leads to lipid peroxidation, which inhibits cellular antioxidant capability, resulting in prominent colonic inflammation. There is a great need to search for safe and tolerable compounds for the management of IBD to reduce patient compliance as well as the adverse effects of conventional treatments. Naringenin (NG) is a naturally occurring flavonoid that can be extracted from citrus fruits, tomatoes, cherries, grapefruit, and cocoa. Like most of the flavonoids, NG was experimentally found to have several pharmacological potentials, including antioxidant, antitumor and anti-inflammatory because of NG has properties to produce sufficient hydroxyl (-OH) substitutions, which give it the capability to scavenge ROS. Thus, it has considered that NG may diminish and/or improve pathological conditions where oxidation or inflammation is deemed to play a vital role, like in case of IBD.

Research frontiers

In the present study, NG was orally (gavage) treated with three doses (25, 50 and 100 mg/kg per day body weight) for 7 consecutive days, 24 h later UC was induced by 4% acetic acid. In colitis tissue, Alcian blue, pro-oxidative and inflammatory biomarkers were estimated. The biochemical alterations were further justified with histopathological changes.

Innovations and breakthroughs

NG pretreatment clearly revealed the protection against acetic acid-induced UC in animal model. Antioxidant and anti-inflammatory properties of NG are suggested to be the key for these effects as NG significantly reduced oxidative stress and inflammatory biomarkers in a dose dependent manner.

Applications

The present data shows that NG has a promising protective effect against experimentally-induced UC in animal model. Thus, NG could be recommended for its use as potential alternative and complementary therapy for IBD after confirmation of the obtained findings by clinical trials.

Peer review

The preclinical preventative properties of NG against UC are outlined in present study. Also the possible pharmacological mechanisms of action responsible for these effects are evaluated. Overall, this study proofed that NG is an effective and safe compound that worth to be investigated in future clinical trials for its colonic anti-ulcerogenic properties.

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