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Genistein and/or sulfasalazine ameliorate acetic acid-induced ulcerative colitis in rats via modulating INF-γ/JAK1/STAT1/IRF-1, TLR-4/NF-κB/IL-6, and JAK2/STAT3/COX-2 crosstalk

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ABSTRACT

Ulcerative Colitis (UC) is a chronic idiopathic inflammatory bowel disease in which the colon's lining becomes inflamed. Exploring herbal remedies that can recover mucosal damage is becoming popular in UC. The study aims to investigate the probable colo-protective effect of a natural isoflavone, genistein (GEN), and/or a drug, sulfasalazine (SZ), against acetic acid (AA)-induced UC in rats, in addition to exploring the possible underlying mechanisms. UC was induced by the intrarectal installation of 1–2 ml of 5% diluted AA for 24 h. Ulcerated rats were allocated into the disease group and three treated groups, with SZ (100 mg/kg), GEN (100 mg/kg), and their combination for 14 days, besides the control groups. The anti-colitic efficacy of GEN and/or SZ was evidenced by hindering the AA-induced weight loss, colon edema, and macroscopic scores, besides reduced disease activity index and colon weight/length ratio. Furthermore, treatments attenuated the colon histopathological injury scores, increased the number of goblet cells, and lessened fibrosis. Both treatments reduced the up-regulation of INF-γ/JAK1/STAT1 and INF-γ /TLR-4/ NF-κB signaling pathways and modulated the IRF-1/iNOS/NO and IL-6/JAK2/STAT3/COX-2 pathways and consequently, reduced the levels of TNF-α and IL-1β. Moreover, both treatments diminished oxidative stress, which appeared by reducing the MPO level and elevating the SOD activity, and hindered apoptosis; proved by the decreased immunohistochemical expression of caspase-3. The current findings offer novel insights into the protective effects of GEN and suggest a superior benefit of combining GEN with SZ, over either drug alone, in the UC management.

1. Introduction

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC), are chronic inflammatory idiopathic intestinal diseases [1]. UC particularly affects the colon and is accompanied by bleeding, diffuse friability, and superficial erosions in the colonic wall. It is the most common form of IBD worldwide with accelerated global incidence [2]. Inflammation often begins in the rectum and spreads proximally to all or part of the colon in a continuous manner [3]. Colonic discomfort, bloody diarrhea, fluid and electrolyte loss, abdominal pain, cramping, and weight loss are all symptoms of UC impairing the quality of life [4]. Moreover, unwell control and treatment of UC result in colectomy and colorectal cancer [5]. The etiology of UC development is complex and multifactorial and involves genetic, microbial, environmental, and other

Abbreviations: AA, acetic acid; COX-2, cyclo-oxygenase 2; CMC, carboxymethyl cellulose; DAB, diaminobenzidine; DAI, disease activity index; GEN, genistein; GCs, goblet cells; H & E, Hematoxylin and Eosin; HRP, horseradish peroxidase; IBD, inflammatory bowel diseases; IFN-γ, interferon-gamma; IL, interleukin; iNOS, calcium-independent nitric oxide synthase; IRF-1, interferon regulatory factor 1; JAK/STAT, Janus kinase/signal transducer and activator of transcription; LTR, long terminal repeat; MPO, myeloperoxidase; MTC, Masson's Trichrome for collagen fibers; MyD88, myeloid differentiation primary response protein 88; NF-κB, nuclear factor kappa B; NO, nitric oxide; PRRs, pattern recognition receptors; RIPA, radioimmunoprecipitation assay; ROS, reactive oxygen species; SOD, superoxide dismutase; SZ, sulfasalazine; TLRs, toll-like receptors; TNF-α, tumor necrosis factor-alpha; UC, ulcerative colitis.

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factors which influence both; the disease onset and progression [6].

Acetic acid (AA)-induced UC is a widely used model that closely mimics human UC [7]. This model is considered one of the most commonly used experimental models for evaluating medicines used in inflammatory bowel illness due to its inexpensive cost and convenience of delivery [6].

The exact etiopathogenesis of UC remains not completely clear. Multiple processes are known to be implicated, including uncontrolled innate immune system activation and sustained overproduction of inflammatory cytokines like interferon-gamma (IFN-γ), activation of toll-like receptors (TLRs), nuclear factor kappa B (NFκB), which in turn, induce the production of tumor necrosis factor-alpha (TNF-α), interleukin (IL) -1β and IL-6, inflammatory cells invasion, massive release of reactive oxygen species (ROS), reduction in colon antioxidant capability along with depletion of the intestine mucosal integrity [4,8]. By inhibiting the antioxidative system and upregulating free radicals, calcium-independent nitric oxide synthase (iNOS) and cyclo-oxygenase 2 (COX-2) further damage the large intestine mucosa [9].

A properly regulated equilibrium between cell proliferation and cell death (apoptosis) maintains the homeostasis of colonic epithelial cells. The excessively high rate of intestinal epithelial cell death in UC causes the epithelial barrier to be destroyed, activates the inflammatory response triggered by the microbiota, and further accelerates tissue damage [10].

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway have been linked to the development of a wide range of human illnesses. It plays a critical role in the IBD pathophysiology, including UC [11]. The cytokines binding to its specific cell surface receptors results in receptors dimerization causing conformational changes of JAKs located in the cytoplasm, and the intracellular part of JAKs becomes activated. Activated JAKs bind with latent cytoplasmic factors STATs resulting in their activation. Subsequently, activated STATs dimerize and then translocate to regulate cytokines gene (target gene) expression in the nucleus [12].

Sulfasalazine (SZ) has been the most widely prescribed agent and standard therapy for IBD treatment and is beneficial in maintaining remission in people with UC. Its mechanism of action is to intrude with eicosanoid synthesis and inhibit prostaglandin synthesis by interfering with arachidonic acid metabolism, resulting in local anti-inflammatory effects in the colon [13]. SZ affects JAK/STAT signaling pathway leading to decreased proinflammatory cytokines linked to this pathway [14]. But, the most frequently reported side effects of sulfasalazine are nausea, vomiting, anorexia, headaches, stomach pain, fever, and rashes [15]. Consequently, new therapeutic approaches are required for IBD treatment [16]. Reinforced this, many IBD patients prefer alternative medical therapies, such as traditional herbal medicines [17]. The approach of using herbal remedies includes plant isoflavones compounds, which retain the ability to act as antioxidants and anti-inflammatory agents, and thus significantly contribute to the treatment of numerous inflammatory and auto-immune disorders [18].

Genistein (GEN) (4, 5, 7 trihydroxy isoflavone) is a natural isoflavone compound found in leguminous plants [19]. It is a major isoflavone in soy and soy-based food products and is considered a potent agent; due to its potential therapeutic, anti-oxidative, and anti-inflammatory effects on multiple degenerative diseases and other chronic conditions, including gastrointestinal diseases, such as UC [20]. GEN inhibits the JAK/STAT signaling pathway by reducing the expression of the proinflammatory cytokines underlying this pathway [21].

This study was planned to investigate the potential beneficial effects of GEN in ameliorating AA-induced UC in a rat model compared to the standard drug, SZ, and explore their underlying possible mechanisms when administrated alone and in combination.

2. Materials and methods

2.1. Drugs and chemicals

SZ, GEN, and AA were purchased from Sigma-Aldrich Chemical Co., St Louis, MO, USA. SZ and GEN were dissolved in 1% carboxymethyl cellulose (CMC), procured from El-Nasr Co. for Intermediate Chemicals, Giza, Egypt, and diluted in distilled water [20]. All solvents and chemicals were of high-grade purity.

2.2. Animals

Adult male Wister rats weighing 200–250 g were obtained from National Research Center, Cairo, Egypt. They were housed in the animal facility of Faculty Pharmacy, Future University in Egypt. Rats were kept in standard polypropylene cages (6 per cage) under appropriate conditions of temperature (25 ∘C ± 2 ∘C), relative humidity (60%–70%), and 12-hour dark/light cycles throughout the experimental period. Rats were fed a normal chow diet and water ad libitum. Standard diet pellets obtained from El-Marwa Feed Company, Al-sharqia, Egypt, were composed of not <5% fiber, 15% protein, 3.5% fat, and a vitamin mixture. Animals were allowed to acclimate for two weeks before the experiment. To minimize variability owing to circadian rhythm, testing was performed roughly at the same time each day. All experiments were performed in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, and experiments were carried out by the National Institutes of Health guidelines for the care and use of laboratory animals (NIH, revised in 2011). The study protocol, all procedures, and manipulations of experimental animals were carried out according to guidelines approved by the Research Ethics Committee for experimental and clinical studies of the Faculty of Pharmacy Cairo University, Cairo, Egypt (PT-2941) and Faculty of Pharmacy Future University in Egypt, Cairo, Egypt (REC-FOPFUE-15/116).

2.3. Induction of colitis

Rats were fasted for 24 h prior to the induction of colitis but were allowed free access to tap water. Later, water and food were available ad libitum. After 24 h of fasting, animals were anesthetized with ketamine (50 mg/kg i.p) [22], obtained from Inresa Arzneimittel GmbH Co., Freiburg, Germany. Colitis was induced by a rectal enema, with instillation of 1–2 ml of 5% AA, diluted in distilled water, administered through a medical-grade polyethylene catheter (external diameter 2 mm) inserted into the rectum at a depth of 4.5 cm. The tip was advanced to 8 cm proximal to the anus verge [23]. Over the years, because perforations are common with enemas with high concentrations of AA in the gastrointestinal lumen, further adjustments and optimizations focused on adjusting the AA concentration and contact time. Thus, we used the newest procedure of selecting the minimum AA concentration of 5 % within 15–30 s of exposure [24]. Rats were maintained in a supine Trendelenburg position or head down for one min to prevent spillage of intracolonic solution from the rectum [6].

2.4. Experimental design

Thirty-six animals were randomly divided into six groups (six animals each). Group I: normal control group received vehicle 1% CMC solution and normal saline solution rectally. Group II: SZ + GEN combination alone group received (100 mg/kg/day) of each drug and normal saline solution rectally. Group III: AA-induced UC "AA Model" group received 1% CMC solution and 1–2 ml of 5% AA rectally. Ulcerated rats were allocated into three groups; Group IV: SZ-treated group received SZ (100 mg/kg/day) [25]; Group V: GEN-treated group received GEN (100 mg/kg/day) [20], Notably, this dose is equivalent to a human dose of 16.13 mg/kg using a conversion factor of 0.162 [26], which lies within the safe therapeutic range of GEN doses in clinical

safety and pharmacokinetics studies [27]; Group VI: SZ + GEN combination-treated group received both treatments in the previously mentioned doses. All treatments were gavaged orally 24 h after the colitis induction, once daily for 14 days.

At the end of the treatment period, animals were anesthetized again with ketamine (50 mg/kg i.p) [22]. Then the rats were humanly sacrificed by cervical dislocation. Colons were collected, washed in normal saline, and blotted on filter paper. The length of the colon was measured, and the colon was weighed. This blotting is used also for macroscopic scoring determination. Afterward, the proximal 8 cm was discarded, and the distal part of the colon was opened longitudinally and divided into two portions. The first portions of the six colon specimens were stored and frozen at −80 °C until assayed for biochemical studies including the ELISA technique, and colorimetric method. Meanwhile, three specimens of the second portion were kept in 10% formalin, purchased from Sigma-Aldrich Chemical Co., St Louis, MO, USA, for histopathological and immunohistopathological studies. The other three specimens were kept at −80 °C to be used in the Western blotting assessment. Blood samples were collected from the rats' *retro*-orbital plexus for serum separation by centrifugation at 2000×*g* for 20 min at 4 °C. Serum samples were divided into two aliquots and stored at −80 °C until serum parameters assay [28]. Dead bodies were frozen until incinerated.

2.5. Assessment of colitis

2.5.1. Determination of change in the body weight

Rats were weighed before induction of colitis (wt_1) and on the day of sacrifice (wt_f) to calculate the difference in body weight. Body weight variation was calculated as follows (wt_f - wt_1).

2.5.2. Determination of colon mass index

The colon mass index was calculated to indicate colon edema in colitis. For each rat, the colon weight and the total body weight were calculated. The colon mass index (mg/g) = colon weight / total body weight [29].

2.5.3. Determination of colonic macroscopic score

Each animal's proximal 8 cm portion of the colon was removed, cut longitudinally, and slightly cleaned in physiological saline to remove fecal residue, then blotted on filter paper. Macroscopic inflammation scores were assigned based on clinical features of the colon; using an arbitrary scale ranging from 0 to 4, as follows: 0: no macroscopic changes (no ulcer or damage), 1: mucosal erythema only (localized hyperemia but no ulcer), 2: mild mucosal edema, slight bleeding, or small erosions, 3: moderate edema, slight bleeding ulcers or erosions, 4: severe ulceration, edema, and tissue necrosis [30].

2.5.4. Determination of disease activity index (DAI)

Weight changes were recorded daily throughout the experiment. Fecal samples of each animal were visually inspected for signs of diarrhea and rectal bleeding. The DAI is calculated based on the cumulative scores of percent loss in body weight (0–4), stool consistency (0–4), and stool occult bleeding (0–4), which was detected by the benzidine test [31]. Colitis was quantified with clinical scores as described in Table 1.

Table 1
Disease activity index (DAI)

Score	% Weight loss	Stool consistency	Occult/gross bleeding
0	None	Normal	Negative
1	1–5%		Occult blood ±
2	5–10%	Loose stool	Occult blood +
3	10–15%		Occult blood ++
4	> 15%	Diarrhea	Gross blood

(±) a very light blue color taking over 30 s to appear; (+) a blue color developing in 30 s or more; (++) an immediate blue color occurring in <30 s.

DAI was calculated according to the following equation [25]:

DAI = (body weight loss score + diarrhea score + rectal bleeding score) / 3

2.5.5. Determination of colon weight/length (W/L) ratio

For each rat, the weight and length of the isolated colons were measured, and the W/L ratios (mg/cm) were calculated to record the severity of colitis [32].

2.6. Enzyme-linked immunosorbent assay (ELISA)

The rat-specific ELISA kits provided by MyBioSource, CA, USA, (Cat#: MBS9711590, #MBS266603, and #MBS825017) were used to determine the content of INF-γ, COX-2, and IL-1β, respectively. The ELISA kits used to assess rat TNF-α and TLR-4 were purchased from CUSABIO, TX, USA, (Cat#: CSB-E11987r and #CSB-E15822r), respectively, whereas the ELISA kit for Interferon regulatory factor 1 (IRF-1) was procured from LifeSpan Biosciences, WA, USA (Cat#: LS-F18007). The ELISA kit used to assess IL-6 was purchased from RnDSystems, CA, USA (Cat#: R6000B), and the ELISA kit used to measure myeloperoxidase (MPO) was purchased from Reddot Biotech Inc., Kelowna, Canada (Cat#: RDR-MPO-Ra). All assessments were done according to the manufacturer's instructions.

2.7. Western blot technique

The portions of the colon used in this technique were kept in radioimmunoprecipitation assay (RIPA) buffer with protease/phosphatase inhibitors, obtained from Sigma-Aldrich Chemical Co., St Louis, MO, USA., then the colon specimens were homogenized and stored at −80 °C. The protein concentration of the lysates was determined using a Bradford Protein Assay Kit obtained from Bio Basic Inc., Markham, Canada (Cat#: SK3041), according to the manufacturer's instructions. Thereafter, the protein expression of different proteins was measured using primary antibodies against JAK 1, STAT 1, JAK 2, STAT 3, and β-actin, purchased from Thermo Fisher Scientific, MA, USA, (Cat#: PA5-105265, #PA5-81911, #AHO1352, #MA5-15712, and #PA1-183), respectively at a dilution of 1:1000. Equal sample protein concentration was added to 2x Laemmli sample buffer, purchased from Sigma-Aldrich Chemical Co., St Louis, MO, USA., and heated at 95 °C for 5 min to ensure denaturation of protein before loading on polyacrylamide gel electrophoresis using sodium dodecyl sulfate (SDS-PAGE), purchased from Sigma-Aldrich Chemical Co., St Louis, MO, USA., to separate the proteins according to their molecular weight. The blots on the gel were transferred to a nitrocellulose membrane blocked in tris-buffered saline with Tween 20 (TEST) buffer and 3% bovine serum albumin (BSA), all were procured from Sigma-Aldrich Chemical Co., St Louis, MO, USA., at room temperature for 1 h. Afterward, the membrane was incubated with the primary antibodies for target proteins diluted in TEST. The targeted proteins were detected using a horseradish peroxidase (HRP)-conjugated goat antirabbit-IgG secondary antibody obtained from Abcam, Cambridge, UK, (Cat#: ab6721). The chemiluminescent substrate, Clarity™ Western ECL Substrate-Bio-Rad, CA, USA (Cat#: 170-5060), was applied to the blot according to the manufacturer's recommendation. The chemiluminescent signals of target protein bands were captured using a CCD camera-based imager and were analyzed using the Chemi Doc MP Bio-Rad imager, CA, USA (Cat#: 170-5060) software. The results were expressed as the relative expression from the control after normalization for the β-actin protein expression.

2.8. Colorimetric assay

Commercial colorimetric kits were used to measure the content of Nitric Oxide (NO) and Superoxide Dismutase (SOD) in colon tissue homogenates. These kits were purchased from BioDiagnostic, Giza, Egypt, (Cat#: NO 25 32 and #SD 25 20).

2.9. Histopathological and histochemical analysis

Specimens from the colon of all rats were harvested, washed in water followed by serial dilutions of alcohol, purchased from El-Nasr Co. for Intermediate Chemicals, Giza, Egypt., for dehydration, fixed in neutral buffered formalin 10%, and processed by paraffin, obtained from Sigma-Aldrich Chemical Co., St Louis, MO, USA., embedding technique at 56 °C in a hot air oven for 24 h. Paraffin blocks were sectioned at 4 μm thickness by sled microtome, purchased from Leica Biosystems, Barrington, USA. The obtained tissue sections were collected on glass slides, deparaffinized, and stained with Hematoxylin and Eosin (H & E). Furthermore, alcian blue was used for goblet cells (GCs) staining and Masson's Trichrome (MTC) for collagen fibers [33]. Sections were inspected blindly, under a light microscope BX43 purchased from Olympus, Tokyo, Japan, by a pathologist.

Quantitative histological assessment of colon lesions was carried out and scored from (0–3) as follows: (0) indicated no changes, (1), (2), and (3) indicated mild, moderate, and severe changes, respectively. Briefly, the six assigned alterations were mucosal necrosis, inflammatory cell infiltration (mainly neutrophils and macrophages), edema, hemorrhage, depletion of mucous-secreting glands, and fibrosis [34]. The number of positive goblet cells and the area percentage of fibrosis were blindly visualized and quantified in five random non-overlapped fields from each sample using CellSens dimensions (Olympus Software, Tokyo, Japan).

2.10. Immunohistochemical analysis

Immunohistochemical analysis of NF-κB, iNOS, and caspase-3 was investigated. 4–5 μm striatal sections were cut into pre-coated (poly-L-lysin) adhesive slides, deparaffinized, and subjected to rehydration followed by heat-induced epitope retrieval steps. Overnight at the refrigerator (4 ∘C), the slides were incubated with a mouse monoclonal primary antibody against NF-κB, iNOS, and caspase-3. All were purchased from Santa Cruz Biotechnology Inc., Dallas, TX, USA, (Cat#: sc-8008, #sc-7271, and #sc-7272), respectively, at a dilution of 1:200. Slides were then incubated with HRP-labeled goat anti-rat secondary antibody was procured from Abcam, Cambridge, UK, (Cat#: ab97057) for 2 h, accompanied by the detection step with 3,3′-Diaminobenzidine (DAB)-Substrate kit purchased from Thermo Fisher Scientific, MA, USA, (Cat#: 34002). Positive staining was represented by a blinded investigator as the area percentage of expression measured by CellSens dimensions (Olympus software, Tokyo, Japan) by calculating the area % of positive cells in five random non-overlapped fields from each sample [35].

2.11. Statistical analysis

For the parametric data, values are presented as mean ± SD (n = 6 per group for the ELISA technique and colorimetric method, n = 3 for Western blotting assessment, and n = 3 for alcian blue, MTC staining, and immunohistochemical analysis in five random fields from each section). Comparisons between two groups were analyzed using the unpaired *t*-test, whereas comparisons between more than two groups were analyzed using the analysis of variance (ANOVA) test, followed by Tukey's post hoc test. The non-parametric data (histological scores) are presented as the median (min–max; n = 3 per group). The values were analyzed using the Kruskal-Wallis test, followed by Dunn's post hoc test. The Level of probability (P value) <0.05 is considered statistically significant. Graph pad prism® software package version 7 (GraphPad Software, CA, USA) was used to calculate and draw the assessed parameters.

Since there is no significant difference between the normal control group and the GEN + SZ combination control group, all the assessed parameters were compared to the normal control.

3. Results

3.1. GEN and/or SZ normalized the AA-induced changes in body weight and colon mass index

(Fig. 1A) displayed a body weight 65 g loss in ulcerated rats relative to the initial weight along, with a 2 folds increment in colon mass index compared to the control group (Fig. 1B). All treatment regimens restored the body weight and colon mass index to normal.

3.2. GEN and/or SZ attenuated the AA-induced macroscopic alterations in the colon

AA caused severe inflammation in the colon, and the mucosa was ulcerated, edematous, and hemorrhagic in appearance (Fig. 1C), with a much higher macroscopic score of colonic damage in comparison to the normal control group (Table 2). Meanwhile, all treatment regimens markedly alleviated the severity of the gross lesion score compared to the UC group. Macroscopically, the highest protective effect was shown in the combination-treated group.

3.3. GEN and/or SZ improved DAI in AA-induced UC in rats

Rats that received AA showed a significant increase in DAI compared to the control group, while administration of all treatment regimens significantly reduced DAI compared to the UC group. There was no significant difference between the treated groups (Table 2).

3.4. GEN and/or SZ reduced the AA-induced increase in the W/L ratio (g/cm)

AA caused a 2.7 folds increase in colon W/L ratio compared with the control group. While GEN, SZ, and combination-treated groups showed a 54%, 55%, and 60% reduction in colon W/L ratio, respectively, when compared to the UC group. There was no significant difference between the treated groups (Table 2).

3.5. GEN and/or SZ amended histopathologic structure and colon morphology in rats with AA-induced UC

Microscopically, colons of normal control rats as well as rats from the combination control group exhibited normal histological architecture (Fig. 2A & B). The colitis model colons showed ulceration characterized by remarkable microscopic damage, summarized as marked mucosal necrosis, inflammatory cells infiltration, edema, and hemorrhage in the mucosal and submucosal layers, associated with fibroblasts proliferation and depletion in GCs (Fig. 2C, D & E). Contrariwise, the colons of rats treated with SZ revealed regression in the previously mentioned histopathological lesions and examined sections exhibited few inflammatory cell infiltration and edema in the lamina propria and submucosa (Fig. 2F). Meanwhile, the colons of rats treated with GEN showed focal necrosis of mucous-secreting glands and inflammatory cell infiltration in the mucosa and submucosa (Fig. 2G). On the other hand, a better recovery in the colon histological structure is detected in the combination-treated group showing marked ameliorative effects and restoration of the histological structure with hyperplasia of mucous-secreting glands and a few focal inflammatory cells in the lamina propria (Fig. 2H). The individual scores of histopathological lesions are summarized in (Panels 2a-2e), and the collective score is shown in (Panel 2f).

3.6. Alcian blue staining showed the GEN and/or SZ-induced increase in the number of GCs in rats with AA-induced UC

The number of alcian blue-stained GCs was visualized in the colon sections of different experimental groups (Fig. 3). The number of GCs in the UC group (Fig. 3C) was significantly decreased compared to the

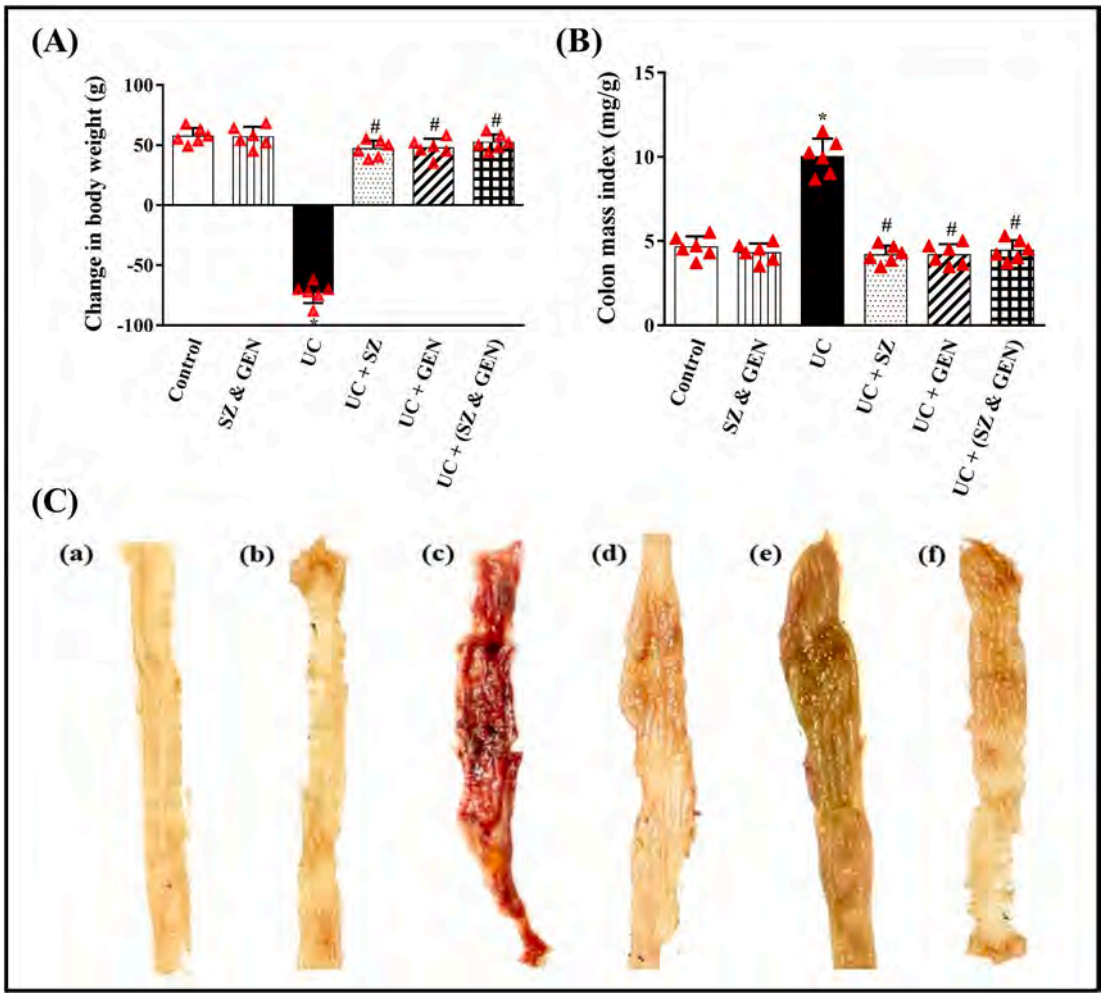

Fig. 1. Effect of GEN and/or SZ on (A) change in body weight, (B) colon mass index, and (C) macroscopic appearance of the colonic mucosa in UC rats. Rats were subjected to one colonic instillation of AA and left for 24 h to induce colitis, then treated orally with SZ (100 mg/kg), GEN (100 mg/kg), and (SZ + GEN) with previously mentioned doses for 14 days: (a) Control group; (b) SZ & GEN combination alone group; (c) UC group; (d) UC + SZ treated group; (e) UC + GEN treated group; (f) UC + (SZ & GEN) combination-treated group. Data are presented as mean ± SD (n = 6 per group; one-way ANOVA followed by Tukey's post hoc test); P < 0.05, as compared to (*) Control group, (#) UC group. GEN: genistein; SZ: sulfasalazine; AA: acetic acid; UC: ulcerative colitis.

Table 2
Effect of genistein, sulfasalazine, and their combination on the macroscopic score, DAI, and W/L ratio in AA-induced UC

Group	Macroscopic Score	DAI	W/L Ratio (mg/cm)
Control	0.00 ± 0.000	0.00 ± 0.000	56.92 ± 5.979
SZ & GEN	0.00 ± 0.000	0.00 ± 0.000	60.73 ± 8.689
UC	3.83 ± 0.408**	3.50 ± 0.182**	155.01 ± 17.050**
UC + SZ	0.50 ± 0.547##	0.27 ± 0.250##	69.11 ± 4.479##
UC + GEN	0.33 ± 0.516##	0.22 ± 0.171##	71.77 ± 4.650##
UC + (SZ & GEN)	0.16 ± 0.408##	0.11 ± 0.172##	62.47 ± 5.137##

For W/L ratio values represent the mean ± SD (n = 6 per group; one-way ANOVA followed by Tukey's post hoc test; ** p < 0.05, vs. the control group; ## p < 0.05, vs. UC group), while for DAI and macroscopic score, the data were analyzed using Kruskal-Wallis test as the median (min–max; n = 6 per group; followed by Dunn's post hoc test; ** p < 0.05, vs. the control group; ## p < 0.05, vs. UC group). GEN: genistein; SZ: sulfasalazine; DAI: disease activity index; W/L: weight/length; AA: acetic acid; UC: ulcerative colitis.

normal control and combination control groups (Fig. 3A & B). On the other hand, an increase in the number of GCs was recorded in the colon of rats treated with either SZ (Fig. 3D) or GEN (Fig. 3E) compared to the UC group. Furthermore, a marked increase in the number of GCs was exhibited in the combination-treated group reaching a synergistic level as evidenced by the observed excessive secretion of mucous compared to the UC group (Fig. 3F). The count of GCs in all groups was summarized in (Panel 3G).

3.7. GEN and/or SZ lessened the AA-induced collagen deposition in the colon of UC rats, which appeared by Masson's Trichrome staining for collagen fibers

Light microscopic examination of the normal control colons as well as the colons of combination control rats exhibited normal weak MTC stained collagen fibers deposition (Fig. 4A & B). A marked increase in collagen fibers deposition was demonstrated in the UC group (Fig. 4C). Treatment with SZ improved colon fibrosis with a slight increase in collagen deposition (Fig. 4D). On the other hand, treatment with GEN exhibited moderate MTC reaction of collagen fibers deposition (Fig. 4E). Interestingly, the combination treatment normalized the collagen fiber deposition, indicated by a weak deposition of normal collagen (Fig. 4F). The area percentage of MTC staining is summarized in (Panel 4G).

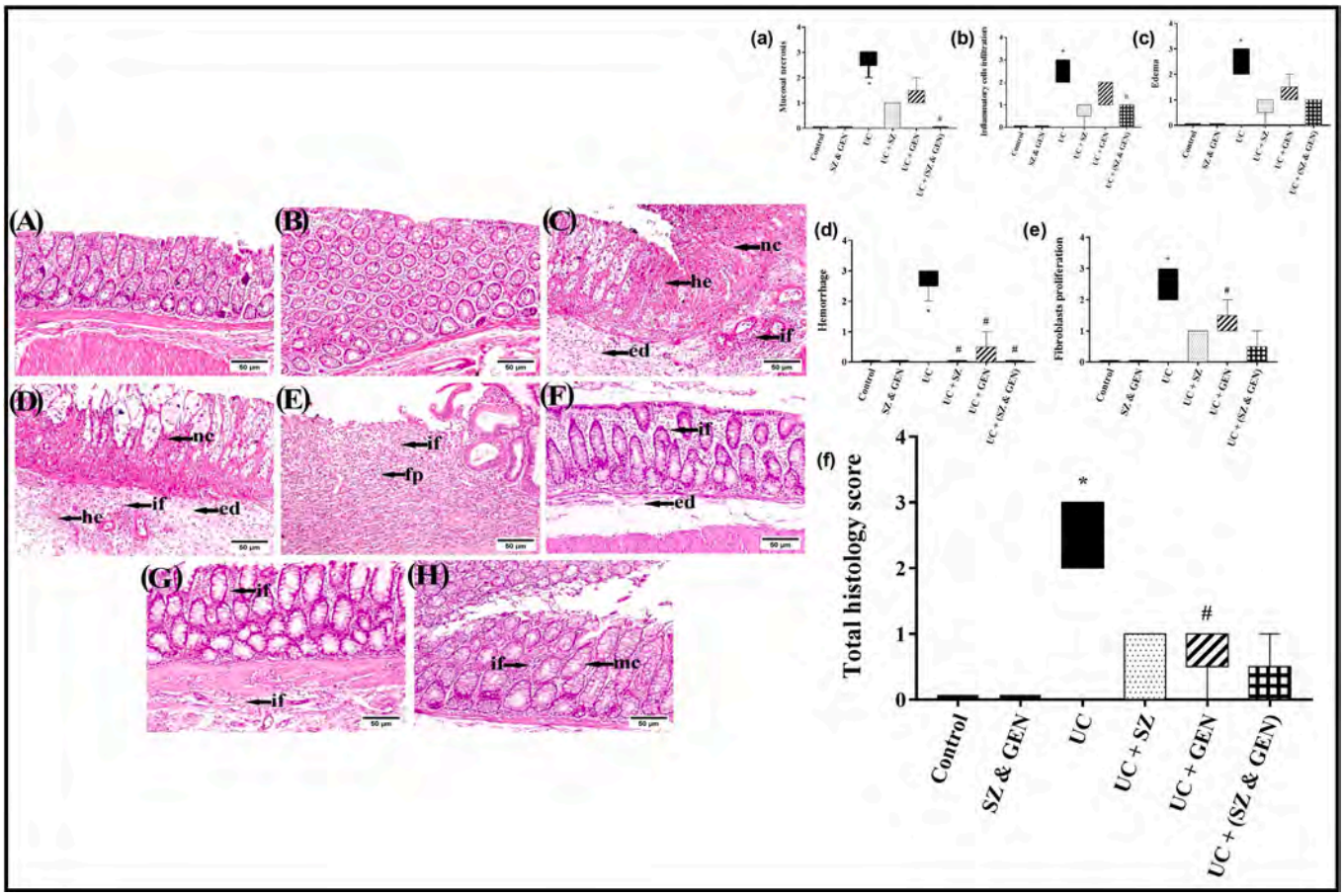

Fig. 2. Effect of GEN and/or SZ on colon histopathological changes and lesion scores in UC rats. Representative photomicrographs exhibited H & E-stained colon sections (scale bar, 50 μm). The normal control group (A) and SZ + GEN combination alone group (B) showed normal histological architecture. UC group model (C), (D), and (E) showed extensive mucosal necrosis (nc), inflammatory cells infiltration (if), edema (ed), hemorrhage in the mucosa and submucosa (he), and fibroblasts proliferation (fp). SZ-treated group (F) showed few inflammatory cells infiltration (if) in the lamina propria associated with submucosal edema (ed). GEN-treated group (G) showed inflammatory cell infiltration (if) in the mucosa and submucosa. SZ + GEN combination-treated group (H) showed hyperplasia of mucous-secreting glands (mc) and few focal inflammatory cells (if) in the lamina propria. The individual scores of histopathological lesions are summarized in (Panels a-e), and the collective score is shown in (Panel f), the data were analyzed using the Kruskal-Wallis test as the median (min–max; n = 3 per group; followed by Dunn's post hoc test; $p < 0.05$, as compared to (*) Control group, (#) UC group. GEN: genistein; SZ: sulfasalazine; UC: ulcerative colitis.

3.8. GEN and/or SZ downregulated the AA-induced increase in the colonic proinflammatory mediators via inhibiting INF-γ/JAK1/STAT1 signaling pathway

AA rectal administration significantly raised INF-γ levels (Fig. 5A), JAK1 expression (Fig. 5B), and STAT1 expression (Fig. 5C) to 3.8, 4.8, and 6.9 folds, respectively, compared to the normal control rats. On the other hand, the administration of treatment regimens SZ, GEN, and combination treatment diminished the production of the pro-inflammatory cytokine INF-γ by 52%, 48%, and 69%, the relative expression of JAK1 by 46%, 55%, and 62% leading to reduction of the relative expression of STAT1 by 44%, 43%, and 64%, respectively, compared to the UC group. Remarkably, the most ameliorative effect was shown in the SZ + GEN-treated rats.

3.9. GEN and/or SZ inactivated IRF-1/iNOS/NO hub in AA-induced UC

As illustrated in (Fig. 6A-G), the expression of iNOS was immunohistochemically measured in the colon. No expression was visualized in the colon tissues of normal control and combination control rats (Fig. 6A & B). Strong positive immune expression was seen in the colon sections of the colitis model (Fig. 6C). On the other hand, treatment with SZ revealed a reduced number of brown staining-positive cells compared to the UC group (Fig. 6D). Furthermore, moderate expression of iNOS was detected in the colon of rats treated with GEN (Fig. 6E). Otherwise, colons of the combination-treated group exhibited weak immune expression (Fig. 6F). The immunohistochemical expression of iNOS was markedly elevated to 30.4 folds in the UC group compared to the normal control rats. Startlingly, treatment with SZ, GEN, and their combination showed a significant decrease in the iNOS expression by 80%, 73%, and 88%, respectively, compared to the UC rats. The area percentage of iNOS immune expression is shown in (Panel 6G).

The level of IRF-1 in the colonic tissues was markedly elevated to 2.6 folds in the UC group compared to the normal control group. While treatment with SZ, GEN, and their combination showed a marked decrease in IRF-1 levels by 50%, 54%, and 63%, respectively, compared to the UC group (Fig. 6H). Furthermore, UC rats showed a 3.1 folds increment increase in the NO content in the colonic tissue compared to the control group. However, treatment with SZ, GEN, and a combination of them lowered the NO levels by 47%, 48%, and 59%, respectively, compared to the UC group (Fig. 6I). Notably, the administration of SZ + GEN-combination treatment normalized both IRF-1 and NO levels.

3.10. GEN and/or SZ suppressed the colonic NF-κB expression and TLR-4 content in AA-induced UC

(Fig. 7A-G) showed NF-κB immune expression in the colon tissues. No expression was visualized in the colon tissues of normal control and

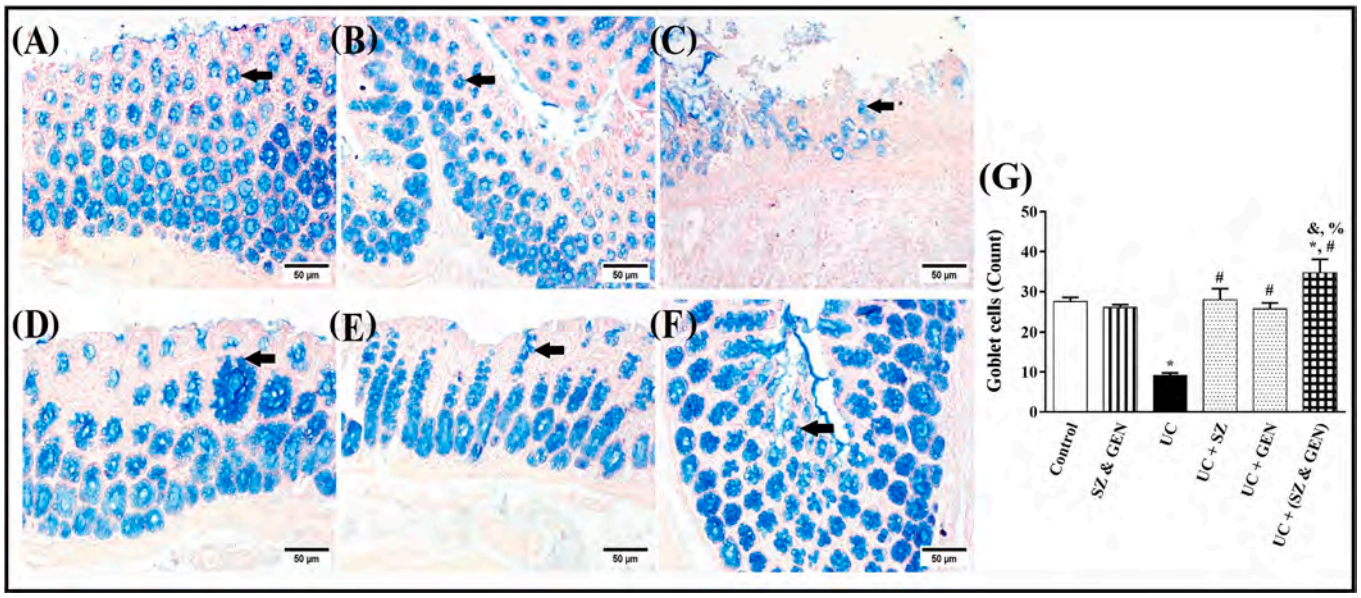

Fig. 3. Effect of GEN and/or SZ on the number of GCs in UC rats. Representative photomicrographs exhibited alcian blue stained colon sections (scale bar, 50 μm). The normal control group (A) and SZ + GEN combination group (B) showed normal positive staining GCs. UC group model (C) showed a marked decrease in the GCs number. SZ-treated group (D) showed an increase in the number of GCs. GEN-treated group (E) showed an increase in the alcian blue-stained GCs number. SZ + GEN combination-treated group (F) showed a significant increase in the GCs number (arrows). (Panel G) summarizes the above results (5 fields in each section). Data are presented as mean ± SD (n = 3 per group; one-way ANOVA followed by Tukey's post hoc test); $P < 0.05$, as compared to (*) Control group, (#) UC group, (&) SZ treated group, (%) GEN treated group. GEN: genistein; SZ: sulfasalazine; GCs: goblet cells; UC: ulcerative colitis.

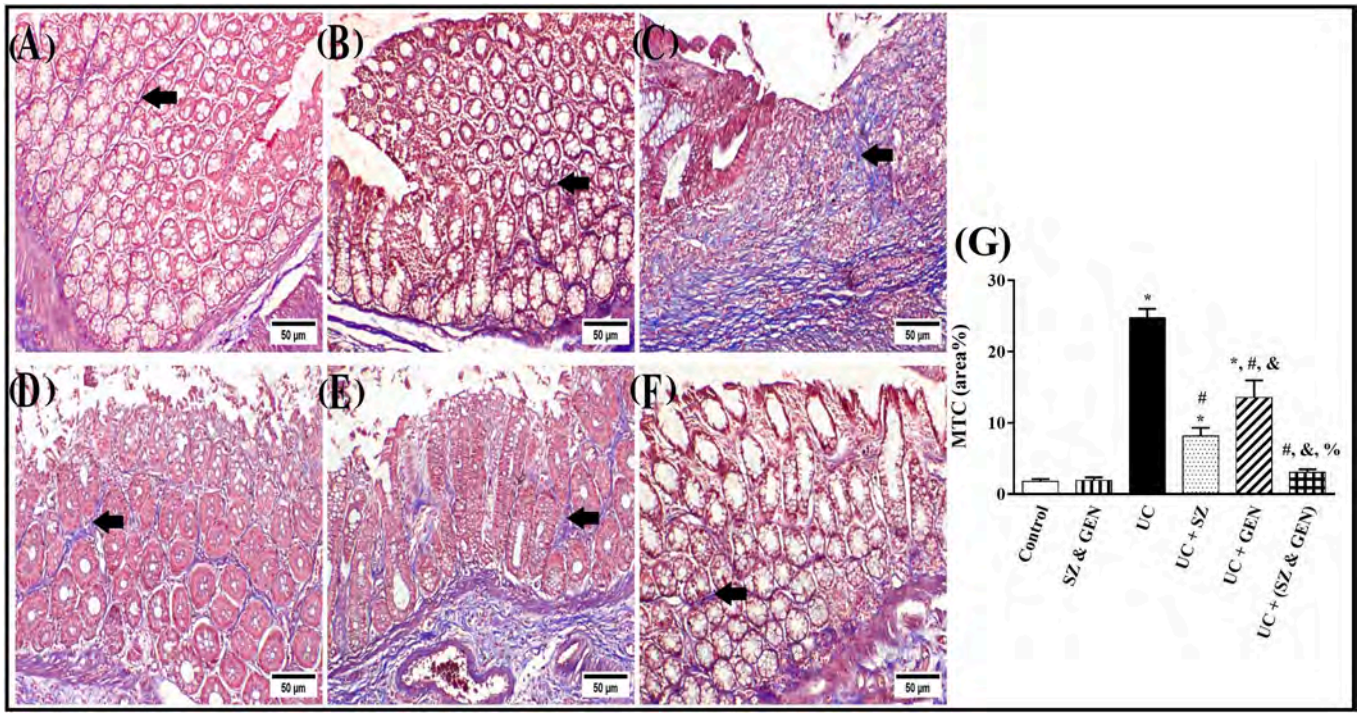

Fig. 4. Effect of GEN and/or SZ on MTC stained collagen fibers in UC rat colon. Representative photomicrographs exhibited Masson's Trichrome (MTC)-stained colon sections (scale bar, 50 μm). The normal control group (A) and SZ + GEN combination group (B) showed normal weak MTC-stained collagen fibers. UC group model (C) showed a marked increase in collagen fibers deposition indicated by a strong positive MTC stain. SZ-treated group (D) showed a slight increase in collagen fibers deposition. GEN-treated group (E) showed a moderate increase in collagen fibers deposition. (F) SZ + GEN combination-treated group showed weak normal collagen fibers deposition (arrows). (Panel G) shows the area % of MTC staining (5 fields in each section). Data are presented as mean ± SD (n = 3 per group; one-way ANOVA followed by Tukey's post hoc test); $P < 0.05$, as compared to (*) Control group, (#) UC group, (&) SZ treated group, (%) GEN treated group. GEN: genistein; SZ: sulfasalazine; MTC: Masson's Trichrome; UC: ulcerative colitis.

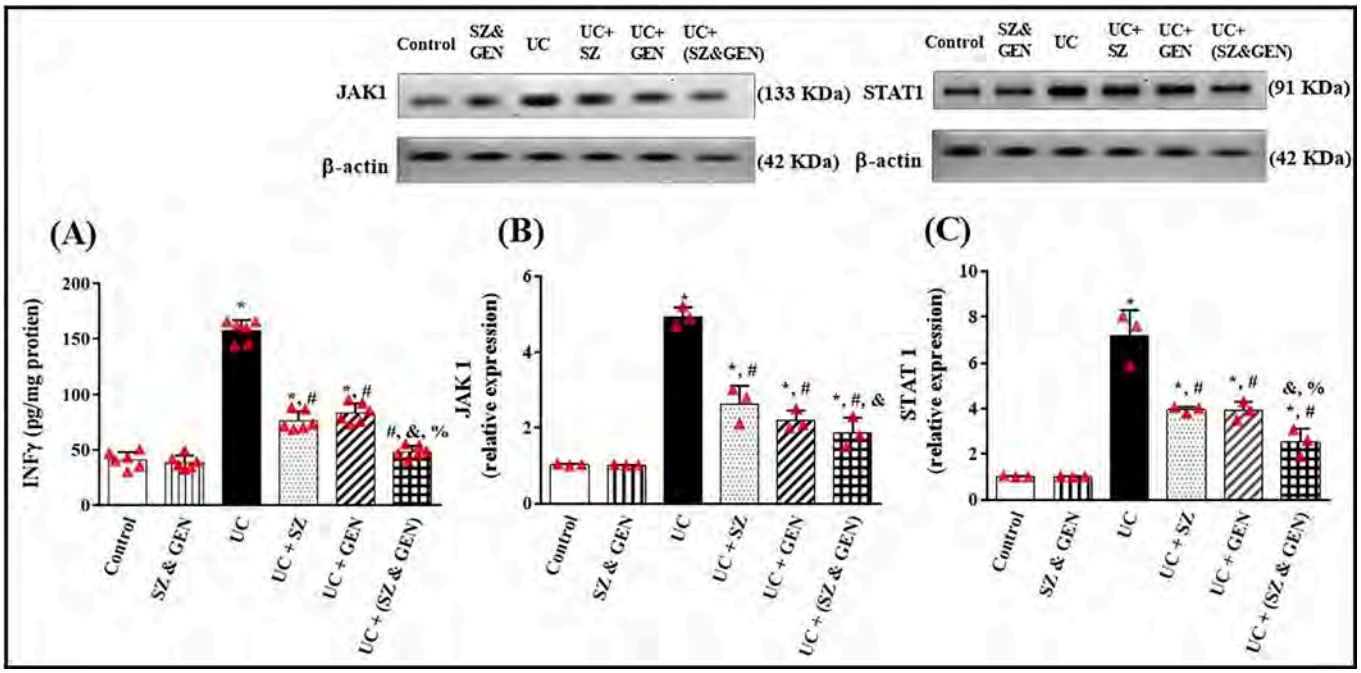

Fig. 5. Effect of GEN and/or SZ on (A) INF-γ, (B) JAK1, and (C) STAT1 in UC rats. Rats were subjected to one colonic instillation of AA and left for 24 h to induce colitis, then treated orally with SZ (100 mg/kg), GEN (100 mg/kg), and (SZ + GEN) with previously mentioned doses for 14 days. Data are presented as mean ± SD (INF- γ: n = 6 per group "ELISA technique"; JAK1 and STAT1: n = 3 per group "Western blotting assessment"; one-way ANOVA followed by Tukey's post hoc test); $P < 0.05$, as compared to (*) Control group, (#) UC group, (&) SZ treated group, (%) GEN treated group. GEN: genistein; SZ: sulfasalazine; INF-γ: interferon-gamma; JAK1: Janus kinase 1; STAT1: signal transducers and activators of transcription 1; UC: ulcerative colitis.

combination control rats (Fig. 7A & B). Strong positive immune expression was detected in the colons of the colitis model and the expression markedly rose following the induction of colitis by AA to 23.5 folds compared to the normal control rats (Fig. 7C). Treatment with SZ reduced the number of brown staining-positive cells (Fig. 7D) by 69% compared to the UC group. Moderate expression of NF-κB was detected in the colon of rats treated with GEN (Fig. 7E), an effect illustrated by a 60% reduction in its expression compared to the AA group. Otherwise, colons of rats treated with combination treatment exhibited weak immune expression (Fig. 7F) by 81% compared to the UC group. The area percentage of NF-κB immune expression is summarized in (Panel 7G).

The content of TLR-4 in the colonic tissues was markedly increased to 3.6 folds in the UC group compared to the normal control group. On the contrary, the administration of SZ, GEN, and their combination hindered the levels of TLR-4 by 48%, 44%, and 63% respectively, compared to the UC group. However, the combination treatment has the maximum effect in normalizing its level (Fig. 7H).

3.11. GEN and/or SZ reduced the levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in AA-induced UC

SZ, GEN, and their combination treatment normalized the AA-induced 5.2, 2.4, and 2.7 folds increase in the TNF-α, IL-1β, and IL-6 levels, respectively. SZ, GEN, and combination treatment significantly decreased the TNF-α level by 47%, 53%, and 70% respectively (Fig. 7I), with a concurrent marked decline in the IL-1β levels by 33%, 34%, and 52% respectively (Fig. 7J), with diminishing the levels of IL-6 by 42%, 44%, and 50%, respectively, compared to the UC rats (Fig. 7K).

3.12. GEN and/or SZ modulated the AA-induced disturbance in JAK2/STAT3/COX-2 trajectory

AA administration significantly elevated the relative expression of JAK2, STAT3, and the levels COX-2 to 7.2, 5.3, and 3.9 folds, respectively, compared to the normal control rats (Fig. 8). On the other hand, administration of SZ, GEN, and SZ + GEN combination treatment showed a marked decline in the relative expression of JAK2 by 63%, 57%, and 76%, respectively (Fig. 8A), and the relative expression of STAT3 by 60%, 59%, and 70%, respectively (Fig. 8B), resulting in the normalization of the COX-2 content by 60%, 61%, and 72% respectively compared to the UC group (Fig. 8C).

3.13. GEN and/or SZ alleviated AA-induced oxidative stress

Interestingly, the co-administration of SZ, GEN, and their combination normalized the AA-induced 2 folds increase in MPO and a 69% decrease in SOD activity in the colon homogenates. Treatment with SZ, GEN, and their combination markedly declined the MPO levels by 43%, 42%, and 49% respectively (Fig. 9A), and augmented the SOD activity by 78%, 82%, and 95% respectively, compared to the UC group (Fig. 9B). Oxidative stress status improvement was remarkable in the combination-treated group among the other treated groups.

3.14. GEN and/or SZ reduced apoptosis in AA-induced UC

As shown in (Fig. 10A-G), Caspase-3 immune expression in the colon tissues was measured. No expression was visualized in the colon tissues of normal control and combination control rats (Fig. 10A & B). Strong positive immune caspase-3 expression was seen in colon sections of colitis model rats, it was markedly elevated to 27.3 folds compared to the normal control rats (Fig. 10C). On the other hand, treatment with SZ reduced the number of brown staining-positive cells by 61%, compared to the UC group (Fig. 10D). Furthermore, moderate expression of caspase-3 was detected in the colon of rats treated with GEN by 50% compared to the UC group (Fig. 10E). Otherwise, colons of rats treated with combination treatment exhibited weak immune expression by 80% compared to the UC group (Fig. 10F). The area percentage of caspase-3 immune expression is shown in (Panel 10G).

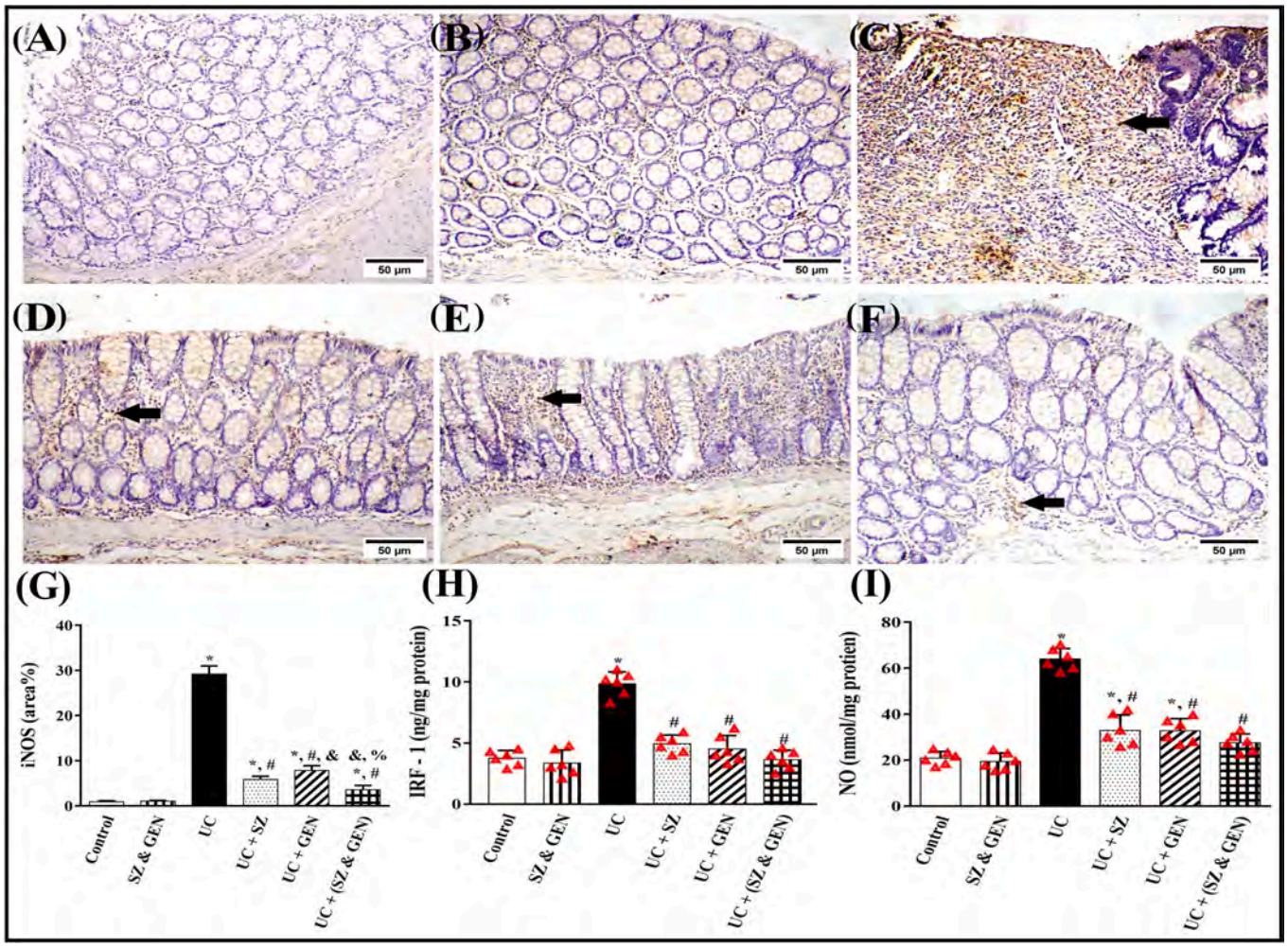

Fig. 6. Effect of GEN and/or SZ on colonic iNOS immunoreactivity and IRF-1 and NO content in UC rats. Representative photomicrographs of iNOS immune stained colon sections (scale bar, 50 µm). The normal control group (A) and SZ + GEN combination group (B) showed normal no immune expression. UC group model (C) showed a significant increase in positive immunostaining cells. SZ-treated group (D) showed a reduced number of brown staining positive cells. GEN-treated group (E) showed moderate expression of iNOS. SZ + GEN combination-treated group (F) showed weak immune expression (arrows). (Panel G) presents the % area of iNOS staining (5 fields in each section). (H) represents the IRF-1 content and (I) showed the NO content. Data are presented as mean ± SD (% area of iNOS staining n = 3 per group "Immunohistochemical analysis"; IRF-1 and NO: n = 6 per group "ELISA technique and colorimetric method respectively"; one-way ANOVA followed by Tukey's post hoc test); $P < 0.05$, as compared to (*) Control group, (#) UC group, (&) SZ treated group, (%) GEN treated group. GEN: genistein; SZ: sulfasalazine; iNOS: calcium-independent nitric oxide synthase; IRF-1: interferon regulatory factor 1; NO: nitric oxide; UC: ulcerative colitis.

4. Discussion

This study is the first to highlight the probable mechanisms implicated in GEN, a natural isoflavone compound, in combination with SZ, the standard therapy against AA-induced UC in a rat model. The protective effect of GEN and/or SZ was shown to be mediated via JAK/STAT signaling pathway. Anti-colitis effects of both SZ and GEN rely on shutting down INF-γ/JAK1/STAT1 and INF-γ /TLR-4/ NF-κB signaling pathways. Besides, they modulate the IRF-1/iNOS/NO hub and IL-6/JAK2/STAT3/COX-2 trajectory and reduce the levels of pro-inflammatory cytokines TNF-α and IL-1β. Furthermore, by decreasing MPO and elevating SOD levels, these drugs preserve the homeostasis between oxidative and anti-oxidative stress biomarkers. Moreover, they alleviate the apoptotic effect of colitis by declining the expression of caspase-3 in the colon mucosa.

Intrarectal administration of diluted AA provides an alternative method for creating a harmful effect on the colon epithelium mucosa that induces a transient phenotype mimicking UC [24]. Massive necrosis disseminated inflammatory leucocyte infiltration and ulcerated mucosa result from the intrarectal injection of AA [6].

As described by various studies, AA-induced UC was accompanied by a decrease in total body weight, and elevation in the colon mass index, with marked macroscopic ulcerative lesions [29,36,37]. The colonic damage was represented in the elevation of DAI and colon W/L ratio, similar results in the Fouad et al. study were shown [25]. As reported by Hagar et al. we ended up finding that histopathological deterioration in the rats' colons appeared in marked mucosal necrosis, edema, diffuse inflammatory cell infiltration in the mucosa, and hemorrhage in the mucosal and submucosal layers associated with fibroblast proliferation [23]. The contribution of mucin-secreted GCs, cells found along the whole length of the small and large intestines, was reported in the pathophysiology of UC [38]. As previously mentioned in different studies, the number of GCs was significantly decreased upon intrarectal administration of AA [39], with a marked increase in collagen fibers deposition [40].

On the other hand, marked improvements in the macroscopic lesions with declined DAI scores and W/L ratio were evidenced after GEN treatment with or without SZ. Comparable GEN ameliorative effects were previously demonstrated [41,42]. In addition to a restoration of the histological structure [20,37] with elevated GCs number that was proven in former studies demonstrating the ability of GEN to alleviate colitis and inhibit GCs loss [43] associated with an ameliorative effect on

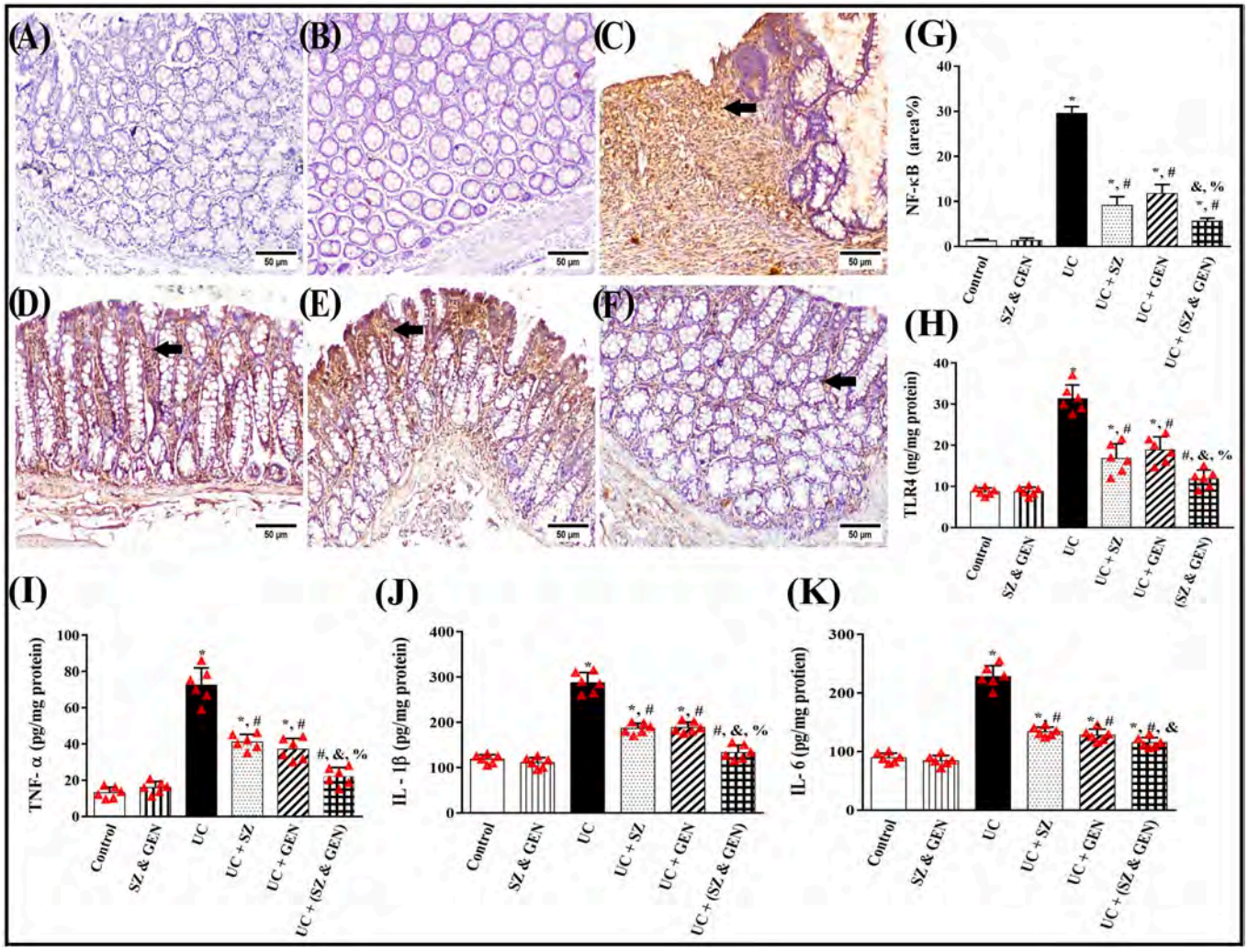

Fig. 7. Effect of GEN and/or SZ on colonic NFκB immunoreactivity and TLR-4, TNF-α, IL-1β, and IL-6 content in UC rats. Representative photomicrographs of NF-κB immune stained colon sections (scale bar, 50 µm). The normal control group (A) and SZ + GEN combination group (B) showed normal no immune expression. UC group model (C) showed a significant increase in positive immunostaining cells. SZ-treated group (D) showed a reduced number of brown staining positive cells. GEN-treated group (E) showed moderate expression of NF-κB. SZ + GEN combination-treated group (F) showed weak immune expression (arrows). (Panel G) presents the % area of NF-κB staining (5 fields in each section). Data are presented as mean ± SD (% area of NF-κB staining n = 3 per group "Immunohistochemical analysis"; TLR-4, TNF-α, IL-1β, and IL-6n = 6 per group "ELISA technique"; one-way ANOVA followed by Tukey's post hoc test); $P < 0.05$, as compared to (*) Control group, (#) UC group, (&) SZ treated group, (%) GEN treated group. GEN: genistein; SZ: sulfasalazine; NF-κB: nuclear factor kappa B; TLR-4: toll-like receptor 4; TNF-α: tumor necrosis factor-alpha; IL-1β: interleukin one beta; IL-6: Interleukin 6; UC: ulcerative colitis.

collagen fibers deposition and fibrosis [44].

It is well known that SZ has a marked effect in restoring colon morphology and reducing lesion scores [37], with decreasing DAI and W/L ratio in the UC model [45] as well as increasing the GCs number [46] and alleviating the deposition of collagen fibers [47].

There is a network of interacting cytokines rather than a single cytokine responsible for the evolution of UC [12]. Most cytokines exert their biological effects via the activation of JAK/STAT signal transduction [48]. Indeed, herein induction of UC by AA was escorted by elevated colonic content of INF-γ, JAK1, STAT1, and IRF-1, whereas post-administration of GEN with or without SZ hindered this axis.

Administration of AA results in excessive release of the inflammatory cytokine INF-γ [49]. The overly produced INF-γ results in turning on the JAK1/STAT1 trajectory [50], whereas binding of the cytokine to its specific cell surface receptor (INF-γ receptor), the receptor dimerizes and causes conformational changes in JAK1 leading to its dimerization and activation [51,52]. Subsequently, the activated JAK1 recruits and activates STAT1 leading to its nuclear translocation and induction of the inflammatory cytokines gene expression [12]. Translocation of STAT1 in the nucleus is accompanied by activation of the transcription of several genes, including IRF-1[53].

Former studies proved that GEN significantly perturbed the secretion of IFN-γ in a model of UC [54], JAK1 in an esophageal carcinoma cell proliferation model [55], STAT1 in a stomatitis model [56], and IRF-1 in lipopolysaccharide-activated microglia model [57]. Moreover, the protective effect of SZ was demonstrated in previous studies that showed its ability to reduce the levels of IFN-γ [58], JAK1 in AA-induced UC model [59], and STAT1, and IRF-1 levels in murine macrophages [53]. Interestingly, the combination of both drugs showed a significant reduction in INF-γ, JAK1, STAT1, and IRF-1 levels rather than each drug individually.

Nitric oxide is an essential intracellular and intercellular signaling molecule acting as an endogenous messenger in most mammalian organs, contributing to vascular integrity, neurotransmission, and protection against infectious agents. Elevated concentration of NO is expressed in a variety of IBD and oxidative stress-induced gut tissue damage [60]. Its production is provoked by iNOS, whose expression may account for the characteristic vasodilatation, edema, and impairment of

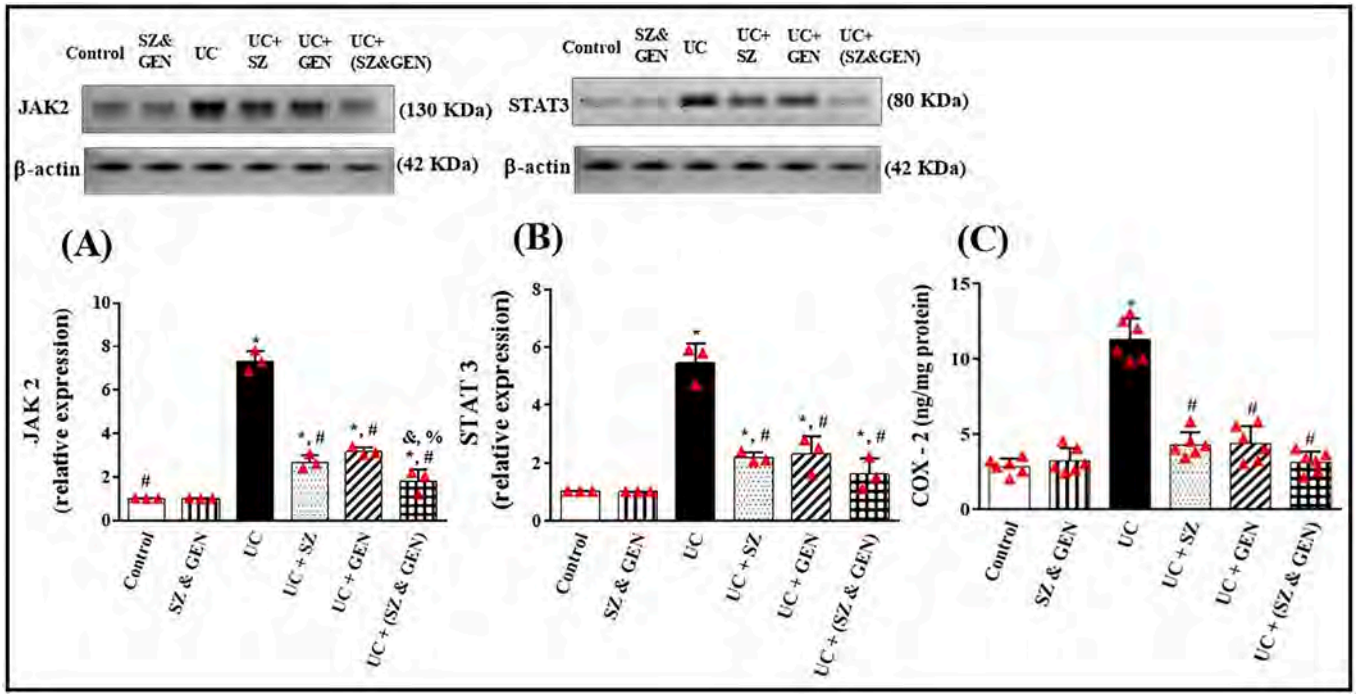

Fig. 8. Effect of GEN and/or SZ on (A) JAK2, (B) STAT3, and (C) COX-2 in UC rats. Rats were subjected to one colonic instillation of AA and left for 24 h to induce colitis, then treated orally with SZ (100 mg/kg), GEN (100 mg/kg), and (SZ + GEN) with previously mentioned doses for 14 days. Data are presented as mean ± SD (JAK2 and STAT3 n = 3 per group "Western blotting assessment"; COX-2n = 6 per group "ELISA technique"; one-way ANOVA followed by Tukey's post hoc test); $P < 0.05$, as compared to (*) Control group, (#) UC group, (&) SZ treated group, (%) GEN treated group. GEN: genistein; SZ: sulfasalazine; JAK2: Janus kinase 2; STAT3: signal transducers and activators of transcription 3; COX-2: cyclooxygenase 2; UC: ulcerative colitis.

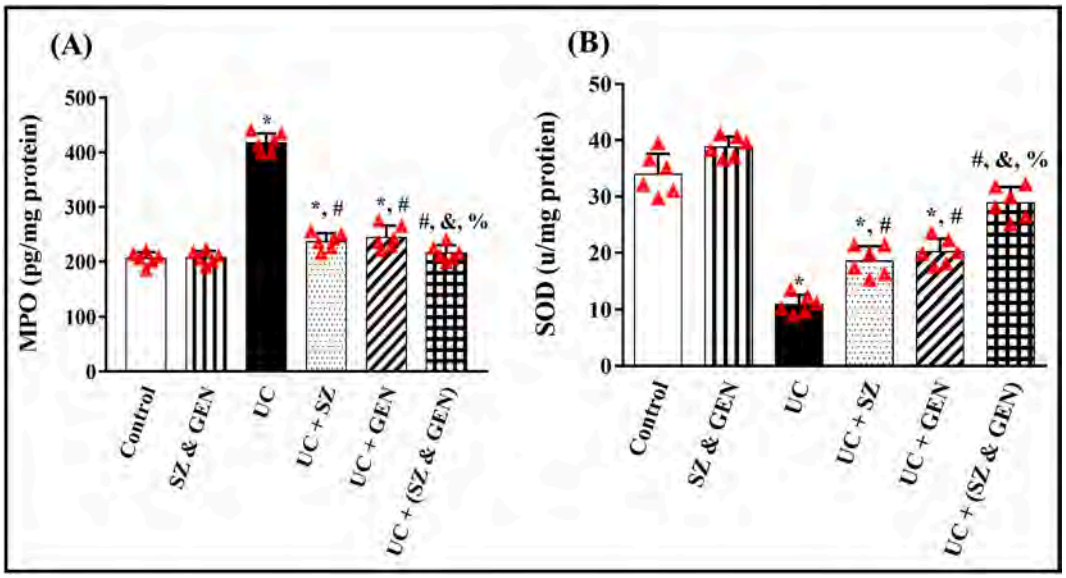

Fig. 9. Effect of GEN and/or SZ on (A) MPO and (B) SOD in UC rats. Rats were subjected to one colonic instillation of AA and left for 24 h to induce colitis, then treated orally with SZ (100 mg/kg), GEN (100 mg/kg), and (SZ + GEN) with previously mentioned doses for 14 days. Data are presented as mean ± SD (n = 6 per group; one-way ANOVA followed by Tukey's post hoc test); $P < 0.05$, as compared to (*) Control group, (#) UC group, (&) SZ treated group, (%) GEN treated group. GEN: genistein; SZ: sulfasalazine; MPO: myeloperoxidase; SOD: superoxide dismutase; UC: ulcerative colitis.

gut motility observed in active UC [61]. Herein, AA rectal installation results in up-regulation in gene expression of iNOS as a consequence of activated IRF-1 transcription [62], with subsequent elevation in colonic NO, as described in Kannan et al. study that evidenced the colonic increase of iNOS gene expression and NO levels in AA-induced UC [63].

Treatment with GEN and SZ reduced iNOS immune expression, with a consequent decrease in NO content in colon tissue; however, their combination tended to normalize them. This ameliorative effect of GEN was shown in a previous study of neurodegenerative diseases [57]. Another study proved the ability of SZ to lessen the colonic immune expression of iNOS and levels of NO in the UC model [64].

Toll-like receptors are among the pattern recognition receptors (PRRs), the essential components of the innate immune system that stimulate multiple inflammatory cascades. TLRs have a crucial role in

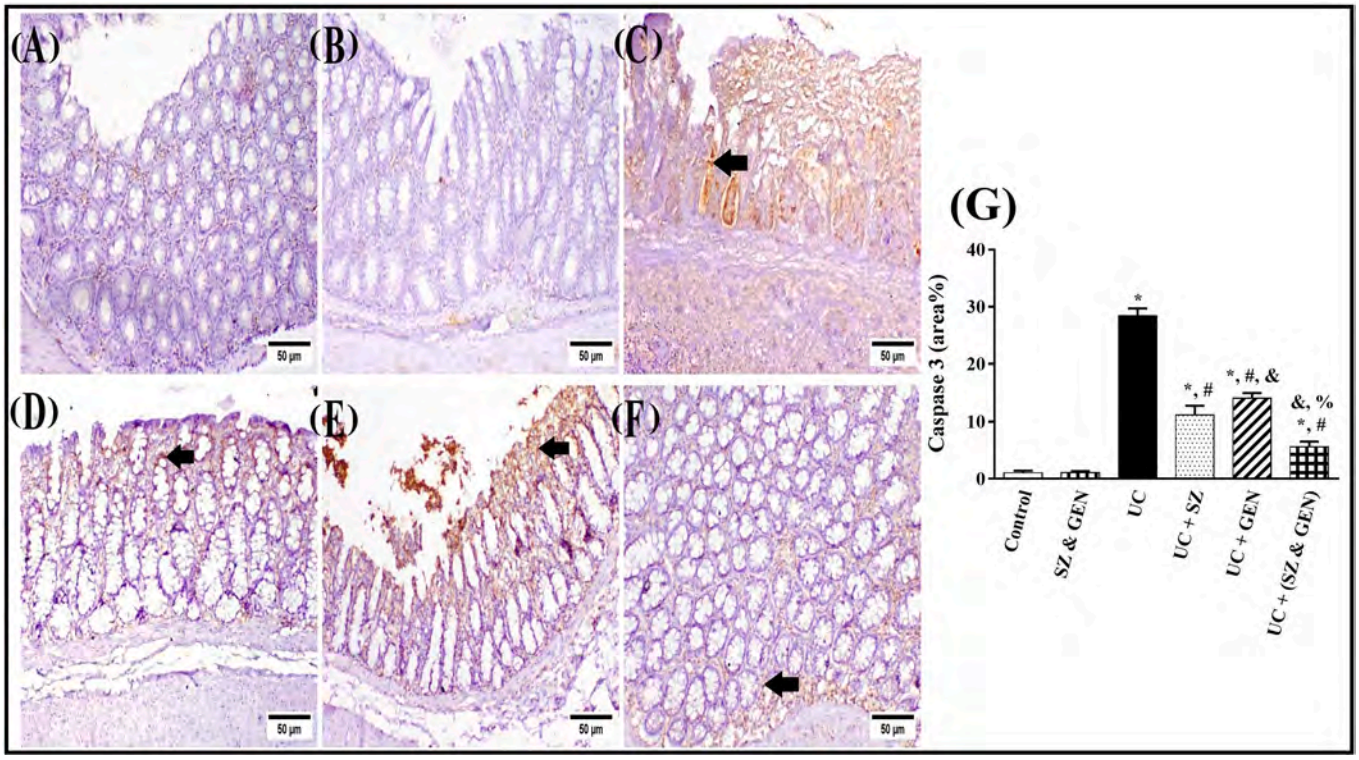

Fig. 10. Effect of GEN and/or SZ on colonic caspase-3 immunoreactivity in UC rats. Representative photomicrographs of caspase-3 immune stained colon sections (scale bar, 50 μm); The normal control group (A) and SZ + GEN combination group (B) showed no immune expression. UC group model (C) showed a significant increase in positive immunostaining cells. SZ-treated group (D) showed a reduced number of brown staining positive cells. GEN-treated group (E) showed moderate expression of caspase 3. SZ + GEN combination-treated group (F) showed weak immune expression (arrows). (Panel G) presents the % area of caspase-3 staining (5 fields in each section). Data are presented as mean ± SD (n = 3 per group; one-way ANOVA followed by Tukey's post hoc test); $P < 0.05$, as compared to (*) Control group, (#) UC group, (&) SZ treated group, (%) GEN treated group. GEN: genistein; SZ: sulfasalazine; UC: ulcerative colitis.

the pathogenesis of UC in colonic mucosa and TLR-4 is expressed in many cells, including enterocytes [65].

As reported in Wang et al. study, the administration of AA elicited amplified inflammatory responses in the colon, evidenced by the upregulation in the colonic levels of TLR-4 and NF-κB [66]. Disturbances in the TLR4 expression are implied to be one of the potential mechanisms responsible for the disease progression [67]. Pivotal cytokine IFN-γ has a critical role in activating and stimulating TLR-4, and in response to TLR-4 activation, the intracellular adaptor proteins are enlisted to activate downstream pathways [68]. Expression of TLR4 is linked by the first and most critical TLR adaptor protein called the helical 'Myddosome' complex for myeloid differentiation primary response protein 88 (MyD88). Subsequently, the downstream NF-κB is activated, further triggering the synthesis and release of pro-inflammatory cytokines [69]. Moreover, IRF-1 is required to enhance NF-κB transcriptional activity by forming a functional complex in long terminal repeat (LTR) κB sites [70].

Administration of GEN with or without SZ effectively abolished these inflammatory signals, as shown by the marked reduction in the colonic contents of TLR-4 and NF-κB. As reported in Yu et al. study, GEN-induced suppression in the activation of the TLR-4/NF-κB signaling pathway [71]. whilst the beneficial effect of SZ in diminishing the levels of TLR-4 and NF-κB was previously reported in the UC model [72]. Notably, the combined treatment of both SZ and GEN markedly attenuated the TLR-4/NF-κB signaling pathway compared to individual treatments.

NF-κB, a central mediator of immune and inflammatory responses, its activation and over-expression have been implicated in UC by increasing the synthesis of inflammatory mediators in inflamed mucosa [73]. It translocates into the nucleus and, in sequence, activates numerous cytokines, such as TNF-α, IL-1β, and IL-6 [74].

By mutual approval with El-Akabawy et al. study, AA-induced colitis showed a marked elevation in the colonic content of TNF-α, IL-1β, and IL-6 [9]. The beneficial effect of GEN with or without SZ has been demonstrated by normalizing the TNF-α, IL-1β, and IL-6 levels in the colon, the results in agreement with a former study that proved the ability of GEN to reduce these pro-inflammatory cytokines levels in murine intestinal epithelial cells [75]. Another study granted the SZ effect in normalizing their content in the UC model [57].

IL-6, a pleiotropic cytokine which considered a regulation wheel in various inflammatory pathways, mediates several trajectories reported to play a fundamental role in the development of IBD [76]. The excessive release of IL-6 in the inflamed mucosa activates the JAK2/STAT3 trajectory after its combination with its specific cell surface receptor, leading to receptor dimerization and subsequent conformational changes in JAK2, leading to its activation. Afterward, the activated JAK2 acts as an upstream activator of STAT3, leading to its nuclear translocation. The activated STAT3 modulates the downstream target gene transcription and regulates inflammation in the colonic tissues. The IL-6-induced JAK2/STAT3 trajectory plays a significant role in the pathogenesis of IBD [77] as the activated STAT3 over-expression is accompanied by a more severe disease state and subsequently increases the transcription of COX-2 in inflamed tissues [78]. COX-2 is considered a crucial inflammatory mediator in the prognosis of UC [79]. In line with this, a marked elevation in JAK2 and STAT3 relative expression in colonic mucosa was observed in our study in agreement with El-Ghannam et al. previous study [80], with a subsequent increase in COX-2 as demonstrated in another former study [9].

Based on the observed effects of GEN and/or SZ on the JAK/STAT signaling pathway we propose that the amelioration noted is mediated through JAK2/STAT3/COX-2 trajectory in the colonic mucosa. The beneficial effect of GEN was proved in former studies that explained its role in suppressing the IL-6, JAK2, and STAT3 levels in a rheumatoid

arthritis model [21] and COX-2 level in the UC model [81]. Indeed, our study is the first to report the effect of GEN on this trajectory in an AA-induced UC model. The SZ-induced significant decrease in the IL-6 levels and JAK2 and STAT3 relative expression was described previously in Tao et al. study [77] and in the COX-2 content as found in Sakthivel et al. study [82].

Oxidative stress is one of the immune-regulatory factors and important mechanisms linked to the pathophysiology of IBD. Increased oxidative stress in the colonic mucosa and impaired antioxidant defense mechanisms were reported in individuals with UC [83]. MPO is one of the essential oxidative stress biomarkers, it increases and upregulates in response to UC, and its contribution to promoting and maintaining IBD is becoming well-recognized [84]. SOD is an anti-oxidative stress biomarker and activates many pathways to scavenge free radicals and regulates the incidence of oxidative stress in response to UC [85]. In the current study, the higher MPO levels and the decreased SOD activity in colon tissues provided further evidence of enhanced oxidative stress homeostasis in the AA-induced colitis model, these findings are previously reported in Fawzy et al. study [86].

Apoptosis and regulation of apoptotic biomarkers contribute to the incidence and development of IBD, and intestinal epithelial cells apoptosis has an essential role in the progression of the disease, thought to occur early in the development of UC [87]. Caspases are cysteine proteases that influence cell growth, cellular proliferation, tissue homeostasis, and elimination of damaged and destructed cells from the gut and other body tissues [88]. As reported earlier in Alsharif et al. study, there was an increase in caspase-3 expression and apoptosis in the colon tissues of the UC group [37].

Treatment with GEN and/or SZ defended against mucosal damage by lessening the MPO levels, reestablishing the SOD activity, and decreasing caspase-3 expression in the colon tissues. The antioxidant activity of GEN in lowering the colonic levels of MPO [89] and increasing the levels of SOD [90], in addition to its antiapoptotic activity [91] has been formerly shown. Consistent with our findings, Dina et al. formerly discussed the SZ protective effect against oxidative stress by restoring the MPO levels to normal and elevating the SOD levels in addition to its antiapoptotic activity in the colonic tissues [92]. Interestingly, the combination of SZ and GEN has an augmented and remarkable effect on oxidative stress and apoptotic markers than using either drug alone.

Our study is the first to document the relationship between INF-γ, JAK1, STAT1, and IRF-1 parameters, and to report the effect of GEN on the JAK2/STAT3/COX-2 trajectory, in an AA-induced UC model.

In conclusion, The present study elucidated the color-protective role of GEN and/or SZ against AA-induced UC in rats, evidenced by improvement in ulcer scores and histopathological architecture in the colon mucosa and modulation of the intersection between INF-γ/JAK1/STAT1 and INF-γ/TLR-4/NF-κB signaling pathways, in addition to modulation of the IRF-1/iNOS/NO hub and IL-6/JAK2/STAT3/COX-2 trajectory, with consequent reduction of the levels of pro-inflammatory cytokines TNF-α and IL-1β and elevation of GCs number, resulting in preserving cell integrity and colon function, and lessening the collagen fibers deposition. Additionally, this anti-colitis effect may be attributed to the inhibition of oxidative stress and apoptosis in the inflamed mucosa. Apart from the studied pathways, additional cascades should be evaluated to reveal other mechanisms by which the examined treatments can act.

Credit Author Statement

Nabila N. El Maraghy, Hala F. Zaki, Enas A. Abd El-Haleim, and Esraa A. Elhefnawy created the conception and design, Esraa A. Elhefnawy carried out the experimental part, Nabila N. El Maraghy and Enas A. Abd El-Haleim supervised the practical part, Enas A. Abd El-Haleim, and Esraa A. Elhefnawy helped in the acquisition of data, Enas A. Abd El-Haleim and Esraa A. Elhefnawy analyzed the results, Kawkab A. Ahmed analyzed and interpreted the histopathological and immunohistochemical data, Esraa A. Elhefnawy wrote the 1st draft of the article, Enas A. Abd El-Haleim revised the 1st draft of the article, Nabila N. El Maraghy and Hala F. Zaki comprised a major revision of the article and all authors participated in revising the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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