ORIGINAL ARTICLE

Cilostazol against 2,4,6-trinitrobenzene sulfonic acid-induced colitis: Effect on tight junction, inflammation, and apoptosis

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Abstract

Background: Inflammatory bowel diseases are immunologically mediated disorders of gastrointestinal tract, characterized by dysregulated immune responses that result in a chronic intestinal inflammation. The antiplatelet cilostazol (CS), a phosphodiesterase-III inhibitor, exerted a beneficial effect on several models of gastrointestinal diseases; however, the full mechanism of action in this context has not been unveiled.

Aim: The current study aimed to elucidate the potential role of CS in a 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model.

Methods: Male Wistar rats were divided into a sham group and groups treated with sulfasalazine (500 mg/kg), CS (50 and 100 mg/kg), and a combination (sulfasalazine/CS 50 mg/kg). All treatments were administered orally 15 days, with TNBS rectal administration on the 11th day.

Results: TNBS-produced colitis manifested as a decrease in the epithelial junctional adhesion molecule-A (JAM-A) and as an increase in trefoil factor-3, ulcerative area, and colon mass index, parameters that collaborate with the gross macroscopic changes in colon tissue. In addition, TNBS increased hemeoxygenase-1, nuclear factor-kappa-B, P-selectin, and myeloperoxidase, as well as the apoptotic ratio of Bax/Bcl-2. Administration of CS alone, especially at the high dose level, attenuated the severity of TNBS-induced colitis in a sulfasalazine-comparable manner. In addition, a better effect was mediated by the combination regimen, which succeeded in normalizing most of the measured parameters.

Conclusion: CS protected the colon against TNBS through its anti-inflammatory and antiapoptotic effects along with maintaining cellular tight junctions (TJs). Furthermore, CS can be beneficial as an add-on drug with the conventional treatments of colitis.

Introduction

Inflammatory bowel disease (IBD) is a multifactorial relapsing remitting disorder characterized by intermittent periods of acute inflammation in the small and large intestines,[1] of which Crohn's disease (CD) and ulcerative colitis (UC) are the two main categories. CD can occur in any part along the gastrointestinal tract[2] but usually affects the distal ileum and colon, whereas UC affects the colon only.[3]

Although the etiopathogenesis of IBD remains unsolved, the widely accepted hypothesis is that it involves a constellation of interacting factors, among which are genetic and environmental ones that trigger an inappropriate mucosal immune response.[4] Once a large number of activated neutrophils and macrophages infiltrate the injured intestinal mucosa, they overproduce oxygen-free radicals, causing damage to target cells in inflamed tissue.[5] In a vicious cycle pattern, the increased oxidative stress maintains active inflammation within the intestinal mucosa, which

subsequently activates nuclear factor-kappa B (NF-κB), as well as the overexpression of pro-inflammatory cytokines and adhesion molecules.[6] These events are associated with a disruption in the epithelial barrier function.[7] The latter has been reported to be regulated by one of the tight junction (TJ) proteins, epithelial junctional adhesion molecule-A (JAM-A),[8] which is altered in IBD patients with active disease as a molecular mechanism of dysregulated epithelial permeability.[9] Moreover, trefoil peptides play a key role in gastrointestinal epithelium repair by promoting epithelial restitution.[10] Trefoil factor (TFF)-3, abundant at the intestinal mucosal surface,[11] is involved in accelerating intestinal epithelial wound healing and maintaining the mucosal integrity.[12] Therefore, these acute-phase proteins are upregulated in response to injured gastrointestinal mucosa.[13]

The current pharmacological treatments of IBD are intended to ameliorate the secondary effects of the disease, as well as induce and maintain remission instead of reversing the

underlying pathogenic mechanism.[14] Although the efficacy of these drugs has been proven in IBD, they do not provide a cure or prevent long-term complications.[15]

Cilostazol (CS) is a well-known phosphodiesterase-III inhibitor (PDE-I), which, besides its antiplatelet effect, has various pharmacological effects, including anti-inflammatory, antioxidant, and antiapoptotic effects via the c-AMP-dependent and -independent pathways.[16] The anti-inflammatory effect of CS has been evidenced in several animal models of inflammation, such as aspirin-induced gastric mucosal inflammation,[17] as well as ischemia/reperfusion-induced hepatic[18] and renal[19] injury.

However, the potential alleviating effects of CS against a 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model has not been evaluated, which is the aim of the present study. The potential anticolitic effect of CS, and its possible beneficial role as an add-on therapy, against TNBS-induced colitis is compared with the standard drug sulfasalazine (SAZ).

Materials and methods

Animals. Male Wistar rats weighing 150–200 g were purchased from "The Modern Veterinary Office for Laboratory Animals" (Cairo, Egypt). Animals were housed at the animal facility of the Faculty of Pharmacy, Cairo University, for 1 week prior to experimentation in an ambient temperature of $22 \pm 2°C$. Rats were maintained on a standard pellet diet and given tap water *ad libitum*. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The study protocol was approved and carried out according to the guidelines of the Research Ethical Committee of the Faculty of Pharmacy (PT 1013), Cairo University, Cairo, Egypt. All efforts were made to minimize animal suffering.

Drugs used. TNBS was purchased from Sigma-Aldrich, (Sigma Chemical MO, USA), CS from Otsuka (Al Sharqia, Egypt), and SAZ from Alexandria Co. (Alexandria, Egypt). CS was suspended in 0.5% Tween 80, while SAZ was suspended in 1% methylcellulose.

Induction of colitis. TNBS colitis was induced according to Morris *et al.*[20] Briefly, animals were housed in mesh-bottom cages to prevent coprophagy, and food-fasted animals (36 h) were lightly anesthetized with ether inhalation. Afterward, a polypropylene tube, 2 mm in diameter and fitted onto a 1 mL syringe, was inserted rectally 8 cm proximal to anus. A total volume of 0.25 mL of TNBS solution in 50% ethanol solution (v/v), calculated as a dose of 50 mg/kg,[21] was introduced intrarectally. The catheter was left in place for 30 s and then gently removed. Rats were retained in the Trendlenburg position for 1 min to avoid anal leakage of the instillate. Animals in the normal control group were handled similarly, but they received normal saline instead.

Experimental design. Rats were randomly assigned to six experimental groups ($n = 8$). Group I (control group) received the vehicle (0.5% Tween 80) rectally, while in group II (TNBS control group), rats received the vehicle for 15 days, and TNBS

was introduced on day 11. Animals in groups III, IV, V, and VI received SAZ (500 mg/kg,[22]) as the reference drug, CS 50 mg/kg (CS_{50}), CS 100 mg/kg (CS_{100}),[19] and SAZ + CS_{50}, respectively. All treatment regimens were gavaged for 10 days before induction of colitis (day 11) and were continued for 4 days thereafter. On day 11, rats were weighed immediately before the administration of TNBS and on the 15th day just before sacrifice in order to assess the effect of TNBS on rat bodyweight (BW).

Blood/tissue collection and preparation. At the end of the experimental duration, animals were anesthetized, and blood samples withdrawn from vena cava and sera were separated and maintained at $-20°C$ until the time of analysis. After blood collection, animals were euthanized by an overdose of thiopental, and a distal 8 cm of the rat colon was excised, opened longitudinally, and rinsed thoroughly with ice-cold normal saline. The distal colon was weighed to calculate colon mass index [CMI; colon weight (mg)/BW (g)]; the parameter reflects the severity of colonic inflammation.[23] After the assessment of the mucosal damage by measuring the ulcerative area (cm^2), the colonic segments were divided into two portions, and the first was homogenized in ice-cold physiological saline (20%) for the determination of myeloperoxidase (MPO) activity. The second portion was homogenized in a phosphate buffer (pH = 7.4) to prepare 10% homogenate for the estimation of hemeoxygenase (HO)-1, NF-κB, P-selectin, JAM-A, and TFF-3.

Determination of colonic MPO activity. Activity of MPO, a marker for neutrophil infiltration, was estimated according to the method described by Bradley *et al.*[24] The colon homogenates were subjected to three cycles of freezing/thawing, were suspended in potassium phosphate buffer (pH 6) with 0.5% (w/v) of hexadectyl-trimethylammonium bromide (HTAB), were sonicated for 30 s, and were then centrifuged at 10 000 rpm for 15 min at 4°C. The supernatant was mixed with o-dianisidine (0.167 mg/mL) and hydrogen peroxide (H_2O_2, 0.0005%) in the phosphate buffer, and the absorbance was measured at 460 nm for 3 min, at 1-min intervals. MPO activity was expressed in U/g, where 1 unit corresponds to the activity required to degrade 1 μmol of H_2O_2 to water in 1 min at 25°C.

Determination of colonic hemeoxygenase-1, NF-κB, P-selectin, JAM-A, and TFF-3. The colonic contents of hemeoxygenase (HO)-1 (Uscn Life Science Inc., Wuhan, China; Cat. No. E90584Ra), NF-κB (ElAabscience, Wuhan, PRC; cat # E-EL-R0674) and P-selectin (Wuhan EiAAB Science Co., Wuhan, China; Cat. No. E0115r), JAM-A (Cloud-Clone Corp., TX, USA; Cat. No. SEB782Hu), and TFF-3 (Kamiya Biomedical Co., WA, USA; Cat. No. KT-51682) were estimated using specific ELISA kits according to the manufacturer's instructions.

Determination of serum apoptosis regulated proteins. The ELISA technique was used to determine the serum level of B Cell Lymphoma-2 (Bcl-2; Cat. No. E0778r) and Bcl-2-associated X protein (Bax; Cat. No. E1343r) using rat-specific

ELISA kits (Wuhan EiAAB Science Co.). The assay was performed following the manufacturer's protocols.

Statistical analysis

Data are expressed as mean $\pm$ SEM ($n = 8$). Differences among groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. For all statistical tests, the level of significance was set at $P < 0.05$. Graphs were presented using a Graph Pad Prism program (V6, San Diego, CA, USA).

Results

Macroscopic inspection. Gross macroscopic examination (Fig. 1b) demonstrates that the colon of the TNBS untreated group was the most edematous and was the greatest damaged area among the tested groups compared to (a) the sham colon that shows an intact normal colon. However, the pretreated groups improved the TNBS-induced damage, with the (e) CS$_{100}$ exhibiting a comparable effect to that of (c) SAZ and (f) the combination regimen showing the best effect.

Effect of different treatments on BW, ulcerative area, and CMI. Intrarectal instillation of TNBS induced severe weight loss compared to the normal control group, an effect that was opposed by the oral administration of all treatments. Moreover, pretreatment with the combination of SAZ and CS$_{50}$ resulted in a further increase in BW compared to single treatments (Fig. 2a). Moreover, TNBS induced (b) colon ulceration of 3.8 cm^2 and increased (c) CMI by 74%, thus supporting the gross findings. All pretreatments protected against TNBS

ulcerative effect to different extents; the high dose of CS was comparable to that of SAZ, while the coadministration of the low dose with SAZ showed the best effect. Meanwhile, only the high dose and the combination regimen reduced CMI significantly.
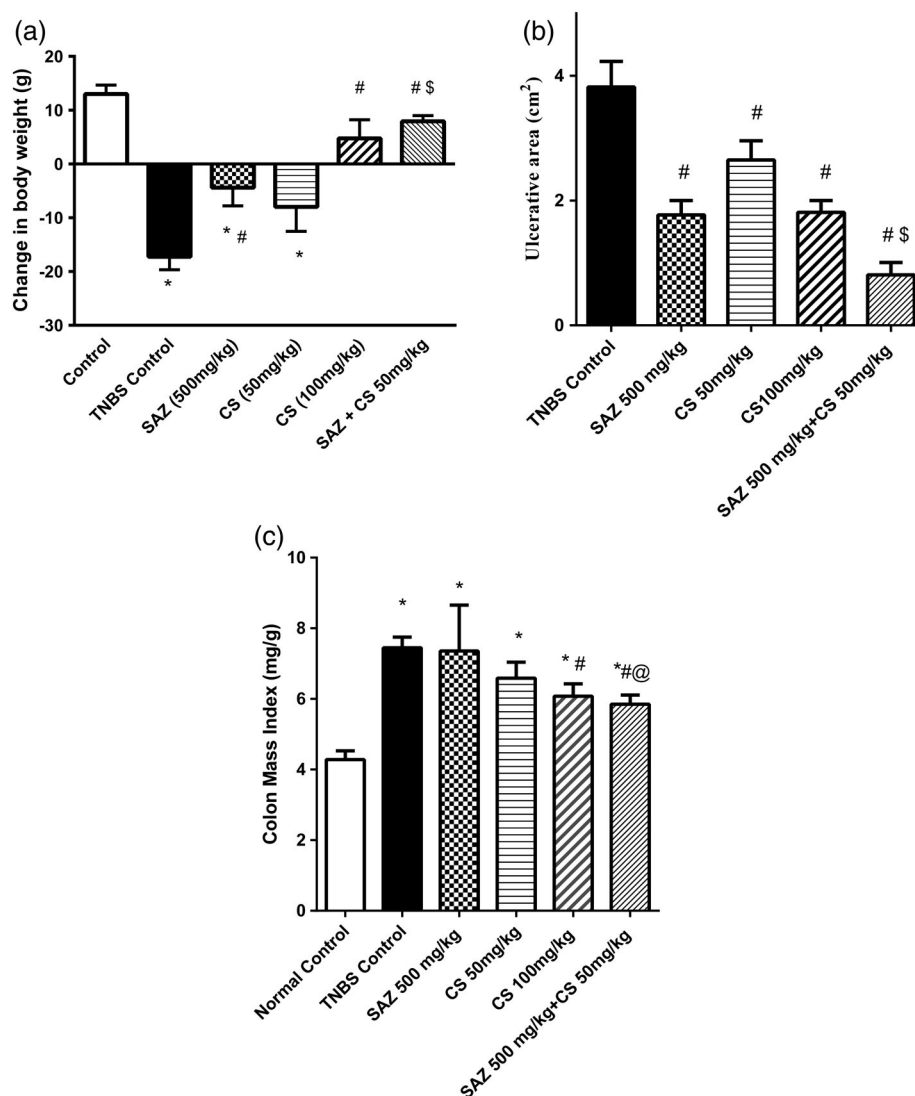
Effect of different treatments on colonic HO-1, NF-κB, P-selectin, and MPO. As depicted in Figure 3, TNBS administration caused 5.8- and 6.9-fold elevation of colonic (a) HO-1 and (b) NF-κB, respectively. In addition, the colitic agent increased neutrophil infiltration documented by the marked elevation of (c) P-selectin and (d) MPO compared to the normal control group. Pretreatment with the two doses of CS caused a dose-dependent decrease in all parameters, and the effects of CS$_{100}$ and SAZ were akin. However, CS50 succeeded in modulating these parameters to a level that was significant from SAZ alone.

Effect of different treatments on colonic barrier. Colonic administration of TNBS altered the colonic barrier (Fig. 4) as manifested by the sixfold increment in (a) colonic TFF-3 level, but with a depletion (b) of JAM-A (83%) compared to the normal control group. Pretreatment with SAZ, CS$_{50}$, and CS$_{100}$ opposed the TNBS effect and leveled off TFF-3 by 70, 36, and 58%, respectively. The positive effect also entailed JAM-A content, which was increased by 3.6, 2, and 2.8-fold, respectively, compared to the TNBS control group. The combination regimen, however, provided further protection against the TNBS injurious effect compared to the standalone parameters.

Effect of different treatments on apoptotic biomarkers. Figure 5 depicts the apoptotic effect of TNBS, displayed as a sharp decline (80%) in the serum level of the
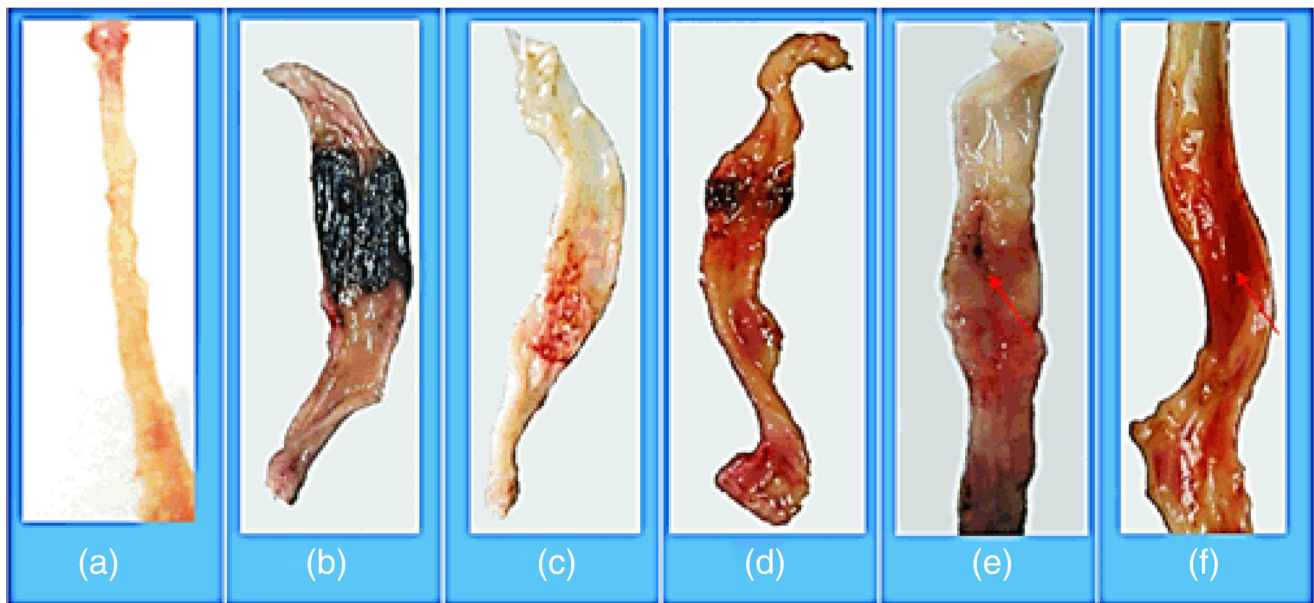
Figure 1 Changes in gross appearance of colon in different groups. (a) Section of normal control group shows a normal appearance of intact colon, while (b) trinitrobenzene sulfonic acid (TNBS)-induced colitis shows the largest area of colonic damage and edema. Preadministration of (c) sulfasalazine (500 mg/kg), (d) cilostazol (50 mg/kg), (e) cilostazol (100 mg/kg), and (f) the combination regimen reduced the ulcerative area and edema to different extents, with the combination-treated group showing the best effect.

Figure 2 Effect of different treatments on (a) bodyweight, (b) ulcerative area, and (c) colon mass index (CMI) in rats with trinitrobenzene sulfonic acid (TNBS)-induced colitis. Values are presented as mean (n = 8) ± SEM. Sulfasalazine (SAZ; 500 mg/kg), cilostazol (CS; 50 and 100 mg/kg), and their combination (CS 50 and SAZ) were gavaged for 15 days, and TNBS was administered on day 11. Compared with the control (∗), TNBS (#). SAZ (@), CS 50 ($), and CS 100 (‡), using one-way ANOVA followed by Tukey's multiple comparisons test, $P < 0.05$.

(a) antiapoptotic marker, Bcl-2, and a boost (7.1-fold) in (b) the pro-apoptotic one, Bax, compared to the normal control group. These effects were significantly hindered by preadministration of SAZ, CS_{50}, CS_{100}, and the combination therapy; these treatments elevated Bcl-2 by 3-, 1.67-, 2.78-, and 4-fold, respectively, and abated Bax by 52, 24, 46, and 67%, respectively, compared to the TNBS control group, indicating the prevalence of the combination treatment. Moreover, the (c) Bax/Bcl-2 ratio emphasizes the positive antiapoptotic effect of the used treatments.

Discussion

In our study, the phosphodiesterase inhibitor amended much of the macroscopic changes associated with the use of TNBS; it increased CMI and diminished TNBS-induced colonic ulceration,

necrosis, and severe colonic edema. These structural changes were provoked by the use of TNBS to concur with earlier reports.[25] The effect of CS, especially the high dose, showed an effect akin to the standard chosen drug, whereas the best effect was by the low dose tested when added to the standard drug used. CS has previously demonstrated its antiulcerogenic effect in a gastric ulcer model.[17] These improvements are, in part, responsible for the increased weight gain reported here.

Indeed, UC is the result of several intersecting factors, with the integrity of the epithelial barrier playing a crucial role; hence, preserving this element can be one mechanism of the anticolitic action of CS as proven by our work. This effect was evidenced by prohibiting the TNBS-induced depletion of JAM-A in a dose-dependent manner, a protein that was reported to decrease in IBD patients.[26] The TJ protein JAM-A regulates
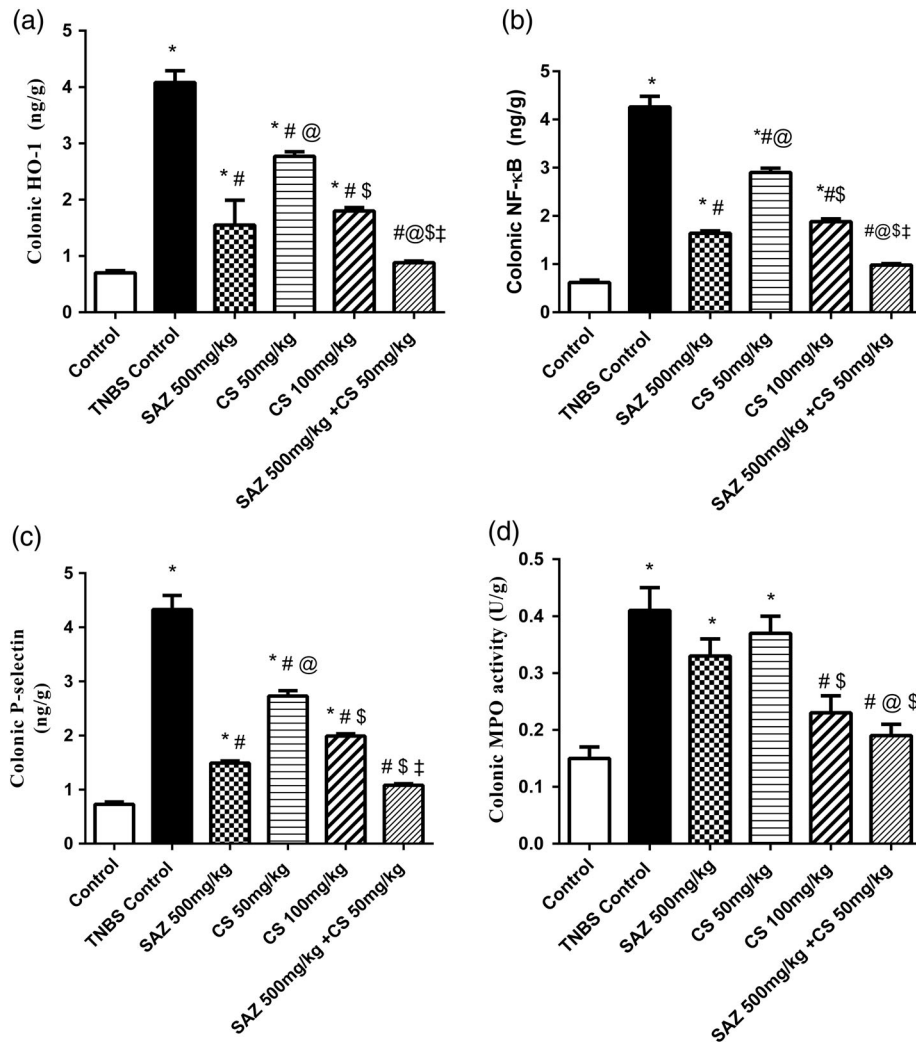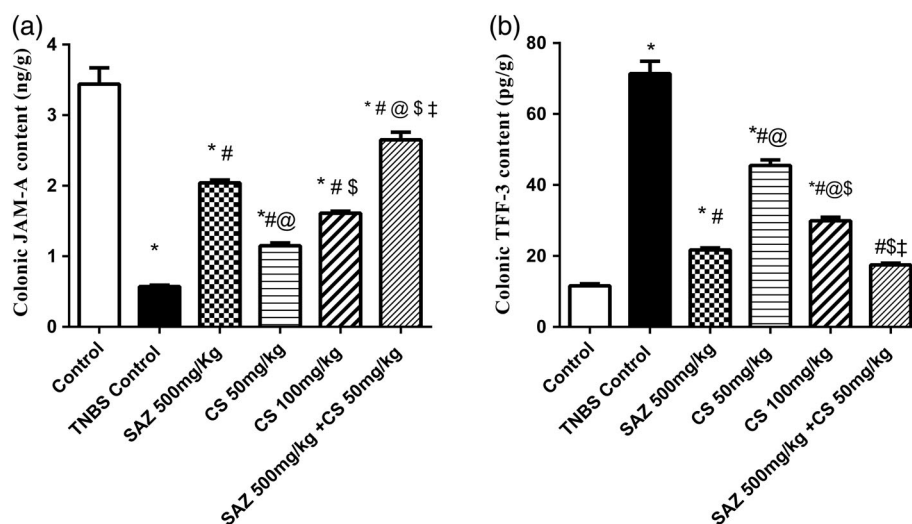
Figure 3 Effect of different treatments on colonic (a) hemeoxygenase (HO)-1, (b) nuclear factor kappa (NF-κ)B, (c) P-selectin, and (d) myeloperoxidase (MPO) in rats with trinitrobenzene sulfonic acid (TNBS)-induced colitis. Values are presented as mean (n = 8) ± SEM. Sulfasalazine (SAZ; 500 mg/kg), cilostazol (CS; 50 and 100 mg/kg), and their combination (CS 50 and SAZ) were gavaged for 15 days, and TNBS was administered on day 11. Compared with control (*), TNBS (#), SAZ (@), CS 50 ($), and CS 100 (‡) using one-way ANOVA followed by Tukey's multiple comparisons test, $P < 0.05$.

epithelial barrier function[7,8] and plays an important role in the healing of the epithelial wound[27] by regulating epithelial TJ assembly[28] and cell migration.[29] Being a phosphodiesterase inhibitor, CS mechanism could be related to increasing cAMP, which is implicated in the activation of protein kinase (PK)A to increase barrier function of intestinal epithelial cells.[30] cAMP-dependent PKA acts by phosphorylating claudin5 at Thr207 to enhance TJ functions.[31] The improved TJ can be one reason behind the aforementioned improvement in colon structure. In addition, the elevation of cAMP is accompanied by a decrease in inflammatory cytokines generation, thus protecting against their negative influence on the barrier junction.[32] This is partly reflected here by the CS-mediated inhibition of NF-κB, a known regulator of inflammatory cytokines. NF-κB has a regulatory role in TJ protein expression[33] and facilitates the TJ opening process.[8] Moreover, NF-κB inhibitors, which prevent tumor necrosis factor

(TNF)-α activation and nuclear translocation of NF-κB, defend against increased TJ permeability. Similarly, the standard drug used opposed the TNBS-induced JAM-A inhibition, an effect that is in line with Wang *et al.*[34] In the same context, a recent study by Kangawa *et al.*[35] documented the anticolitic effect of CS in a dextran-induced colitis model in mice depending on its anti-inflammatory effect.

Another epithelial element that was examined in the present study was the colonic level of TFF-3. This peptide is efficient in promoting epithelium healing after mucosal injury and triggers the protective barrier properties of the mucus layer to defend against enteropathogens.[36] In the present work, the peptide was increased following TNBS administration to mimic its response in patients with active UC and to correlate with disease activity indices.[37] Therefore, the increased amount of TFF-3 in colitic tissues may play an advantageous compensatory role in

Figure 4 Effect of different treatments on (a) colonic junctional adhesion molecule (JAM)-a and (b) trefoil factor (TFF)-3 in rats with trinitrobenzene sulfonic acid (TNBS)-induced colitis. Values are presented as mean (n = 8) ± SEM. Sulfasalazine (SAZ; 500 mg/kg), cilostazol (CS; 50 and 100 mg/kg), and their combination (CS 50 and SAZ) were gavaged for 15 days, and TNBS was administered on day 11. Compared with control (*), TNBS (#), SAZ (@), CS 50 ($), and CS 100 (‡) using one-way ANOVA followed by Tukey's multiple comparisons test, P < 0.05.

healing mucosal wounds and coping against TNBS-induced colonic damage[23] because, despite lacking a direct antibacterial effect, TFF-3 binds to bacteria to guard the host against infection.[36] Pretreatment with SAZ and CS, and especially their combination, reversed this elevation in an injured colon, indicating their ability to curtail the injurious effect of TNBS, which was associated with elevated levels of TFF-3.

NF-κB is considered a major transcription factor in the complex scenario of IBD that is known to be activated in IBD patients[38] and TNBS-induced colitis model,[39] facts that support the present findings. The activation of NF-κB induces the transcription of several molecules relevant to the pathogenesis of IBD, including factors involved in the inflammatory response and costimulatory molecules.[40] In fact, currently used IBD treatments, such as corticosteroids, SAZ, methotrexate, and anti-TNF-α antibodies, are known to mediate their therapeutic effect depending, in part, on mediating anti-inflammatory effects that, at least, depend on the inhibition of this transcription factor.[41] Likewise, CS in the present study inhibited colonic NF-κB content dose-dependently, an effect that stands for previous findings reporting its ability to inhibit NF-κB activation via cAMP-dependent[42] and -independent pathways.[43]

OS is implicated as a key factor in IBD pathogenesis in human beings and different experimental models.[44] TNBS is considered a source for the generation of ROS as it is metabolized in the colonocytes into the parent oxidants superoxide anion and hydrogen peroxide,[45] thus initiating and perpetuating colonic inflammation.[46] HO-1, which is a stress-response protein to several stimuli, including NF-κB[47] or inflammation and ROS, may be increased in the present colitic rats as a defense tactic against the TNBS inflammatory insult.[23,48] This result coincides with previous findings regarding the TNBS colitic model[23] and pins down the fact that HO-1 expression is upregulated in samples of human colitic tissue.[48] The redox-sensitive inducible protein HO-1 has been identified as being a cytoprotective gene because of

its antioxidant, anti-inflammatory, and antiapoptotic actions.[49] CS and SAZ antagonized the effect of TNBS and reduced the HO-1 to reach its normal level in the combination pretreated group. This inhibitory effect reported here may be attributed to the antioxidant characters of both CS[20] and SAZ,[50] which does not necessitate the elevation of the antioxidant/anti-inflammatory cytoprotective molecule HO-1.

Another important source of superoxide production is neutrophils, which infiltrate the damaged mucosa[51] using several adhesion molecules. This again was obvious in the current study where, along with the perturbed epithelial integrity, neutrophils can escape the blood stream into the tissue. The elevated MPO activity points to increased neutrophil infiltration as reported previously in a study.[52] This invasion is facilitated by adhesion molecules, such as P-selectin, which plays a key role in the migration of leukocytes by mediating their adhesion and 'rolling' on the endothelial cells during the inflammatory process.[53] In the present study, P-selectin was concomitantly elevated with MPO in the TNBS-treated rats and were both leveled off by CS, thus proving its anti-inflammatory effect. CS earlier inhibited MPO in different models of gastrointestinal tract (GIT) diseases[18,54] and abated monocytes conscription to inflamed intestinal microvessels after treatment with lipopolysaccharide,[54] which nominates it as a promising drug for the treatment of IBD. Again, the addition of CS to SAZ kept both parameters at their normal level, thus proving their ability to hinder the injurious effect of TNBS and supporting their effect on the epithelial barrier.

In parallel with a previous study,[48] intrarectal TNBS administration induced colon apoptosis as confirmed by elevation of the pro-apoptotic Bax, with concomitant reduction in the pro-survival Bcl-2 level. Previous reports have shown that CS can reduce apoptotic cell death by inhibiting the mitochondria-dependent apoptotic signaling pathway.[55] In the current study, CS alone or in combination with SAZ strongly reversed TNBS-induced apoptosis, thereby skewing the balance in favor of cell
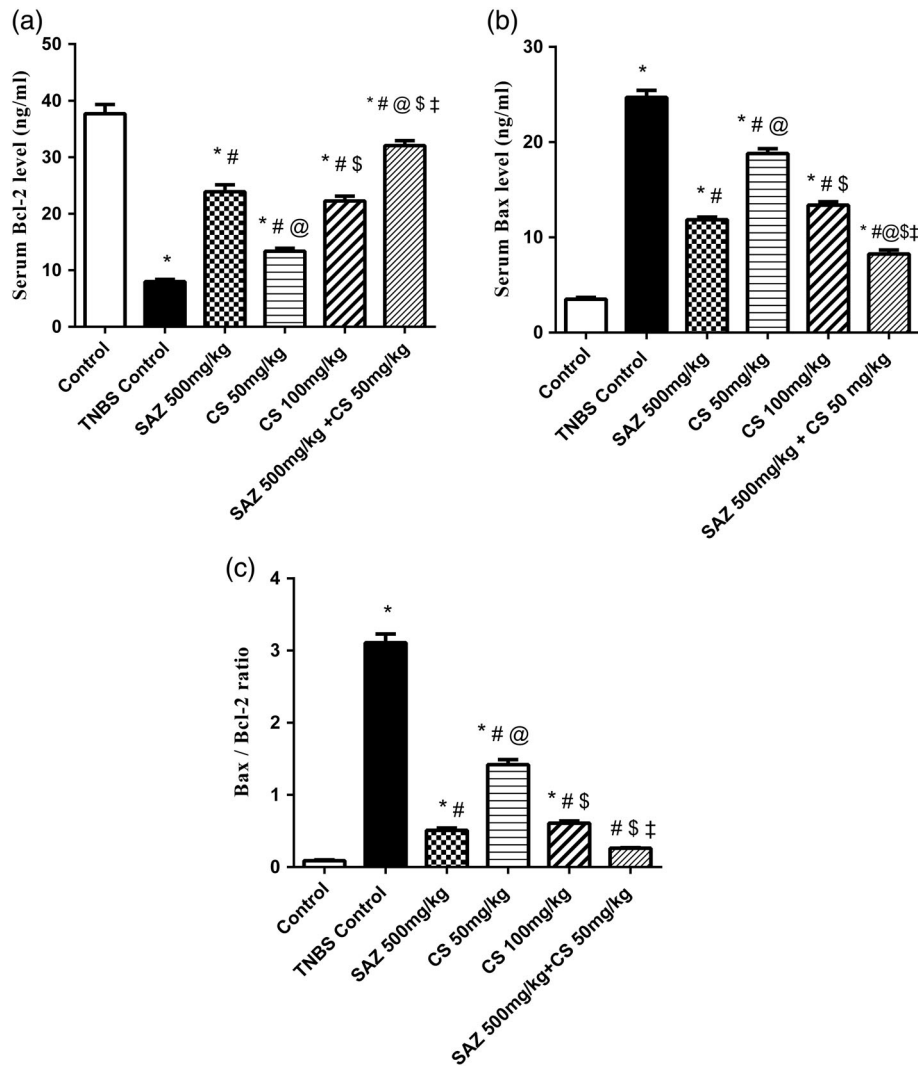
Figure 5 Effect of different treatments on serum level of (a) B cell lymphoma (Bcl)-2, (b) Bcl-2-associated X protein (Bax), and (c) Bax/Bcl-2 ratio in rats with trinitrobenzene sulfonic acid (TNBS)-induced colitis. Values are presented as mean ($n = 8$) ± SEM. Sulfasalazine (SAZ; 500 mg/kg), cilostazol (CS; 50 and 100 mg/kg), and their combination (CS 50 and SAZ) were gavaged for 15 days, and TNBS was administered on day 11. Compared with control (*), TNBS (#), SAZ (@), CS 50 ($), and CS 100 (‡) using one-way ANOVA followed by Tukey's multiple comparisons test, $P < 0.05$.

survival. This was further confirmed by the inhibition of the Bax/Bcl-2 ratio, which is well known to be a parameter of apoptotic cell death. SAZ was also found to reduce the apoptotic colonic epithelial cells in TNBS-induced colitis as was documented in earlier study.[56]

Finally, the current study documented the valuable effect of CS in the TNBS model of rat colitis. CS exerted a protective anticolitic effect by ameliorating the reflected mucosal barrier damage by opposing the TNBS effect on JAM-A and TFF-3 besides modulating NF-κB, P-selectin, HO-1, and apoptosis. Moreover, a combination of low doses of CS and SAZ resulted in better mucosal protection against inflammatory changes, indicating a possible beneficial effect of this combination. Therefore, these data represent a rationale for the use of the PDE-I CS in the management of IBD and describes it as a helpful add-on therapy.

Acknowledgments

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