Research Article

Possible protective effects of sulfasalazine on acetic acid-induced colitis in rats through its effect on oxidative stress and proinflammatory mediators

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Abstract
There is no clear data addressing the role of oxidative stress and proinflammatory mediators in acetic acid (AA)-induced colitis model. This study was aimed to study the effect of sulfasalazine (SLZ) on AA-induced colitis in rats. Rats were allocated into 3 groups: group 1: control, group 2: AA group (received 1 ml 4% acetic acid transrectaly single dose at 13th day), group 3: SLZ+AA group (recieved SLZ 250 mg/kg/day orally for 14 days and 1 ml 4% acetic acid transrectaly single dose at 13th day). Rats were sacrificed after 2 weeks. The colonic oxidative damage and inflammatory effects of AA were evaluated by measuring colonic levels of malonaldehyde (MDA), total antioxidant capacity (TAC), superoxide dismutase (SOD), histamine, interleukin-1β (IL-1β), interleukin-18 (IL-18) and histopathological assessment. Colitis induced by AA revealed statistically significant improvement in rats treated with SLZ compared with AA alone group. These results suggest that SLZ can protect against AA- induced colitis. SLZ effects rely, at least partially, on its antioxidant and anti-inflammatory effects.
Key words: acetic acid, colitis, sulfasalazine, oxidative stress, histamine, IL-1β, IL-18.

Introduction
Immune-mediated inflammatory diseases (IMIDs) are systemic diseases of complex and multi-factorial etiology. The most prevalent IMIDs include inflammatory bowel disease (IBD) (Agrawal et al., 2019). IBD is a group of chronic auto-inflammatory intestinal diseases which is divided into two major distinctive entities as ulcerative colitis (UC) and crohn's disease (CD) (Yanna et al., 2014). Several immunological, environmental, and genetic factors are believed to be involved in the etiology of IBD (Katz et al., 1999).

Ulcerative colitis is a chronic relapsing disease, with the greatest reported incidence in mainland Europe and Scandinavia of 9.2 to 20.3 per 100,000 people, totalling approximately 2.2 million sufferers in Europe alone (Kaur et al., 2020). Inflammation and oxidative stress are thought to play key roles in the pathophysiological process of UC (Wang et al., 2019). Free oxygen radicals are considered to be a causal factor for IBD as oxygen radicles result in mucosal injury pathogenesis and initiation of apoptosis. Therefore, the majority of studies have focused on substances with anti-oxidant, antiapoptotic and anti-inflammatory properties (Cagin et al., 2016).

Ulcerative colitis experimentally induced by intra-rectal administration of low concentration of AA. This a well-known model for the study of IBD (Aleisa et al., 2014). Though AA-induced ulcerative colitis and human IBD may differ in aetiology, the two diseases share common pathophysiological features as well as sensitivity to drug treatment. For instances, colonic changes such as mucosal inflammation, ulceration, hemorrhage, and weight loss, which occur following intrarectal administration of AA in rats are also common in human IBD (Hartmann et al., 2012).

Sulfasalazine, the oldest medication in this class, consists of 5-ASA bonded to sulfapyridine. Sulfasalazine is converted to the sulfapyridine and 5-ASA moieties by colonic bacteria. The 5-ASA moiety is thought to be the

active compound for treatment of UC, while sulfapyridine is thought to contribute to adverse effects. The exact mechanism of sulfasalazine is not fully understood. Furthermore, it is not known whether sulfasalazine or its metabolites such as sulfapyridine and 5-aminosalicylic acid are responsible for its anti-inflammatory effects (LU, and Zhao, 2020). Topical and oral 5-ASA compounds have remained the backbone of therapeutic management in mild-to-moderate UC patients, either for induction or main-tenance therapy. Conversely, aminosali-cylates are not recommended in patients with moderate-to-severe UC, in whom systemic corticosteroids are the first-line induction treatment (Iacucci et al., 2010).

Materials and Methods

Drugs, chemicals, and kits

Acetic acid (El-Nasr Pharmaceutical Co., Egypt), Histamine kit (Enzo Life Sciences, Switzerland), Interleukin-1β (IL-1β) kit (Elabscience, USA), Interleukin-18 (IL-18) kit (abcam, UK), MDA kit (Spectrum Diagnostic, Egypt), Saline 0.9% (El-Nasr Pharmaceutical Co., Egypt), Sulphasalazine (Acdima Co, Egypt), Total antioxidant kit (Biodiagnostic, Egypt).

Animals

The present study was conducted on adult male albino rats weighing 180–225 g. They were obtained from the National Research Centre, Giza, Egypt. They were housed in laboratory cages with free access to water. They were fed a standard diet of commercial rat chow and left to accommodate to the environment for one week before the start of the experiments.

Experimental protocol

Rats were weighed and randomly divided into five groups (n=6-8). Group 1: Control group: received 1ml/rat distilled water as a vehicle orally for 14 days and saline intra-rectally at the 13th day of the experiment; group 2: AA model group: received 1ml/rat distilled water as a vehicle orally for 14 days with induction of UC by AA on the 13th day. Group 3: SLZ+AA group: received SLZ 250 mg/kg orally for 14 days, dissolved in distilled water, and AA on the 13th day.

The above mentioned doses of AA and SLZ were selected on the basis of our preliminary studies, as well as previously published results (Millar et al., 1996; Araujo et al., 2016), respectively.

Induction of colitis

Colitis was induced on the 13th day of the experiment using AA via a method that was previously described by Millar et al., 1996 (Millar et al., 1996). Animals fasted for 16 h with free access to water then rats were anesthetized by ketamine (50mg/kg) and xylazine (10mg/kg) (Mustafa et al., 2006). Each rat was infused with a single intra-rectal dose of AA as 1 ml (4%, v/v, in 0.9 % saline) using a polyethylene tube (2 mm in diameter). The tube was inserted through the rectum into the colon to a distance of 6 cm. The AA was retained in the colon for 30 s after which the fluid was withdrawn and animals' heads were kept in a downward position for another 30s then returned to cages.

Samples collection and preparation

At the end of the experimental period (14 days), the animals were scarified. Colon specimens were collected.

For each rat, the colon was obtained and washed. Then, one part from this excised colon was kept in 10% formalin and embedded in paraffin for histopathological evaluation. The remaining parts of colon were homogenized in approximately 1:5 wt/volumes of ice-cold phosphate buffer (prepared by dissolving 8.01g NaCl, 0.20g KCl, 1.78g $Na_2HPO_4.2H_2O$ and 0.27g KH_2PO_4 in 1 liter of distilled water and pH was adjusted at 7.4) using a polytron homogenizer (Tri-R Stir-R homogenizer, Tri-R Instruments, Inc., Rockville Centre, NY). The homogenate was centrifuged at 5000 rpm for 15 min and then the supernatant was divided in aliquots. Aliquots were prepared and stored at -80oC until estimation of MDA.

Assessment of the biochemical parameters

Determination of colonic oxidative stress parameters

Determination of lipid peroxides in the form of malonaldehyde (MDA) in the colon:

The MDA is a reactive aldehyde that is a measure of lipid peroxidation. Colonic contents of MDA were determined using the thiobar-bituric acid method described by Mihara and Uchiyama method (1978).

Determination of total antioxidant capacity (TAC) in the colon:
TAC was measured colorimetrically using commercial kit according to the manufacturer's instructions (Biodiagnostic, Egypt).

Determination of superoxide dismutase (SOD) in the colon:
Superoxide dismutase activity was evaluated in tissue homogenate chemically as previously described by Marklund (Markuland and Markuland, 1974). SOD activity in tissue homogenate was detected by spectrophotometry at 420 nm.

Determination of colonic proinflammatory mediators

Determination of histamine level in the colon:
Histamine level in colonic tissue homogenate was assessed by using its ELISA kit (catalogue numbers ENZ-KIT140) according to manufacturer's instructions.

Determination of interleukin-1β (IL-1β) and interleukin-18 (IL-18) level in the colon:
IL-1β and IL-18 levels in colonic tissue homogenate were assessed by using enzyme-linked immunosorbent assay (ELISA) kits (Catalog # E-EL-R0012 and ab213909 respectively) according to the manufacturer's instructions.

Statistical Analysis of the data
Results were expressed as means±SEM. Results were analyzed by one-way ANOVA followed by Tukey's test. Differences with p value < 0.05 were considered significant. Graph Pad Prism was used for statistical analysis (version 5.01 for Windows, Graphpad Software, San Diego California USA; www.graphpad.com).

Histopathological study
Colons were made as Swiss rolls for the histological analysis as previously prescribed (Whittem et al., 2010). Swiss-rolled colonic specimens were fixed in 10 % neutral-buffered formalin, dehydrated in a graded alcohol series, and cleared with xylene then embedded in paraffin wax. Then, sections were cut into 5µm-thick. Then, sections were subjected to either H&E stain for studying the general histological structure (Bancroft at al., 2013).

Results

Assessment of the biochemical parameters

Determination of oxidative stress parameters

Effect of SLZ on colonic MDA, TAC and SOD levels in AA-colitis model in rats:
In the current study, colonic MDA was significantly increased in AA group compared to control. Treatment with SLZ significantly attenuated the effect of AA on colonic MDA in comparison to AA group. On the other hand, colonic TAC and SOD levels were significantly decreased in AA group compared to control. Treatment with SLZ significantly attenuated the effect of AA on colonic TAC and SOD in comparison to AA group (Table 1).

Determination of colonic proinflammatory mediators

Effect of SLZ on colonic histamine level in AA-colitis model in rats:
AA group showed a significant increase in colonic histamine level as compared to control group. SLZ caused a significant decrease in colonic histamine level when compared to AA group (table 2).

Effect of SLZ on colonic IL-1β and IL-18 levels in AA-colitis model in rats:
Rats treated with AA showed a significant increase in colonic IL-1β, and IL-18 levels when compared to the control group. On the other hand, pretreatment with SLZ caused a significant decrease in colonic IL-1β, and IL-18 levels in comparison to the AA group (table 2).

Histopathological assessment

Effect of SLZ on colonic histopathology in AA-colitis model in rats:
Sections of the control group stained with H&E showed normal colonic histology formed of mucosa, submucosa, muscularis externa, and serosa. The mucosa was intact, continuous, and formed of closely packed simple tubular glands (crypts of Lieberkuhn). They were seen extended down to the muscularis mucosa. The goblet cells (most conspicuous cell type) and columnar absorptive cells were seen lined the crypts. The basal parts of the crypts were seen lined by columnar cells with basal oval vesicular nuclei (Figure 1a).
Regarding, AA group, it showed focal necrotic mucosa, loss of mucosal architecture, loss of

surface columnar epithelial lining, and diffuse cryptal distortion. In most sections, the crypts were seen lined by flat epithelial cells with less numerous goblet cell lining. Additionally, intraluminal cellular debris was frequently noticed. Furthermore, their lumina propria contained numerous inflammatory cells among the sections (Figure 1b).

The SLZ+AA group exhibited surface discontinuity in certain areas but the epithelial lining started to appear, the crypts appeared with wide lumen and were widely separated (Figure 1c).

Table (1): Effect of sulfasalazine (SLZ) on colonic level of MDA, TAC and SOD in acetic acid (AA)-induced colitis in rats

Groups	Colonic MDA (nmol/g tissue)	Serum TAC (mM/L)	Colonic SOD (U/mg tissue)
Control	5.566±0.53	2.648±0.06	7.900±0.93
AA	17.42±0.50[a]	1.688±0.08[a]	2.120±0.45[a]
SLZ+AA	10.09±0.73[b]	2.883±0.13[b]	6.220±0.47[b]

Results represent the mean ± S.E.M (n= 6-8). [a] Significant difference from control group, [b] significant difference from AA group ($P < 0.05$). AA; Acetic acid, SLZ; sulfasalazine, TAC: total antioxidant capacity, SOD: superoxide dismutase, MDA: malonaldehyde.

Table (2): Effect of sulfasalazine (SLZ) on colonic level of histamine, IL-1 β and IL-18 in acetic acid (AA)-induced colitis in rats

Groups	Histamine (ng/ mg tissue)	IL-1β (pg/ml)	IL-18 (pg/mg tissue)
Control	6.03±0.44	19.98±0.73	105.5±6.33
AA	18.80±0.77[a]	55.25±2.82[a]	340.8±15.19[a]
SLZ+AA	5.77±0.15[b]	23.27±0.53[b]	115.2±5.57[b]

Results represent the mean ± S.E.M. (n=6-8). [a] Significant difference from control group, [b] significant difference from AA group ($P < 0.05$). Interleukin 1β; IL-1β, IL-18; interleukin 18, AA; Acetic acid, SLZ; sulfasalazine.

Figure (1): Effect of SLZ on colonic histopathology in AA-colitis model in rats:

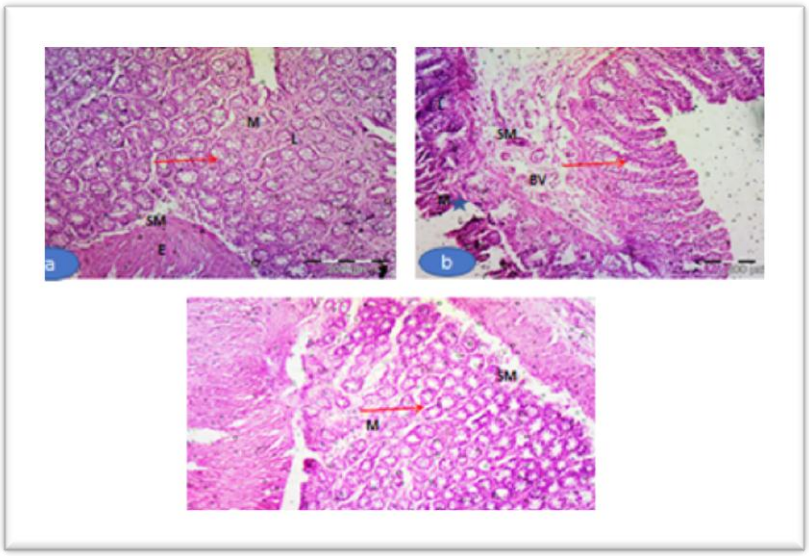

Figure (1): Effect of SLZ on colonic histopathology in AA-colitis model in rats:

a) Control group showing its different layers, the mucosa (M), submucosa (SB), and the muscularis externa (E). Crypts of Lieberkuhn (L) are seen extending down to the muscularis mucosa.
b) AA group showing areas of ulceration (star) and areas of diffuse cryptal distorsion (L) Notice the submucosal dilated blood vessels are seen (BV).
c) SLZ group showing apparent normal histological appearance but still some distortion is seen. H&E, × 400

Discussion

UC is one of the common prevalent inflammatory bowel diseases (IBD) distressing the quality of life (Gajendran et al., 2019). Even with several genetic and immunological factors involved in the pathogenesis of UC, the exact etiology still under investigation. Various inflammatory mediators and reactive oxygen species are attributed to the generation of UC (Arafa et al., 2020, Oliveira et al., 2021).

Acetic acid (AA)-induced UC is a well-studied, and easily used experimental model (Rashidian et al., 2009). This model is associated with inflammatory (Wu et al., 2020) and oxidative reactions that mimic the pathogenesis of human IBD (Esiringü et al., 2016). Therefore, AA-induced colitis may be a suitable model for evaluating agents with possible anti-inflammatory and antioxidant action.

The current study evaluated the effects of SLZ on AA-induced colitis in rats. The design of this study offers the advantage that it allowed us to record the evidences of colonic oxidative stress in the form of elevation of MDA, decrease of TAC and SOD in addition to record the evidence of inflammatory colonic damage in the form elevation of proinflammatory mediators as histamine, IL-1β and IL-18 in addition to histopathological changes.

Oxidative stress is an indicator of the damage that results from a change in the balance between oxidants and antioxidants in favor of oxidants. If the delicate balance between oxidants and anti-oxidants cannot be maintained in tissues, many pathological changes extending to cellular damage occur (Mukherjee et al., 2013). MDA is a byproduct of lipid peroxidation occurring in the tissue. In ulcerative colitis, levels of MDA in the plasma increases significantly and this is used as important diagnoses of patients with inflammatory bowel disease (Ali et al., 2017). The sum of endogenous and food-derived antioxidants represents the total antioxidant (TAO) activity. In healthy rats, SOD plays important role as a protective antioxidant enzyme. In UC, levels of this enzyme in colonic tissues become

exhausted as a consequence of oxidative damage caused by free radicals. SOD protects the cells against ulcerative damage by mediating dismutation of superoxide anion and preventing lipid peroxidation. SOD also prevents leukocyte rolling and adhesion in colonic tissues (Baldo, and Serrano, 2017).

Our data showed that AA-induced colitis was manifested by significant elevation in the level of MDA. Motawea et al., (2020) reported that AA increased MDA through its injurious colonic effect. The current study showed that administration of SLZ in AA group improved the colonic damage, as evident by significant reduction in colonic level of MDA and normalization of TAC and SOD levels as compared to AA group. Our data are in good agreement with the previously reported study of Liu et al., (2020) who found that SLZ attenuated AA-induced colitis in rats via inhibition of oxidative stress.

Histamine, the main mast cell mediator, known to increase vascular permeability, smooth muscle contraction, and leucocyte infilteration, has been suggested to be a contributing factor in intestinal inflammation (Nosál'ová et al., 1999). In the current study, AA administration caused increase in histamine level in colonic tissue and this is supported previously by Nosál'ová and his associates (1999) who studied the effects of H1 antagonist, dithiaden, on AA-induced colitis in rats. Additionally, administration of sulfasalazine inhibited histamine release and this is consistant with previous study on the use of sulfasalazine in treatment of mild and moderate IBD underlying on its histamine release inhibitory effect (Peh et al., 2007).

It was previously demonstrated that inflammatory mediators specifically, NF-κB and IL-1β are considered the critical mediators of the pathogenesis of colonic inflammation induced by AA in numerous studies (Chamanara et al., 2019). IL-18 is generally a pro-inflammatory mediator, and its production may be a key etiological factor for patients with IBD (Mukherjee et al., 2020). In this study, intrarectal administration of AA resulted in significant elevation in colonic IL-1β and IL-18 levels indicating severe inflammation and mucosal damage, this is previously reported by Serrya et al., (2021). In the current study, SLZ significantly normalized colonic IL-18 level. This is in line with a study reported that sulfasalazine treatment modulates the expression of mRNA IL-18 and decreased IL-1β and IL-18 production in HIV patients (Feria-Garzón et al., 2019).

In the present study, intra-rectal administration of AA caused a significant histopathological changes in the form of colonic thickening, hyperemia, goblet cell hyperplasia, and inflammatory infiltrations. Similar findings were reported by other investigators (Ahmed et al., 2018, Ercan et al., 2020) that confirm the current pictures. The current study successfully revealed a significant improvement in the histopathological images in groups pretreated with SLZ.

Taken together, the present study concluded that oxidative stress may largely participate in the mechanism of pathogenesis of colonic injury related to AA administration. In addition, SLZ can ameliorate AA-induced colitis.

Conclusion

From the above data, it is clear that oxidative stress may be one of the mechanisms by which AA may cause colonic damage. SLZ was able to attenuate the colitis induced by AA, at least in part through anti-oxidant mechanisms.

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