Research Article

The effect of diacerein versus sulfasalazine on acetic acid-induced ulcerative colitis in rats

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Abstract
Ulcerative colitis is a serious premalignant condition has a confusing multifactorial pathogenesis. Researchers have attention for exploration of new therapeutic or prophylactic drugs targeting its pathophysiology. The current study was aimed to study the possible role of diacerein (50mg/kg/day) in ulcerative colitis rat model, induced by acetic acid. Twenty four male Wistar albino rats were classified into 4 groups: control group, acetic acid (AA)-ulcerated group, AA-ulcerated + diacerein (50 mg/kg/day) and AA-ulcerated + sulfasalazine (100 mg/kg/day) groups. Colonic malondialdehyde (MDA) and superoxide dismutase (SOD) were determined. Also, caspase-3 gene expression was measured as an indicator of apoptosis. Histopathological and COX2 immunohistochemical studies of the colonic tissue were also done. In addition, serum levels of interleukin (IL)-1β and tumour necrosis factor (TNF)-α were measured. In AA-ulcerated group, there was significant elevation in tissue MDA levels with upregulation of caspase 3 gene expression. Meanwhile, AA caused decreases in the SOD activities. Also, AA induced elevation in the serum IL-1β and TNF- α levels. Pretreatment with diacerein 50 mg/kg/day improved changes in these parameters. In conclusion, diacerein has beneficial effects against AA-induced damage of the colon, possibly by exerting anti-inflammatory, antioxidant and anti-apoptotic effects.
Key words: diacerein, ulcerative colitis, acetic acid, anti-apoptotic.

Introduction
Inflammatory bowel disease (IBD) is a life threatening and debilitating disease affecting the colon including ulcerative colitis (UC) which is a premalignant disease, including oxidative and inflammatory factors in its pathogenesis (Karakoyun et al., 2011). Production of proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α) is a corner stone inflammatory mediator in UC. The pathogenesis of IBD also involves activated T-cells, which release various cytokines responsible for production of free radicals and destructive enzymes, with injurious effect on the gastrointestinal tract (Wan et al., 2014). This occurs through apoptosis and can be progressed to colon cancer (Salari-Sharif and Abdollahi 2010). The sites of inflammation in UC have defective apoptotic functions (Sturm et al., 2008). Caspase-3, a key enzyme in apoptosis, is activated in apoptotic cells through both extrinsic and intrinsic pathways which lead to colonic destruction with disturbed functions (Qiu et al., 2011).

Almost all drugs used in treatment of UC, aim at reducing symptoms or maintaining remission. Such as corticosteroids, immunosuppressants, aminosalicylates and biologic medications, but they can have side effects as hepatitis, nephritis, fluid retention, immunosuppression and others (Head and Jurenka 2003). Ongoing researches aim at exploring new remedies which may be of further benefit in ameliorating the disease or have additive or synergistic effects to the already used therapies for better disease prognosis with less side effects.

The interleukin-1 receptor antagonist is a member of the interleukin 1 cytokine family. IL1RA is secreted by various types of cells including immune cells, epithelial cells, and adipocytes, and is a natural inhibitor of the pro-inflammatory effect of

IL1β. This protein inhibits the activities of interleukin 1, alpha (IL1α) and interleukin 1, beta (IL1β), and modulates a variety of interleukin 1 related immune and inflammatory responses (Perrier et al., 2006).

Diacerein is an antiinflammatry drug that is well established in treatment of osteoarthritis. It acts mainly via inhibition of interleukin-1β (IL-1β) synthesis (Domagala et al., 2004). It may act at the pre cell membrane level and the post cell membrane level but does not affect gene levels of IL-1β. Diacerein downregulates the activity of IL-1β by significantly decrease the number of IL-1 receptors (IL-1r) on the cell surface and by significantly increase the synthesis of IL1RA (Domagala et al., 2004).

Rhein, the active metabolite of diacerein, inhibits the production of IL-1β in *vitro* and in *vivo*. Rhein also decreases the number of urokinase receptors to near normal levels and reduced fibrinolytic activity. Leukocyte migration, lysosomal enzyme release, super oxide production and chemotaxis, are also inhibited by rhein in dose dependent manner (Verbruggen, 2006).

The aim of this study was to extend the work of other researchers on the interleukin-1 receptor antagonist role in colitis and investigate the potential effects and mechanisms of action of diacerein in a rat model of AA-induced UC.

Materials and Methods
Drugs, chemicals and kits
Sulfasalazine (Pfizer, Australia) and diacerein (Eva pharma,Egypt) were used. Acetic acid glacial (CID pharmaceutical Co, Egypt) 4% (volume/volume), interleukin (IL)-1β and tumor necrosis factor (TNF)-α enzyme-linked immunosorbent assay (ELISA) kits *(*Nanjing jiancheng Bioengineering, China) were used for analyses. Superoxide dismutase (SOD) colorimetric kits (Biodiagnostic, Egypt)**.** Cyclo-oxygenase-2 (COX2) (Lab Vision Laboratories, Fermont, USA).

Animals
A total of 24 adult healthy male Wistar-albino rats weighing 180 to 220 g were used in this study. Rats were purchased from the National Research Center, Cairo, Egypt. Rats were harbored on a 12-h light/dark cycle (lights on from 08:00 am) at a constant temperature (24±1°C) and humidity with normal rat chow and water was available *ad libitum*. The study followed the guidelines for animal welfare and was approved by the Institutional Reviewer Board of Faculty of Medicine, Minia University.

Experimental design
Four groups of rats were studied (six rats in each). Group I: Normal control group that received only distilled water; Group II: AA-ulcerated, non-treated group, animals that received AA for UC induction (Mascolo et al., 1995); Group III: diacerein (50 mg/kg/day; PO)-treated group (Malaguti et al., 2008) + AA; Group IV: sulfasalazine (100 mg/kg/day; PO)-treated group (Thippeswamy et al., 2011) + AA.

Induction of colitis
After fasting overnight, rats were anasthesized with ether then intrarectally infused with 2 ml AA (4%) using a lubricated paediatric catheter inserted 8 cm proximal to the anus. Rats were kept in a horizontal position for 30 s to avoid AA leakage. The normal control group received an equal volume of 0.9% saline instead of AA (Mascolo et al., 1995).

The pretreated groups received diacerein or sulfasalazine orally daily for 7 days before induction of colitis and for another 3 days following colitis-induction. Blood samples were collected from the tail vein. Serum was separated by centrifugation and stored at -80°C until used for measuring serum IL-1β and TNF-α. Then, rats were sacrificed. After dissection, the colonic specimens were kept in 10% formalin for histopathological examination. The remaining colonic tissues were maintained at -80°C till homogenized and used for assessment of MDA, SOD activities and caspase 3 gene expression.

Biochemical studies
Sera stored at -80°C was used to determine TNF-α and IL-1β levels. Homogenized tissue samples were used for the measurement of MDA, SOD activities and caspase 3 gene expression.

Measurement of tissue MDA

Colonic content of malondialdehyde (MDA) was measured according to the thiobarbituric acid method, previously described by Buege and Aust (1978). It depends on measuring MDA, the break-down products of lipid peroxides.

Measurement of tissue SOD activity

The enzymatic activity of SOD was esti-mated according to the manufacturers' guidelines using commercially available kits.

Measurement of serum levels of TNF-α and IL-1β

Serum levels of IL-1β and TNF-α were measured using enzyme-linked immune-sorbent assay (ELISA) kits according to the manufacturer's instructions.

Gene expression of caspase 3

According to the manufacturer instructions, isolation of RNA from 100 mg of tissue was done by the aid of an RNA extraction kit (Qiagene, USA). After synthesis of first-strand complementary DNA from 2 µg total RNA (Invitrogen Inc., Carlsbad, California, USA) and denaturing the template RNA and primers (25 pmol of each reverse oligonucleotide primer) at 70°C for 10 min, 40 U reverse transcriptase was added in the presence of RT buffer, 4 µl dNTP mix (250 µmol/l each), 40 U RNase inhibitor, and RNase-free water to achieve the final volume. Incubation of the mixture (50 µl) was done for 1 h at 43°C, then stopped at 4°C, and used on the spot for polymerase chain reaction (PCR) or kept at -80°C until use. Reactions were carried out in triplicate. Conditions of the reaction were: an initial 15 min at 95°C, followed by 40 cycles of 15 s at 94°C, 30 s at 55 to 60°C, and 30 s at 72°C. Real-time PCR was carried out in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, California, USA). Calculation of relative gene expression was done using the comparative threshold cycle (Ct) method (Livak and Schmittgen, 2001).

Assessment of colitis
Macroscopic colonic damage scoring

According to the scoring system of Millar (Millar et al., 1996), mucosal damage was assessed using the microscope.

Inflammation scores were assigned using a scale ranging from 0 to 4: 0 indicates no macroscopic changes, 1 indicates mucosal erythema only, 2 indicates mild mucosal edema, slight bleeding, or small erosions, 3 indicates moderate edema, bleeding ulcers or erosions, and 4 indicates severe ulce-ration, erosions, edema and tissue necrosis.

Histological and immunohistochemical examination

Cross sections of colonic tissues were fixed in 10% formaldehyde, embedded in paraffin blocks, and cut into fine sections. Samples were collected on glass slides, stained with hematoxylin and eosin (H&E) and examined under the microscope by a pathologist in a blinded manner. An Olympus (U.TV0.5XC-3) light microscopy was used. Slides were photographed using an Olympus digital camera.

Histopathological slides were examined for destruction of the epithelium and glands, dilatation of glandular crypts, depletion and loss of goblet cells, inflammatory cells infiltration, edema, hemorrhagic mucosa and crypt abscesses (Bancroft and Garble, 2007). Immunohistochemical detection of COX2 was performed using COX2 monoclonal mouse antibody (Lab Vision Laboratories, Fermont, USA) according to the manufacturer's protocol. The slides were then counterstained, dehydrated, and mounted (C^ote et al., 1993).

Statistical analysis

Data were represented as means ± standard error of the mean (SEM). Graph pad Prism 5 software was used to perform the statistical analysis and we used one-way ANOVA to do the significant difference between different groups, followed by Tukey-Kramar post hoc test for multiple comparisons with a value of P ≤ 0.05 considered statistically significant.

Results
Effect of diacerein and sulfasalazine on oxidative stress parameters

There was significant increase of colonic MDA with significant decrease in colonic SOD in AA-ulcerated group when com-pared to normal control group. Meanwhile, diacerein and sulfasalasine pretreated

groups significantly improved these parameters.

Effect of diacerein and sulfasalazine on inflammatory parameters

Acetic acid ulcerated group significantly increased serum TNFα and IL-1B. Diacerein and sulfasalasine pretreated groups significantly decreased serum TNFα and IL-1B.

Effect of diacerein and sulfasalazine on apoptosis

Caspase 3 gene expression was upregulated by AA-ulcerated group. Meanwhile, diacerein and sulfasalasine pretreated groups significantly reduced caspase 3 gene expression.

Macroscopic examination

Acetic acid caused severe edematous inflammation in the colon, with a high macroscopic scoring of colonic damage as compared to the control group. Sulfasalazine (100 mg/kg/day) significantly reduced the severity of gross lesion scores as compared to the AA group. There was an improvement in colonic damage score by diacerein (50mg/kg/day).

Histopathological and immunohisto-chemical changes

Normal control group showed normal colon with preserved mucin secreting glands. Meanwhile AA-ulcerated group showed mucosal ulceration and transmural inflammation. Diacerein pretreated rats appeared near normal mucosa, with ulcer healing and preserved mucin secreting glands. In sulfasalasine pretreated group, the colon is near normal structure with mild inflammation infiltrating the mucosa.

Normal control group showed negative COX2 in normal colon (with weak focal staining that didn't reach the positive cut-off). AA-ulcerated group showed strong positive COX2 (Diffuse dark staining of the mucosal inflammatory cells and the few scattered preserved colonic glands). Meanwhile, diacerein apeared near normal mucosa, with mild COX2 staining (weak staining in almost all cells). Sulfasalasine pretreated group showed normal mucosal glands with mild COX2 staining in addition to the positively stained inflammatory cells in the stroma (weak staining in almost all cells).

Table 1: Effect of diacerein and sulfasalazine on oxidative stress parameters (colonic MDA and SOD) in rats

Groups	Colonic MDA (nmol/g tissue)	Colonic SOD (U/g tissue)
Normal control group	33.54 ± 2.34	152.72 ± 4.12
AA-group	94.12 ± 3.70[a]	68.53 ± 3.31[a]
Dia+AA-group	42.91 ± 2.71[b]	136.52 ± 3.08[b]
Sulfa+AA-group	39.04 ± 1.99[b]	144.68 ± 2.89[b]

Results represent the mean ± SE (n = 6), [a] Significant ($p < 0.05$) difference from normal control group, [b] significant ($p < 0.05$) difference from AA-group.[AA= acetic acid; Dia=diacerein; Sulfa=sulfasalazine; MDA= malondialdehyde; SOD= superoxide dismutase].

Table 2: Effect of diacerein and sulfasalazine on inflammatory parameters (serum TNFα and IL-1B) in rats

Groups	Serum TNFα (pg/ml)	Serum IL-1B (pg/ml)
Normal control group	10.22 ± 1.05	99.34 ± 3.45
AA-group	24.08 ± 2.26[a]	132.27 ± 2.44[a]
Dia+AA-group	14.45 ± 1.24[b]	107.81 ± 3.15[b]
Sulfa+AA-group	12.70 ± 1.09[b]	102.63 ± 4.26[b]

Results represent the mean ± SE (n = 6), [a] Significant ($p < 0.05$) difference from normal control group, [b] significant ($p < 0.05$) difference from AA-group.[AA= acetic acid; Dia=diacerein; Sulfa=sulfasalazine; TNF = tumor necrosis factor; IL=interlukin].

Table 3: Effect of diacerein and sulfasalazine on apoptosis (Caspase 3) in rats

Groups	Caspase 3 gene expression
Normal control group	1.50 ± 0.12
AA-group	6.03 ± 0.56^a
Dia+AA-group	1.80 ± 0.14^b
Sulfa+AA-group	1.60 ± 0.09^b

Results represent the mean $\pm$ SE (n = 6), [a] Significant ($p < 0.05$) difference from normal control group, [b] significant ($p < 0.05$) difference from AA-group. .[AA= acetic acid; Dia=diacerein; Sulfa=sulfasalazine].

Table 4: Effect of diacerein and sulfasalazine on macroscopic scoring of colonic tissue in rats.

Groups	Macroscoping scoring
Normal control group	00 ± 00
AA-group	3.5 ± 0.4^a
Dia+AA-group	1.3 ± 0.5^b
Sulfa+AA-group	1.00 ± 0.3^b

Results represent the mean $\pm$ SE (n = 6), [a] Significant ($p < 0.05$) difference from normal control group, [b] significant ($p < 0.05$) difference from AA-group.
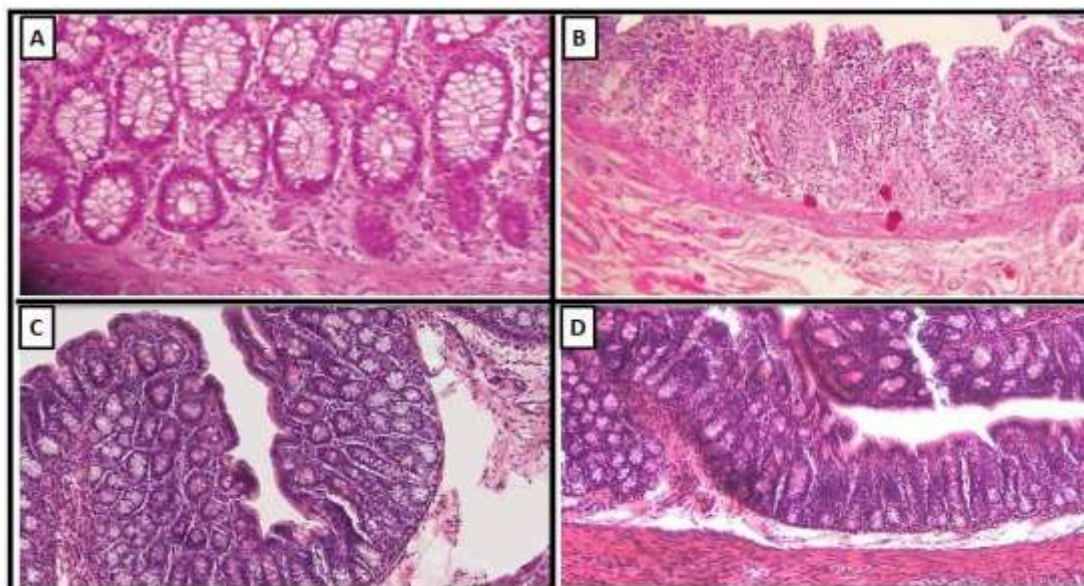

Figure 1. Histopathological sections of colons stained by H&E representing different studied groups. A, Group I: showing normal colon with preserved mucin secreting glands (200x). B, Group II: it shows mucosal ulceration and transmural inflammation (200x). C, Group III: showing near normal mucosa, with ulcer healing and preserved mucin secreting glands (x100). D, Group IV: showing the colon which is near normal structure with mild inflammation infiltrating the mucosa (H&E 100x)
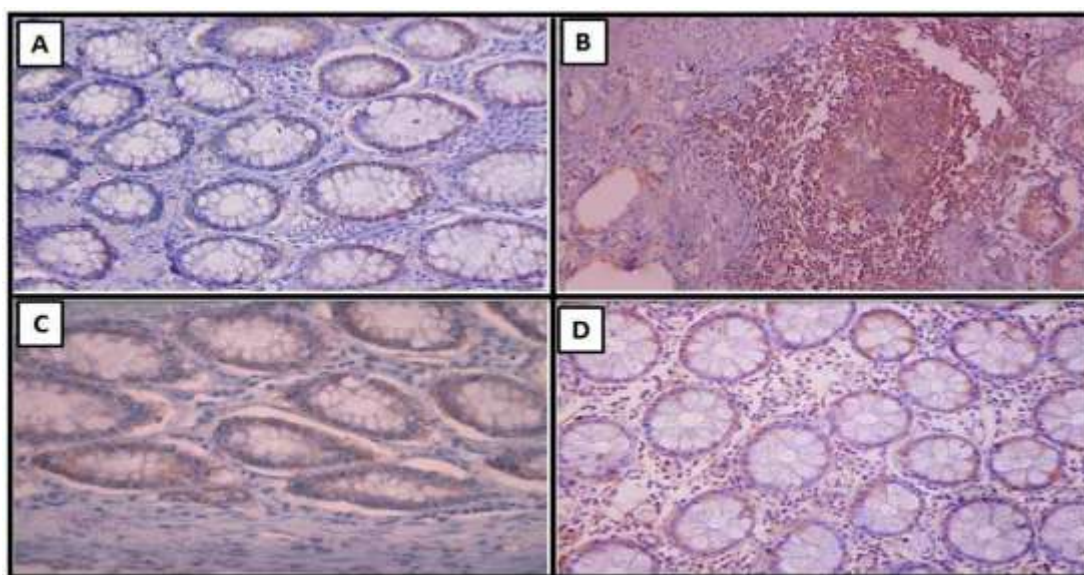
Figure 2. Immunohisochemical stained sections of colons for COX2 antibody representing different studied groups. Staining is considered positive when dark brown cytoplasmic staining is detected in more than 10% of cells. A, Group I: showing negative COX2 in normal colon (with weak focal staining that didn't reach the positive cut-off) (200x). B, Group II : it shows strong positive COX2 (Diffuse dark staining of the mucosal inflammatory cells and the few scattered preserved colonic glands) (200x). C, Group III: showing near normal mucosa with mild COX2 staining (weak staining in almost all cells) (x200). D, Group IV: showing normal mucosal glands with mild COX2 staining in addition to the positively stained inflammatory cells in the stroma (weak staining in almost all cells) (x200).

Discussion

Inflammatory bowel disease is a chronic inflammatory disease with highly expressed enzymes such as COX-2 and inflammatory cytokines such as TNF-α, IL-1β (Karakoyun et al., 2011). Tissue injuries in all inflammatory conditions like colitis caused by reactive oxygen metabolites (ROM). ROM increases the oxidative stress and leads to impairment of the antioxidant defenses which contributes to the patho-genesis of colitis (Iseri et al., 2009). Thus results in increased colonic MDA contents. (Girgin et al., 2000; Ek et al., 2007).

There is a strong relation between the systemic protection against inflammation and the status of antioxidant enzymes e.g., SOD as it is responsible for the conversion of superoxide to peroxide. This guards against lipid peroxidation in colon by eliminating free-radicals. Decreasing SOD activity in the colonic tissue leads to mucosal injury due to decreased ability of scavenging oxidative radicals (Barazzone and White 2000; Krieglstein et al., 2001).

The current study showed that colonic MDA level were significantly increased with significantly decreased colonic SOD in the AA group and this in agreement with Cetinkaya and his co-workers in 2005; Al-Rejaie et al., 2013. Meanwhile, the administration of diacerein (50mg/kg/day) showed a significant decrease in MDA level. This is consistent with Refaie et al., (2015).

Serum TNF-α and IL-1β levels significantly increased in the AA group. Diacerein limited the up-regulation of them which are believed to play a significant role in the pathogenesis of IBD. In accordance with the current findings, the clinical study of Pasin et al., (2012) demonstrated that diacerein decreases TNFα and IL-1β levels in peritoneal fluid and prevents Baker's yeast-induced fever in young rats.

In the AA group, caspase 3 gene expression was upregulated as previously described by Kaushal et al., (2001). The current study results showed that diacerein- pretreated rats reduced caspase 3 gene expression. Similarly, Torina et al., (2015) in their study found that diacerein is able to reduce caspase 3 activity, as an index of apoptosis.

Also, histological and immune histochemical data also match the biochemical changes. Microscopical examination confirmed the previous results, where AA group showed ulceration of the colonic mucosa with inflammation. Rats pretreated with diacerein showed amelioration of AA-colitis. The scoring of AA group significantly increased as compared to the control group, whereas that of diacerein and sulfasalazine significantly decreased as compared to AA. COX2 immunoexpression showed strong positive in AA group. Meanwhile, rats pretreated with diacerein showed near normal mucosa with mild COX2 staining

Conclusion
Overall, the findings of the present study demonstrated that diacerein pretreatment (50mg/kg/day) exerted significant ameliorating effect against AA-induced UC model. This is most probably attributed to its anti-oxidant, anti-inflammatory and anti-apoptotic effects.

Conflict of interest
The authors declare that there is no conflict of interest.

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