The therapeutic effects of ambrisentan on experimentally induced colitis in a male rat's models
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Abstract
Ulcerative colitis is a chronic, intermittent illness. The current treatment failed to cure the disease which made a need to investigate another drug with minimal side effects. The present research was conducted to assess the antioxidant and anti-inflammatory effects of ambrisentan in comparison with that of sulfasalazine in experimental colitis in rats. Acetic acid 4% (vol/vol) was used rectally to induce experimental colitis in rats. After induction, rats were administered either sulfasalazine 100mg/kg or ambrisentan 5 mg/kg orally for one week. There was the estimation of histopathological and macroscopical parameters also the expression of cytokines (TNF-α and IL-4), oxidative stress markers, malondialdehyde (MDA) and myeloperoxidase (MPO), and adhesion molecules (ICAM-1 and E-Selectin) in the colonic tissue. Both ambrisentan and sulfasalazine significantly reduced the macroscopical and histological injury in the colon induced by acetic acid. In addition to the downregulation of the increased colonic proinflammatory cytokines, MDA, MPO parameters and adhesive molecules. In conclusion: Ambrisentan had an effective role in experimental colitis in rats through anti-inflammatory and antioxidant actions with downregulation of the colonic adhesive molecule.

Keyword: Ambrisentan, Ulcerative colitis, Acetic acid, Oxidative stress, Adhesion molecules.

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Introduction
Ulcerative colitis (UC) is one of the chronic, recurrent and inflammatory intestinal illnesses. Although its etiology is unknown, several studies indicated that altered immunity, genetic and environmental factors are intercepted with the pathogenesis of colitis. In the past years despite the high awareness of that disease, the utilization of remedies is still insufficient [1, 2]. The induction of colitis by using an acetic acid universally used experimental pattern [3]. It is effective for the investigation of the pathogenesis of ulcerative colitis and novel options for treatment [4]. This pattern is similar to ulcerative colitis in histological appearance and sulfasalazine responsiveness [5]. Aminosalicylates, immunomodulators, glucocorticoids, and monoclonal antibodies are a pharmacological treatment for UC. Regardless, the increased proportion of undesirable effects at the same time with insufficient therapy get by necessary to inspect novel drug with high effectiveness [6].

Ambrisentan is a selective inhibitor of the endothelin A (ETA) receptor which inhibits phospholipase C-mediated vasoconstriction and protein kinase C-mediated cell proliferation, while it preserves the production of nitric oxide and prostacyclin [7]. A previous study showed that endothelin-1 (ET-1) is implicated in the expression of proinflammatory cytokines and the production of reactive oxygen species [8]. Nevertheless, no adequate data are accessible with reference to the therapeutic outcome of ambrisentan in induced colitis. The current study assesses the protective effect of ambrisentan versus experimental colitis.

Materials and methods

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Forty-eight Wistar albino adult male rats (200-220g) were used throughout this study (supplied from the animal house of the national center for drug control and researches). Prior to the experiment, the animal was placed five per cage that was supplied with a large wire mesh floor for 7 days and were allowed to administer water and laboratory chow pellet. The study protocol was approved by the institutional animal ethics committee from Al-Nahrain University in the college of medicine.

Chemicals and Drugs: Acetic acid and diethyl ether (BDH Chemical Ltd., England), immunohistochemistry kits (Abcam/UK), sulfasalazine and ambrisentan (Sigma –Aldrich) were purchased.

Experimental design
Rats were classified into four groups (n=12 in each group). The Group I received no treatment and served as control while the other groups, colitis was induced intrarectally by 4% acetic acid (v/v). The group II was administered orally normal saline; while group III was administered ambrisentan 5mg/kg orally and lastly group IV treated orally with sulfasalazine (Salazosulfapyridine) 100mg/kg, thirty-minute after the induction of colitis for 7 days. The duration of treatment was depended on previous studies of experimental colitis [9, 10].

Induction of ulcerative colitis
Before the colitis induction, rats were fasted for at least 24hrs to get proper induction of colitis by evacuation the colon from feces but were be permitted to tap water. Experimental colonic ulceration was accomplished after interruption of water (2 hours) in accordance with that procedure suggested by Mousavizadehet al.[11] with modification. Briefly, rats were received a single intrarectal infusion of 4% acetic acid in a dose 5ml/kg solution for the 30s (8cm into the colon) under light ether anesthesia by flexible plastic tube (2 mm extrinsic diameter). Rats were positioned in a horizontal direction for 2 min to prevent the discharge of acetic acid. Control rats go through the identical procedure by using the same amount of normal saline as an alternative to acetic acid.

Preparation of drugs
The sulfasalazine and ambrisentan were freshly prepared before administration. Estimated drugs were prepared as suspensions in the distilled water. Ambrisentan was used at a dose of 5mg/kg according to the study reports that the no observed adverse effect levels (NOAEL) dose in the 26-week study for males and females based on osseous hyperplasia of the nasal turbinates [12]. Sulfasalazine (100 mg/kg) was served as standard therapy [13, 14].

Assessment of colitis
After the experiment ending, rats inhaled an excessive dose of diethyl ether in order to sacrifice them. The colon was removed rapidly after dissection of the abdomen. The specimen of the colon was opened longitudinally and gently cleaned with normal saline. Then, the assessment of macroscopical features was achieved by normal observation. Finally, the samples were assessed for histopathologic changes and immunohistochemical analysis.

Macroscopic evaluation
Disease Activity Index (DAI)
The Disease activity index defined by Niu et al [15] was used to assess the disease clinically which involve bodyweight reduction {0=weight gain or no reduction, 1= 1-5 % reduction, 2=6- 10% reduction, 3=11-15 % reduction, 4=more than 15% reduction}; the consistency of faeces {0=normal, 2=loose faeces, 4=diarrhea}; and bleeding of rectum {0= normal, 2= mild bleeding, 4=severe bleeding}. The calculation was made by a combination of the total scores of DAI.

Colon edema
It was utilized as an indicator of edematous tissue and the intensity of colitis. After the incision was done along the mesenteric margin of each colonic specimen and washed gently, the colon edema was determined by measuring the colon weight by sensitive balance [16].

Macroscopic colonic score
The colonic samples were examined visually. The macroscopic score based on the clinical features of the colon according to scoring system ranging from 0-6 as follows: 0= absence of inflammation; 1= redness or swelling; 2= swelling and redness; 3= one or two ulcers; 4= one large ulcer or more than two ulcers; 5= initial necrosis; 6, severe necrosis [17].
**Histopathological evaluations**

Formalin (10%) was used to fix the colonic samples. Dehydration, paraffin embodiment, and deparaffinization were done on the samples. Colonic samples were cut into sections (4µm) and dyed with Hematoxylin and eosin (H&E). The histopathological changes were assessed by examining and scoring the slides. Experienced histopathologist tested the tissue sections in a blinded manner and results evaluated according to scoring system ranging from 0-3 (0=normal; 1=focal; 2=zonal; 3=sever) which assessed the extent of destruction of glands and epithelium; glandular crypts dilation; depletion of goblet cells; infiltration of inflammatory cells; edema; dysplasia; mucosal hemorrhage and crypt abscesses \[18\].

**Immunohistochemistry**

Immunohistochemistry (IHC) demonstrated directly the cells in the affected tissue directly.[19] The immunohistochemical reactions were produced by the presence of specific antibodies, simultaneously the evaluation of the production of a number of biochemical markers in intestinal samples that were paraffin-embedded in order to measure the colonic cytokines, adhesion molecules, and oxidative stress parameters. "Quantification of IHC was performed in accordance to the following semi quantitative scores [20] which was based on the percentage of positively stained cells as following: 0, no staining; 1, ≤ 25%; 2, 26-50%; 3, 51- 75 %; and 4, 76-100%.

**Statistical analysis**

Statistical package for social science version 23 software program was used to summarize, analyze and present the data. Quantitative (numeric) variables were expressed as mean and standard deviation. One-way ANOVA was used to study the difference in mean of quantitative variables among groups; then followed by post hoc least significant difference (LSD) test to evaluate mean difference within groups. The significant level was considered at p ≤ 0.05 \[21\].

**Results**

**The influence of ambrisentan on macroscopical score**

The colonic mucosa was extensively ulcerated and showed necrotic tissue in untreated rats in contrast to control normal group. Even so, after the induction of colitis, the rats that received ambrisentan or sulfasalazine elicit orally significant reduction to the disease activity index and colonic weight as displayed in the table (1). Additionally, two drugs significantly (p<0.01) reduce the macroscopical score (table 1).

**The influence of ambrisentan on histopathological score**

The current study exhibits the histological changes in colitis group, primarily loss of architecture in the intestinal crypt and extensive mucosal colonic damage, ulcerations and necrosis as displayed in figure (2). Furthermore, both sulfasalazine and ambrisentan administered groups elicit significant (p<0.01) reduction in the microscopical score as specified by colonic mucosa epithelization, reduction of neutrophil infiltration and edema as displayed in the figure (3). Even so, ambrisentan exhibited a higher significant reduction in the scoring of the microscopical parameter displayed in a table (2).

**The influence of ambrisentan on proinflammatory cytokines (IL-4 and TNF-α).**

Oral treatment of ambrisentan or sulfasalazine to the colitis group was significant (p<0.01). It was diminished by IL-4 and TNF-α in comparison with colitis (Table 2). However, ambrisentan exhibited better significant (p<0.05) reduction in the TNF-α level compared with the sulfasalazine group as displayed in a table (2).

**The influence of ambrisentan on oxidative stress markers(MPOand MDA)**

After sulfasalazine and ambrisentan treatment, the high colonic MDA level in the induced group was found to be significantly (p<0.01) diminished (Table 3). Even so, ambrisentan elicits a better significant (p<0.05) reduction in the level of MDA in comparison to the sulfasalazine group. Also, both drugs produce significant (p<0.05) declination in MPO in comparison with the colitis group as displayed in a table (3).

**The influence of ambrisentan on adhesion molecules (ICAM-1 and CD62)**

As displayed in table (3). There were elevated level of ICAM-1and CD62 after the induction by acetic acid in comparison with that of normal group. While these findings elicit significant (p<0.01)
declination in the ambrisentan and sulfasalazine treatment. However, ambrisentan produced a better significantly (p<0.05) declination in the ICAM-1 in comparison to sulfasalazine administered group (table 3).

**Table 1:** Macroscopic parameters in study and healthy control groups.

<table>
<thead>
<tr>
<th>Macroscopic Parameters</th>
<th>Groups (n=12/group)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy control</td>
</tr>
<tr>
<td>Disease activity index</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Colonic weight (gram)</td>
<td>1.5±0.22</td>
</tr>
<tr>
<td>Macroscopic score</td>
<td>0.0±0.0</td>
</tr>
</tbody>
</table>

Comparison expressed by letters; dissimilar letters denotes significant difference. The expression of values as mean ±Standard deviation (SD).

**Table 2:** Cytokines immunohistochemical score and histopathological parameters.

<table>
<thead>
<tr>
<th>Cytokines and histopathological parameters</th>
<th>Groups (n=12/group)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy control</td>
</tr>
<tr>
<td>Histopathology</td>
<td>0.0 ±0.0</td>
</tr>
<tr>
<td>Interleukine-4</td>
<td>1.0 ±0.0</td>
</tr>
<tr>
<td>Tumor necrosis factor-α</td>
<td>1.0 ±0.0</td>
</tr>
</tbody>
</table>

Comparison expressed by letters; dissimilar letters denotes significant difference. The expression of values as mean ±Standard deviation (SD).

**Table 3:** Immunohistochemical score for oxidative stress and adhesive molecules.

<table>
<thead>
<tr>
<th>Oxidative stress and Adhesive molecules Parameters</th>
<th>Groups (n=12/group)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy control</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>1.00 ±0.0</td>
</tr>
<tr>
<td>Malondialdehyde</td>
<td>1.0 ±0.0</td>
</tr>
<tr>
<td>Intercellular adhesive molecule-1</td>
<td>1.0±0.0</td>
</tr>
<tr>
<td>E-selectin</td>
<td>1.0±0.0</td>
</tr>
</tbody>
</table>

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Comparison expressed by letters; dissimilar letters denotes significant difference. The expression of values as mean ± Standard deviation (SD).

**Figure 1:** Histological section throughout the wall of colon reveals the normal mucosa and submucosa without any inflammatory signal and conservation of goblet cells in colon (yellow arrow); H and E stain; A: 10X; B: 40X.

**Figure 2:** Histological section throughout the wall of colon reveals extensive ulceration; necrosis with no gland; and mononuclear inflammatory infiltrate (arrow) in experimentally colitis in rat.

**Figure 3:** Histological section throughout the colonic wall reveals the effects of treatment after 7 days and exhibits (1) the regeneration of mucosa and formation of glands; (2) least severe inflammation and (3) regeneration of goblet cells; H and E stain; A: 10X; B: 40X.
Figure 4: Immunohistochemical expression of interleukine-4 reveals secretory pattern (brown color in the stromal cells); A: 10X; B: 20X.

Figure 5: Immunohistochemical expression of tumor necrosis factor-α (TNF-α) reveals membranous and secretory pattern (brown color in the stromal cells); A: 10X; B: 20X.

Figure 6: Immunohistochemical expression of myeloperoxidase (MPO) reveals cytoplasmic pattern (brown color in the stromal cells); A: 10X; B: 20X.

Figure 7: Immunohistochemical expression of malondialdehyde (MDA) reveals cytoplasmic pattern (brown color in the stromal cells); A: 10X; B: 20X.
Figure 8: Expression of ICAM-1 by Immunohistochemistry reveals membranous pattern (brown color in the stromal cells); A: 10X; B: 20X.

Figure 9: Immunohistochemical expression of CD62 reveals membranous pattern (brown color in the stromal cells); A: 10X; B: 20X

Discussion

The present study showed that ambrisentan significantly reduces the mean DAI, which is consistent with the finding of Lee et al. [22] who showed endothelin converting enzyme inhibitor (SM-19712) attenuated the inflammatory response in a mouse model of colitis and decreased the extent of rectal bleeding and loose feces. However, the current study showed that ambrisentan has decreased the weight of colonic tissue in the colitis group which was comparable to the suggestion of Eibl et al. [23] who showed that endothelin-1 receptor antagonist reduced the capillary permeability in the colon. Moreover, ambrisentan in the present study reduced macroscopic and the scoring of histopathological changes in colitis. The proposed protective mechanism of ambrisentan is through the selective blocking effect of the ETA receptor that prevents vasoconstriction and cell proliferation [7]. Moreover, ambrisentan inhibits the oxidative stress and mucosal dysfunction caused by ET-1 in the colonic rat [8].

There was a close relationship between inflammation and ulcerative colitis [24]. Tumor necrosis factor-α (TNF-α) is a crucial pro-inflammatory cytokine liberated from the lymphocytes and macrophages in the initial step of inflammation, also, it can produce different mediators which responsible for severing inflammation [25]. It has been noted that TNF-α has great participation in the pathogenesis of colonic ulceration [26]. Interleukin-4 is a key immunoregulatory cytokine (Th2-cytokine) [27] which can assist in inflammation and pathogenesis of intestinal ulceration by reducing resistance in intestinal epithelial cells [28], goblet cell hyperplasia [29] and eotaxin expression in colonic mucosa [30]. Kasaian et al. [27] showed the involvement of IL-4 in experimental colitis induced by oxazolone, a model of UC in mice. The present study explained the administration of ambrisentan causes significant reduction in the expression of immunohistochemistry for colonic cytokines (interleukine-4 and tumor necrosis factor-α) in experimentally induced colitis in rats and this finding is in accordance with the findings of the previous study that has been shown that ETA receptors but not ETB receptors have a great role in induced colitis [31]. Endothelin-1 has been found to be associated with an inflammatory response through the activation of proinflammatory cytokines and the expression of nuclear factors such as NF-kB [32].

Malondialdehyde produced from the peroxidation process of lipid which causing destruction of colonic tissue in ulcerative colitis and cell necrosis [33, 34]. Myeloperoxidase is an enzyme which is situated in the neutrophil granules has been exceedingly beneficial as an indicator for neutrophil infiltration to the inflammatory site [35]. Studies have indicated that oxidative stress results from the shift of equilibrium between the pro-oxidant and anti-oxidant systems in favor of the pro-oxidant system which results in excessive production of free oxygen radicals and neutrophil infiltration [36]. The study has also shown that the administration of ambrisentan significantly reduces the expression of immunohistochemistry for markers of oxidative stress (myeloperoxidase and malondialdehyde) in the colonic mucosa of experimentally induced colitis in rats. Studies have shown that activation of ETA receptors by ET-1 is associated with the production of free oxygen particles and lipid peroxidation [32].
Inter Cellular adhesive molecule (ICAM-1) engages the leukocytes association to endothelial cells that simplify their penetration to the inflammatory site \[37\]. Selectins take part in the initial phases of rolling of leukocytes to the blood vessel epithelium, and endothelial selectin plays a major role in the chemotaxis of leukocytes to the vascular wall and their adhesion to the endothelium \[38\]. The present study showed that ambrisentan significantly reduced immune histochemical expression of adhesive molecules in rat colonic mucosa in contrast to colitis group and this evidence is corresponding with Anthony et al. \[39\] that showed endothelin receptor antagonist (bosentan) significantly reduce the inflammation and leukocyte adhesion in a murine model of induced colitis. Other studies indicate that ET-1 contributes to inflammation and endothelial dysfunction; it enhances the adhesion molecules expression on vascular endothelial cells and stimulates the neutrophils aggregation \[8\].

In conclusion, ambrisentan has a therapeutic effect through the anti-oxidant and anti-inflammatory effects which is comparable to that of sulfasalazine in experimentally induced colitis.

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Conflicts of interest: There are no conflicts of interest.

References

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