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Comparative Study between Effect of Simvastatin (5 mg/Kg) and Simvastatin (50 mg/Kg) in an Early Treatment of Experimentally Induced Colitis in Mice

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Authors' contributions

This work was carried out in collaboration between all authors. Authors RE, KA and AH designed the study, wrote the protocol, and wrote the first draft of the manuscript. Author RE managed the literature searches, analyses of the study performed the spectroscopy analysis and author RE managed the experimental process. All authors read and approved the final manuscript.

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ABSTRACT

Inflammatory bowel disease (IBD) (ulcerative colitis and Crohn's disease) is a chronic intestinal inflammatory disease characterized by tissue edema, increased gut epithelial permeability, and extensive infiltration of the gut by leukocytes. Statins, in addition to their cholesterol-lowering activity, have pleiotropic effects, including immune-modulatory and anti-inflammatory effects. Simvastatin is a commonly prescribed statins with antioxidant and anti-inflammatory properties Thus; the aim of this study is to compare effect of simvastatin (5 mg/kg) and simvastatin (50 mg/kg) as an early treatment of experimentally induced ulcerative colitis in mice. For the first time in the current study, Simvastatin was administered after the appearance of signs and symptoms of the disease as an early treatment model. Twenty four mice were divided into four groups; control group, non treated DSS-induced colitis group, simvastatin (5 mg/kg/d) -treated DSS-induced colitis group, simvastatin (50 mg/kg/d) -treated DSS-induced colitis group. simvastatin at dose of (5

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mg/kg/d) reduced MDA and TNF-α .While simvastatin at dose of (50 mg/kg/d) showed a significant increase in colon length of mice,a significant decrease in NO and MDA levels and a significant increase in r GSH level. Simvastatin (5 mg/kg/d) and (50 mg/kg/d) reduced the percentage of DAI by 25% and 41% respectively. The sums of histopathological scores were improved after simvastatin treatment.
It can be concluded that effects of simvastatin treatment was mostly dose dependant. Unfortunately the high dose has no clinical application in human due to toxicity. So it is advised to use simvastatin with a dose of 5mg/kg as an early treatment of dss induced colitis model.

Keywords: Simvastatin; inflammatory bowel disease; TNF-α; malondialdehyde; nitrite.

1. INTRODUCTION

Inflammatory bowel disease (IBD), ulcerative colitis and Crohn's disease, is a chronic intestinal inflammatory disease characterized by tissue edema, increased gut epithelial permeability, and extensive infiltration of the gut by leukocytes [1]. Although great advances have been made in the management of the disease a curative therapy does not yet exist [2].

It has been demonstrated that increased production of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and IL-8 in IBD that are known to play a key role in the modulation of intestinal immune system [3].

Reactive oxygen species (ROS) and nitric oxide synthase (NOS), as well as pro-inflammatory cytokines have a long-standing implication in both the etiology and the progression of ulcerative colitis [4]. A disturbance of the anti-inflammatory cytokine profile in favor of pro-inflammatory cytokine over production leads to disease states, such as that observed in IBD [5]. overexpression of cytokines such as TNF-α, interferon-γ (IFN-γ), interleukin-1β (IL-1β), IL-12 as well as IL-6 were found in patients with IBD[6]. The increased expression of TNF-α has been demonstrated by several studies in intestinal biopsies, both in Crohn's disease patients and in those with ulcerative colitis versus healthy controls; in addition, mucosal biopsies from affected areas showed significantly higher levels than those of mucosal biopsies from macroscopically unaffected areas. Finally, serum levels of TNF-α correlate with clinical and laboratory indices of disease activity such as the erythrocyte-sedimentation rate (ESR), C-reactive protein (CRP), and disease activity index (DAI) [7].

3-hydroxy-3-methyl glutaryl coenzyme A reductase inhibitors (statins), in addition to their cholesterol-lowering activity, have pleiotropic effects, including immune-modulatory and anti-inflammatory effects [8]. Sasaki used the dextran sulfate sodium (DSS-) model of colitis to demonstrate that intestinal injury and symptoms were ameliorated by statins [9,10].

Simvastatin which has tested in this study is a commonly prescribed statin with antioxidant and anti-inflammatory properties [11,12].

Thus, the aim of this study is to compare effect of simvastatin (5 mg/kg) and simvastatin (50 mg/kg) as an early treatment of experimentally induced ulcerative colitis in mice.

2. MATERIALS AND METHODS

2.1 Animals

Twenty four female C57BL/6 mice were purchased from (Tudor Bilharz Institute, Cairo, Egypt). Mice were separated into four groups (n = 6/group) and housed under standard conditions for at least 1 week to acclimate before starting the experiments. Throughout the experiments mice were fed with standard pellet diet ad libitum. All protocols were approved by our local committee of Animal Care and Use. At day 5 of the experiment all animals receiving 3% dextran sulphate sodium (DSS-) showed clear clinical signs of acute colitis. On showing signs, treatments started from day 5 till day 14.

2.2 Induction of DSS-colitis

Acute colitis was induced by administration of 3% (w/v) dextran sulphate sodium dissolved in sterile filtered drinking water. The animals had free access to the DSS- solution, which was changed every day for 7 days then return to plain water drinking at day 8 [13]. DSS--induced colitis is a well established experimental model that mimics many of the features of human ulcerative colitis including diarrhea, bloody feces [14] and colonic shortening [15]. DSS- can promote inflammation by many biological pathways including direct

cytotoxic effects [16] as well as apoptotic damage of colonic epithelial cells [17].

2.3 Drugs and Chemicals

- ***Dextran sodium sulphate (DSS-)*:** 3% (w/v) dextran sulphate sodium (purchased from TDB consultancy, Sweden; MW 40,000) dissolved in sterile filtered drinking water. The animals had free access to the DSS- solution, which was changed every day for 7 days.
- ***PBS (phosphate buffer solution)*** It was prepared by dissolving the following chemicals into 1000 ml distilled water, used for washing the blood and in tissue homogenization.

Sodium dibasic phosphate	2.17 gm
Potassium dihydrogen phosphate	0.2 gm
Sodium Chloride	7.1 gm

- ***Simvastatin (Zocor®):*** was obtained from MERCK in form of 20 mg tablets.

2.4 Animal Grouping and Experimental Design

Mice were divided into the following groups (6 in each group):

2.4.1 Group 1 (control group)

The animals received plain filtered water.

2.4.2 Group 2; non treated DSS--induced colitis group

The animals had free access to the DSS-solution, which was changed every day for 7 days then return to plain water drinking at day 8 [18].

2.4.3 Group3; Simvastatin (5 mg/kg/day) - treated DSS--induced colitis group

DSS--induced colitis mice treated with Simvastatin orally once daily (5 mg/kg/d) starting from day 5 to day 14. Tablets were suspended in 0.5% methyl-cellulose solution as vehicle [19].

2.4.4 Group4; Simvastatin (50 mg/kg/d)-treated DSS--induced colitis group

DSS--induced colitis mice treated with Simvastatin orally once daily (50 mg/kg/d) starting from day 5 to day 14. Tablets were suspended in 0.5% methyl-cellulose solution as vehicle [19].

For scoring colitis activity, weight changes were recorded daily throughout the experiment. Fecal samples of each animal were visually inspected for signs of diarrhea and rectal bleedings. The disease activity index (DAI) was calculated by summarizing the scores for weight loss, stool consistency, hemoccult positivity (detected by Benzidine test) or gross bleedings (Table 1).

DAI value is the combined scores of weight loss, stool consistency, and bleeding divided by 3[20].

At day 14; after the end of each group treatment protocol, animals were anasthesized by thiopental, dissected and postmortem blood was collected by cardiac puncture then allowed to stand for clotting, centrifuged and serum was separated.

The entire colon was removed. Each colon was gently stretched; the length of colon was measured from the colo-cecal junction to the anus as indirect marker of inflammation (rate of colon shortening) for each mouse.

2.4.5 Preparation of colonic homogenates

Immediately after sacrificing the mice, colons were homogenized in 5 ml PBS per gram tissue, centrifuged at 4000 r.p.m for 15 minutes. Supernatant was removed divided into aliquots and frozen at - 80ºC until assayed.

2.5 Histopathological Examination

Colon segments were fixed in 10% formalin solution for 24-h. Paraffin wax tissue blocks were prepared. Slides were H&E stained, and scored according to the criteria listed in Table 2. Individual scores and the sum of all scores were calculated [21].

2.5.1 Biochemical assay

2.5.1.1 1-Determination of TNF-α concentration in mouse serum as an inflammatory marker (Mouse TNFα ELISA Kit, Boster immunoleader) According to Brenner et al. [22].

2.5.2 2- Oxidative stress markers

A. Colorimetric determination of colonic *homogenates* reduced glutathione (GSH) according to Beutler et al. [23].
B. *Colorimetric determination of colonic homogenates Nitrite* according to Montgomer & Dymock, [24].
C. *Colorimetric determination of colonic homogenates lipid peroxide (Malondialdehyde)* according to Ohkawa et al. [25].

2.6 Analytical Statistics

In the statistical comparison between the different groups, the significance of difference was tested using one of the following tests:

1. ANOVA (analysis of variance):- Used to compare between more than two groups of numerical (parametric) data followed by post-hoc tukey for multiple comparisons.
2. Kruskal-Wallis test: Used to compare between more than two groups of numerical (non- parametric) data.

A *P* value <0.05 was considered statistically significant in all analyses.

3. RESULTS

3.1 Effect of Simvastatin on DAI

DAI is a useful index for the degree of colitis. Mice receiving DSS- showed a significant increase in DAI compared to control group ($p < 0.01$). Administration of Simvastatin (5 mg/kg/d) and (50 mg/kg/d) reduced the percentage of DAI by 25% and 41% respectively compared to DSS- control group (Fig.1). No mortality was observed among the experimental animal groups.

3.2 Effect of Simvastatin on Colonic Length

DSS- treatment led to highly significant shortening in colon length of mice (6.10±0.56) as compared to the control (8.25±0.23) group ($p < 0.001$). Simvastatin treated groups (5 mg/kg/d) showed a non significant increase (6.72±0.33) in colon length ($p > 0.05$) compared to DSS-induced colitis (6.10±0.56) group .While simvastatin treated group in doses of (50 mg/kg/d) showed highly significant increase ($p< 0.001$) in colon length of mice compared to DSS-induced colitis group (Table 3).

3.3 Effect of Simvastatin on Inflammatory Markers

DSS- induced a significant increase in serum TNF-α ($p < 0.001$) compared with the normal group. Furthermore, treatment of DSS- induced colitis by simvastatin in doses of (5 mg/kg/d) and (50 mg/kg/d) reduced TNF-α level significantly ($p < 0.001$) as compared to DSS- induced colitis group (Table 3).

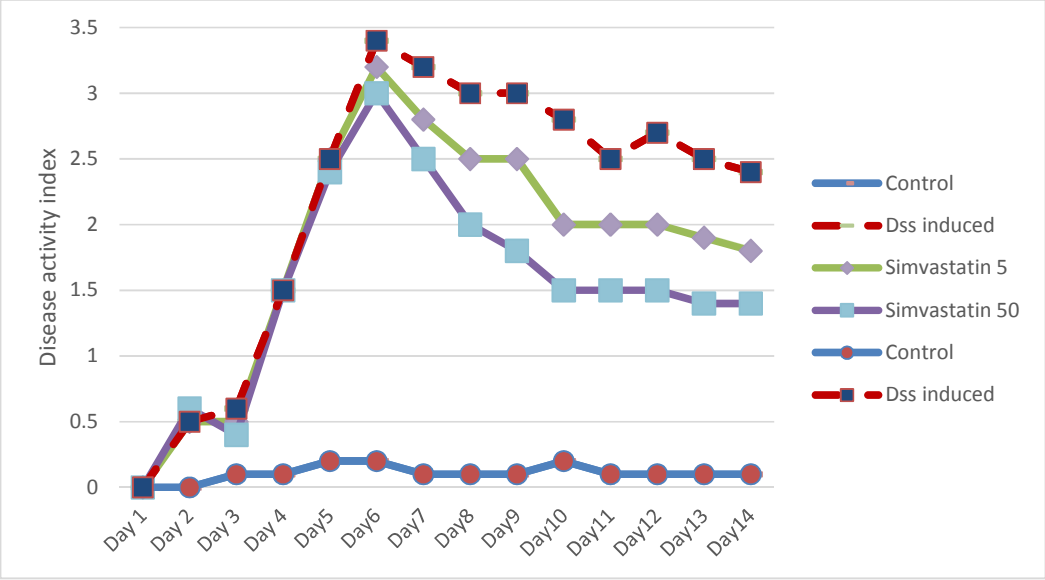

Fig. 1. Effect of different doses of simvastatin on disease activity index (DAI) in DSS-- induced colitis in mice

3.4 Effect of Simvastatin on Oxidative Stress

DSS- treatment led to a significant increase in the MDA (7.63±0.66), NO (45.75±10.47) and decreased reduced glutathione (0.67±0.19) levels in the colon ($p < 0.001$) as compared to normal group (2.95±0.38; 11.12±5.43 & 1.57±0.36, respectively), thus indicating that elevated oxidative stress is involved in the DSS--mediated colitis in mice.

Treatment of DSS- induced colitis by simvastatin at dose of (5 mg/kg/d) reduced MDA level ($p < 0.001$ as compared to DSS- induced colitis group) and showed a non significant change in NO level and r GSH level ($p < 0.05$ as compared to DSS- induced colitis control group).

Treatment of DSS- induced colitis by simvastatin in dose of (50 mg/kg/d) showed a significant decrease in NO and MDA levels and significant increase in r GSH level ($p < 0.01$ and $p < 0.001$ respectively as compared to DSS- induced colitis group) (Table 4).

Table 1. Scoring of disease activity index

Score	Weight loss (%)	Stool consistency	Occult/gross bleeding
0	None	Normal	Normal
1	1-5%		
2	5–10%	Loose stools	Occult bleeding
3	10–15%		
4	>15%	diarrhea	Gross bleeding

3.5 Effect of Simvastatin on Histopatho-logical Lesion

As compared to Fig. 2A (normal histological appearance of the mouse colon), Fig. 2B showing that histological signs of colonic inflammation were: multifocal mucosal infiltrations of predominantly neutrophils and lymphohistocytes (grade 2); diffuse submucosal oedema (grade 3). The extent of inflammation involved mucosa, submucosa and muscle layer (grade 4). Extent of crypt damage included multiple ulcerations characterised by complete loss of the mucosal epithelium (grade 4).

As shown in Fig. 2D: Treatment of DSS- induced colitis by simvastatin at dose of (50 mg/kg/d) ($p < 0.01$ as compared to DSS- control group) reduced the extent of inflammation that involved mucosa and submucosa (grade 2) with focal mucosal infiltrations of neutrophils and lymphohistocytes (grade 1). Basal one third of the crypts were lost (grade 1).

While Fig. 2C demonstrated that simvastatin at dose of (5 mg/kg/d) showed non significant enhancement of DSS- induced colitis ($p > 0.05$ as compared to DSS- induced colitis group) with multifocal mucosal infiltrations of neutrophils and lymphohistocytes (grade 2). Inflammation extended to mucosa and submucosa (grade 2) with lost basal two thirds of crypts (grade 2), but showed no or focal submucosal oedema (grades 0-1).

The sums of histopathological scores were between 9 and 16 in the vehicle treated DSS-groups. The sums of histopathological scores were between 5.00 -11.00 in small dose of Simvastatin - treated group, while those in large dose of Simvastatin -treated group were between 4.00-9.00 which is statistically significant (Table 5).

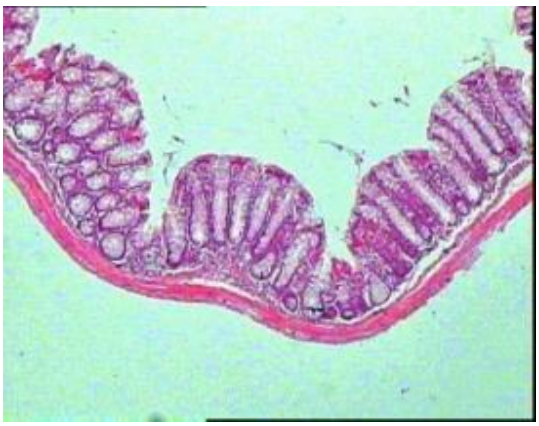

Fig. 2A. Histology of the mouse colon in vehicle control tissues, showing normal histological appearance of the mouse colon

4. DISCUSSION

In the present study, DSS- induced colonic and systemic inflammation in mice when administered for 7 days in drinking water as evident from the DAI. DSS--induced colitis is a well established experimental model that mimics many of the features of human UC, including diarrhea, bloody feces [14] and colonic shortening [15]. These effects of DSS- on the colon were explained by the fact that DSS- can promote inflammation by many biological pathways including direct cytotoxic effects [16] as well as apoptotic damage of colonic epithelial cells [17].

Table 2. Histopathological scores

Grade	Extent of inflammation	Infiltration neutrophils+ Lymphohistio-cytes	Extent of crypt damage	Crypt abscesses	Sub-mucosal oedema	Loss of goblet cells	Reactive epithelial hyperplasia
0	*None*	*None*	*None*	*None*	*None*	*None*	*None*
1	*mucosa*	*focal*	*Basal one third*	*focal*	*Focal*	*focal*	*focal*
2	*Mucosa+ submucosa*	*multifocal*	*Basal two thirds*	*multifocal*	*multifocal*	*multifocal*	*multifocal*
3	*Mucosa+submucosa+ muscle layer*	*diffuse*	*Entire crypt damage*		*diffuse*	*diffuse*	*diffuse*
4	*Transmural*		*Crypt damage+ ulceration*				

Table 3. Effect of different doses of Simvastatin on colonic length & serum TNF alpha in DSS- -induced colitis in mice

Groups *(n=6)*	Control group	DSS-- induced colitis group	Simvastatin (5 mg/kg/d) treated group	Simvastatin (50 mg/kg/d) treated group
Colonic length *(cm)* Mean ±SD	8.25±0.23	6.10±0.56 ^{A}	6.72±0.33 ^{C}	7.18± 0.28 ^{B} ***
Serum TNF alpha(Pg/ml)	30.83±9.91	191.62±58.86 ^{A}	102.52±37.31 ^{B}***	81.03±38.86 ^{B}***

^{A}, p < 0.001 compared with the normal group; ^{B}, p < 0.001 compared with DSS- induced colitis control group; ^{C}, p > 0.05 compared with DSS- induced colitis control group

Table 4. Effect of different doses of Simvastatin on oxidative stress markers activity in colonic tissue in DSS-- induced colitis in mice

Groups *(n=6)*	Control group	DSS-- induced colitis group	Simvastatin (5 mg/kg/d) treated group	Simvastatin (50 mg/kg/d) treated group
Colonic tissue Nitric oxide (µmol/L)	11.12 ±5.43	45.75±10.47 ^{A}	37.10±11.75 ^{C}	24.82±6.73 ^{B}*
Colonic tissue malondialdehyde (MDA)(nmol/mg)	2.95±0.38	7.63±0.66 ^{A}	5.78±1.00 ^{B}***	4.57±0.85 ^{B}***
Colonic tissue *GSH*	1.57±0.36	0.67±0.19 ^{A}	1.19±0.65 ^{C}	1.43±0.41 ^{B}*

*^{A}, p < 0.001 compared with the normal group; ^{B} Significantly different from DSS- group at *p < 0.05 ** p < 0.01 and *** p < 0.001; ^{C}, Non significantly different p >0.05 as compared to DSS- induced colitis group*

Table 5. Effect of different doses of Simvastatin on histopathological score in DSS--induced colitis in mice

Groups *(n=6)*	Control group	DSS-- induced colitis group	Simvastatin (5 mg/kg/d) treated group	Simvastatin (50 mg/kg/d) treated group
Histopathological score	0.00	9.50±8.00-16.00 ^{A}	5.50±2.00-11.00 ^{C}	4.00±2.00-9.00 ^{B}**

*Data are presented in form of median and range (Minimum – maximum) and were analyzed by non parametric Kruskal–Wallis test; ^{A,B} Significantly different as compared to DSS- group (at ** p < 0.01 and *** p < 0.001) ^{C} Non significantly different (p >0.05) as compared to DSS-- induced colitis group*

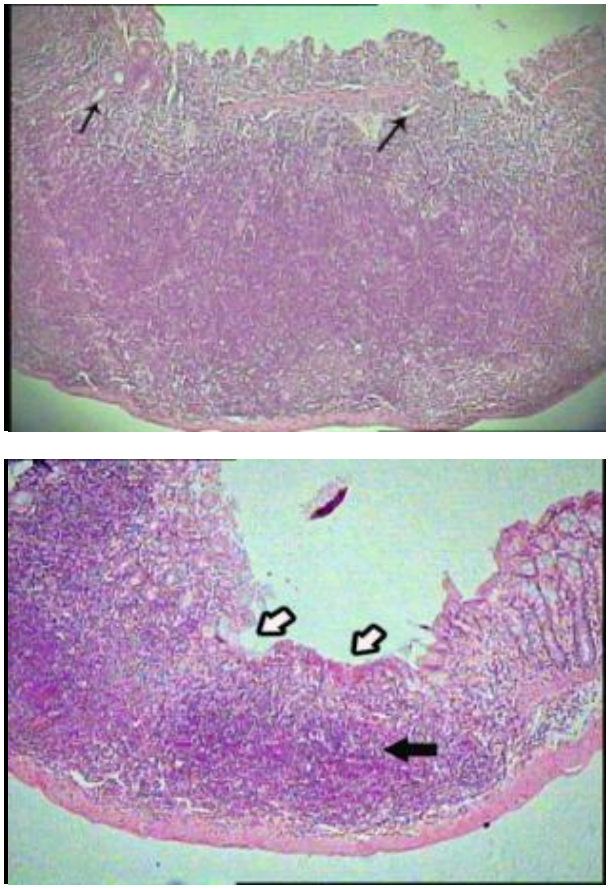

Fig. 2B. Histopathological findings of hematoxylin and eosin-stained colonic tissue sections from DSS--treated mice

Open arrows show lost entire crypts with ulceration and diffuses loss of goblet cells. Bold black arrow shows diffuse inflammatory infiltrate involved mucosa, submucosa and muscle layer. Thin arrows show submucosal edema

In the present study, colitis led to a significant increase in the pro-inflammatory markers in the plasma of mice as apparent from increased levels of TNF-α in the plasma of DSS- treated animals as compared to the control animals.

This is in consistent with study of Nishiyama et al. [26] who showed an elevation of the disease activity index score and histological damage score induced by DSS-. Based on the changes in tumor necrosis factor-alpha in plasma. Furthermore, Oz et al. [27] found that Inflammatory cytokine levels like TNF-*α* was considerably increased in DSS--induced moderately severe colitis in wild type mice. In the present study, inflammation in the colon led

to the generation of oxidative stress as indicated from a significant increase in the MDA and NO parameters in the colonic tissue as compared to the control group. Similar findings have been previously reported [28,29]. The production and release of ROS species by immune cells appear to play an important role in the pathophysiology of colitis [30]. Increased MDA level in stress condition is responsible for lipid membrane destruction and tissue injury [31]. NO reacts with O2 -produced by activated neutrophils- to form another potent oxidant, peroxynitrite (ONOO). ONOO administration to the colon results in tissue injury [32]. However, inducible nitric oxide synthesase (iNOS)-derived NO stimulates TNF-α production in the middle and distal colon, which promotes the infiltration of neutrophils for example through stimulation of synthesis of intracellular adhesion molecule (ICAM) and P-selectin, therefore leading to colonic tissue damage [33]. Furthermore, in the current study, there was a reduced GSH level in the colonic tissue when compared to the control group. Depletion of GSH is considered a crucial event of colonic damage occurring both in human IBD and in animal models [34]. This depletion could be a consequence of enhanced production of free radicals and could represent a specific disorder due to an impaired colitis activity of GSH synthesizing enzyme [35].

Furthermore, the sums of histopathological scores were between 9 and 16 in the non-treated DSS- groups in all studies [21]. Histological signs of colonic inflammation were focal mucosal infiltrations of predominantly neutrophils and lymphohistocytes (grade 2), multifocal submucosal oedema (grade 2). The extent of inflammation affected mucosa, submucosa and muscle layer (grade 3). These were evident in Fig. 2B.

Treatment of DSS- induced colitis by simvastatin at dose of (50 mg/kg/d) reduced the extent of inflammation that involved mucosa and submucosa (grade 2) with focal mucosal infiltrations of neutrophils and lymphohistocytes (grade 1). Only basal one third of the crypts were lost (grade 1). These were evident in Fig. 2C.

In the present study, simvastatin in small and large dose reduced serum level of TNF- α in mice with experimentally-induced colitis. Park et al. [36] suggested that simvastatin inhibited monocyte adhesion to endothelial cells induced by TNF-alpha. Simvastatin protects endothelial progenitor cells from TNF-α- mediated apoptosis [37]. Furthermore, Lee et al. [19] reported that simvastatin inhibits NF-κB signaling in intestinal epithelial cells leading to suppression of multiple pro-inflammatory cytokines as TNFα and ameliorates acute murine colitis. Statins also inhibit interactions between leukocytes and endothelial cells; intravital microscopy confirms that statins inhibit leukocyte-endothelial cell interaction in post-mesenteric venules of rats [38].

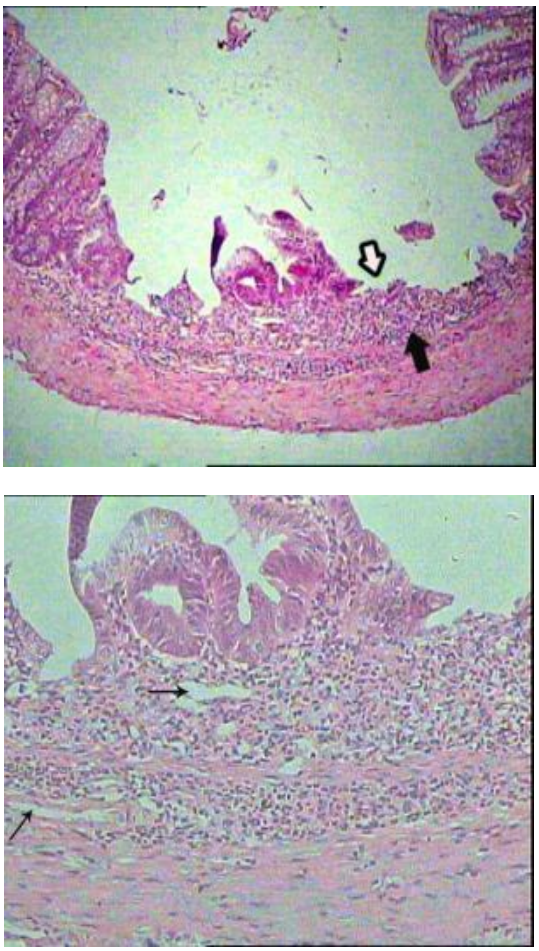

Fig. 2C. Histopathological findings of hematoxylin and eosin-stained colonic tissue sections from DSS- induced colitis group treated with simvastatin 5mg/kg/d

Open arrow show lost entire crypts with ulceration and diffuses loss of goblet cells. Bold black arrows shows diffuse inflammatory infiltrate involved mucosa and submucosa. Thin arrows show submucosal edema

The result of the present study showed a significant reduction of MDA levels by simvastatin early treatment in small and large dose. Haendeler et al. [39] showed that

simvastatin pretreatment caused significant reduction in MDA level in peptic ulcer model and it was reported that simvastatin possesses free radicals scavenger activity. This suggests that simvastatin afforded part of its gastroprotective effect in peptic ulcer model via antioxidant activity [40].

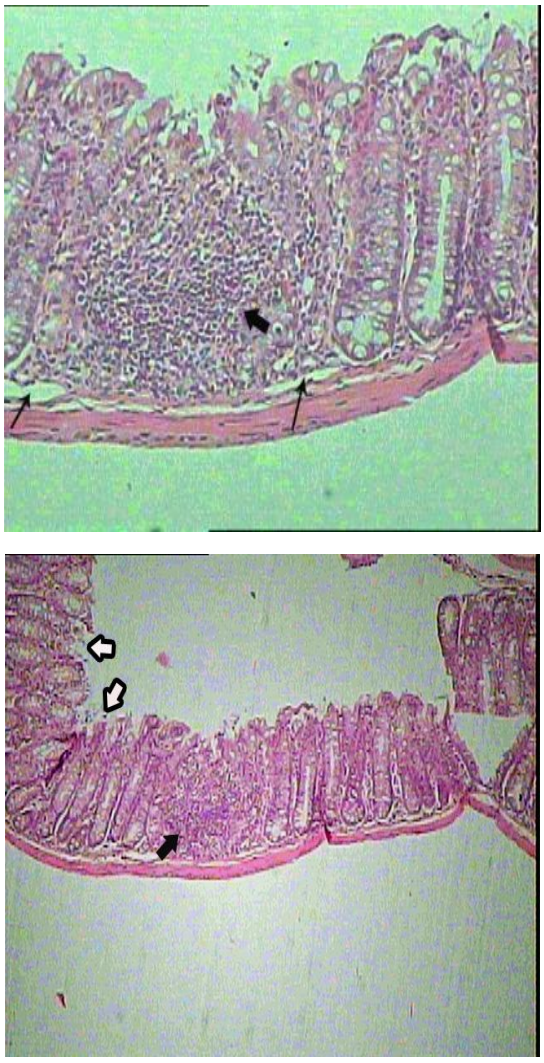

Fig. 2D. Histopathological findings of hematoxylin and eosin-stained colonic tissue sections from DSS- induced colitis group treated with simvastatin 50mg/kg/d

Open arrows show lost basal one third of the crypts. Bold black arrow show focal infiltrates involving mucosa only. Thin arrows show focal submucosal edema

In the present study, large dose of simvastatin produced a significant reduction of NO levels. Simvastatin reduces ischemia–reperfusion injury and prevents coronary endothelial cell and cardiomyocyte damage by NO-dependent mechanisms as simvastatin decreased nitrite production after ischemia–reperfusion.NO reacts with superoxide to form peroxynitrite which is considered a strong cytotoxic agent; promoting peroxidative damage of cell membranes. NO is increased by the induction of iNOS activity by various factors such as tumor necrosis factor- α [41]. Furthermore, simvastatin decreases nitrate plasma concentration and recovers vascular responsiveness during an experimental endotoxic shock [42].

5. CONCLUTION

It can be concluded that effects of simvastatin treatment were mostly dose dependant as simvastatin (50 mg/kg/d) showed highly significant effects on colon length, DAI, histopathological score, NO, MDA and TNF-α levels indicating that the latter dose has a more significant anti-inflammatory and antioxidant role in early treatment of DSS- induced colitis model. Clinically, simvastatin (50 mg/kg/d) can be used as an early treatment of IBD. However, simvastatin (100 mg/kg/d) has limited clinical application in human due to toxicity. So, in clinical application it is advised to use simvastatin with a dose of 5 mg/kg as an early treatment IBD. Further studies are needed to synergize this effect with another drug as an early treatment IBD.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate our local ethics committee with No. MS 437 Faculty of Medicine, Mansoura university.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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