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## Effect of acute Atorvastatin treatment in an experimental model of colitis

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<i>Article History:</i>	ABSTRACT	
Received on: 12.11.2018 Revised on: 07.04.2019 Accepted on: 10.04.2019	<p>Acute atorvastatin treatment in an experimental model of colitis was studied using the acetic acid induction modality for colitis in rats. This study was aimed to evaluate possible therapeutic effects of atorvastatin against acetic acid-induced colitis in a rat model and to find out the correlation between severity index with oxidative stress parameters and inflammatory markers. Experimental colitis was induced in rats by rectal administration of 4% acetic acid (vol/vol). Rats with colitis were received either atorvastatin 10mg/kg or sulfasalazine 100mg/kg orally for 7 days. Macroscopical and microscopical assessment and the measurement of the colonic cytokines (IL-6 and TNF-<math>\alpha</math>), oxidative stress markers; myeloperoxidase (MPO) and malondialdehyde (MDA), and adhesion molecules (E-Selectin and ICAM-1). Both the macroscopical lesion area and histological colonic injury induced by acetic acid were reduced significantly by both atorvastatin and sulfasalazine. These were associated by attenuation of the increased colonic MPO activity, MDA and proinflammatory cytokines. In addition, there is the downregulation of the adhesion molecules. In the present study, Atorvastatin had shown therapeutic effects in experimental colitis. The anti-inflammatory actions involve antioxidant effect related to phenolic moiety along with inhibition of adhesion molecule synthesis in the colonic tissues.</p>	
<i>Keywords:</i>		
Atorvastatin, Phenolic moiety, Ulcerative colitis, Oxidative stress		

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### INTRODUCTION

Inflammatory bowel diseases (IBD) is a chronic idiopathic inflammatory disorder of two types; ulcerative colitis and Crohn's disease (Qureshi *et al.*, 2010). They are increasingly worldwide in their incidence and prevalence, causing significant morbidity and poor quality of patient life (Molodecky *et al.*, 2012). Despite the fact that little is known about etiology of these diseases, it is believed that

initial tissue damage is due to amplification of abnormal host response to endogenous or environmental or immunological factors (Hendrickson *et al.*, 2002). IBD is also associated with extensive inflammatory infiltrates in the lamina propria characterized by extensive inflammatory infiltrates consisting of polymorphonuclear neutrophils, eosinophils, and plasma cells, leading to a remarkable production of unstable chemical species such as nitrogen and reactive oxygen species, significantly involved in injury (Oz *et al.*, 2005, Martin and Wallace, 2006). Activation of these inflammatory infiltrates results in the production of different pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin-1  $\beta$  (IL-1  $\beta$ ), an IL-6, plays a crucial role in tissue disruption and ulceration (Kolios *et al.*, 1998). Although extensive development has been made in the treatment of ulcerative colitis, side effects, and incomplete therapeutic effects of currently used medications is a continuous challenge (Esraa *et al.*, 2016). For this reason, there is a need to develop new strategies that could restore the altered immune response that emerges in the inflamed intestine. One of the important

strategies is targeting adhesion molecules that expressed on the surface of endothelial cells and lymphocyte; which in fact recruits immune and inflammatory cells from the periphery into the site of inflammation. On the other hand, there is evidence that nitric oxide (NO) inhibits the expression of adhesion molecules on endothelial cells, which is an important step in neutrophil migration (Santos *et al.*, 2005). Atorvastatin is one member of hypolipidemic agents called statins that are 3-hydroxy-3 methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors (Mary *et al.*, 2015). Statins have a potent anti-inflammatory effect and endothelial cell protective actions independent of their hypolipidemic action (Rajesh *et al.*, 2015). Cardioprotective effect of statins has been linked to elevated markers of systemic inflammation, which may occur through sterilization of inflamed atherosclerotic plaques, prevent plaque rupture and coronary occlusion (Ridker *et al.*, 2001). Moreover, statins can increase endothelial nitric oxide synthase (eNOS) expression by blocking Rho geranyl granulation (Endres and Laufs, 2004). Statins also can antagonize the lymphocyte function-associated antigen-1 (LFA-1)-ICAM -1 interaction by binding to the L-site (Weitz-Schmidt *et al.*, 2001). On the other hand, statins also reduced highly sensitive C-reactive protein levels and vascular pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 (Maheshwari *et al.*, 2015). Moreover, the cytoprotective effect of statins against ischemic injury has been documented as it reduced neutrophil -induced cardiac contractile dysfunction (Mohsen *et al.*, 2015). Furthermore, experimental studies showed that statins attenuate thrombin-induced leukocyte traffic by potentiating the endothelial release of NO and down-regulation endothelial P-selectin (Stalker *et al.*, 2001). Interesting atorvastatin has a fluorophenyl moiety in its structure, which is mediate its strong reducing power and subsequent antioxidant effect (Istvan *et al.*, 2003).

## MATERIALS AND METHODS

Adult male albino rats (200-220g) were purchased from the animal house of the national center for drug control and researches (NCDCR). The animal was housed five per cage for one week prior to the experiment and had access to laboratory chow pellet and was allowed to drink water *ad libitum*. Experiments were performed after getting prior permission from the Ethics Committee, College of Medicine, Al-Nahrain University.

Drugs: Atorvastatin and sulfasalazine were purchased from Sigma -Aldrich company.

**Experimental Design:** This study was conducted on 40 adult male albino Wistar rats weighing 200-220g previously submitted to starvation for at least 24hrs. Animals were divided into four groups

(n=10/group). Group, I kept as control and received no treatment. Group II, III, IV were subjected to the induction of colitis by rectal administration of 4% acetic acid (AA) (v/v). Thirty minutes after the induction of colitis group II was given normal saline orally; group III and IV were treated orally with atorvastatin 10mg/kg and sulfasalazine 100mg/kg respectively for 7 days.

## Induction of colonic inflammation

Since prior feeding has been shown to prevent the ulcerogenic action of certain drugs and chemical (Robert and Dale, 1971). Rats were starved for at least 24hrs before the induction of colitis but were be allowed free access to tap water, during starvation, rats were kept in cages provided with a wide wire-mesh floor to avoid coprophagy. On the day of the experiment, water was held two hours before the procedure. Experimental injury in colonic tissue was done according to the method described by Mousavizadeh *et al.* (2009). In brief, under ether anaesthesia rats were administered 5ml/kg of 4% acetic acid solution (BDH Chemical Ltd., England) by transrectally using a silicone plastic tube with an external diameter of 2mm was inserted rectally into the colon to 8cm. After acetic acid administration, rats were holed horizontally for 2 min to prevent AA leakage. Control animals undergo the same procedure using an equal volume of saline instead of the acetic acid solution.

## Preparation of drugs

All drugs were freshly prepared before administration on the day of the experiment. Investigated drug (Atorvastatin) and the standard sulfasalazine were prepared as suspensions in distilled water using sodium Carboxymethyl cellulose (s CMC) 0.3% W/V. Atorvastatin was used at a dose 10mg/kg (these doses were chosen depending on previous studies that have been showing their gastroprotective activity at a concentration 10mg/ml (Matloub *et al.*, 2012). Sulfasalazine was used as standard therapy in a dose of 100mg/kg (Paula *et al.*, 2012).

## Assessment of colitis

After the end of the experiment, rats were killed by an overdose of diethyl ether inhalation, and then rapidly, the abdomen was dissected and open, and the colon was removed. The pieces of colons were cut open in an ice bath cleansed gently using saline and observed for macro and microscopic assessment. Then samples were cut into two pieces, one piece for histopathologic assessment (maintained in neutral formalin 10% as a fixator) and one piece for the immunohistochemistry study.

## Macroscopic evaluation

**Colonic mucosal damage** (mean area of colonic mucosal damage)

The excised colonic segment (8 cm proximal to anus) was immediately immersed in normal saline, cleaned from adherent tissues and then opened longitudinal and rinsed with 0.9% sodium chloride solution to discard the faecal materials. Then the segment was fixed with pins on a dissecting board, and the area of mucosal damage was measured using a computerized planimeter in accordance with the method described earlier (Warzecha *et al.*, 2012).

**Colon oedema:** The colon specimen of each animal was incised along its mesenteric border and gently washed. This is measured through colon weight (CW). It was used as an index of tissue oedema, which reflected the severity of colitis (Mohsen *et al.*, 2015).

## Disease activity index (DAI)

To quantify the clinical evaluation of the disease we used the DAI described by Meerveld and Tyler (Meerveld and Tyler, 2006) that based which include body weight loss stool consistency, rectal bleeding (gross or occult) we used five grades of weight loss {0, no loss or weight gain; 1, 1-5% loss; 2, 6-10% loss; 3: 11-15% loss; 4: greater than 15% loss, three grades of stool consistency {0: Normal; 2: loose; 4: diarrhea}, and three grades of bleeding {0: normal; 2 occult blood –positive; 4: gross bleeding}. The presence of occult blood in faces was determined using the benzidine test. The total score of DAI was calculated as combined of these scores divided by 3 (Mao *et al.*, 2012).

## Macroscopic colonic score

The macroscopic colonic score was assessed by the scoring system (Kuralay *et al.*, 2003) as following: score is assigned based on the clinical features of the colon using a scale ranging from 0-4 as follows: 1, intact epithelium with no damage; 2, patch type superficial hyperemia; 3, generalized patch type hyperemic regions; 4, generalized hyperemic and haemorrhage.

## Histological evaluations:

The colonic samples were fixed in 10% formalin, dehydrated, embedded in paraffin, deparaffinized with xylene, cut into 4  $\mu$ m sections and stained by Hematoxylin and eosin (H&E). Slides were examined and scored for histopathological evaluation. The slides were coded to prevent observer bias during evaluation. All tissue sections were examined in a blinded fashion by an experienced histopathologist, and results scored, according to Cooper *et al.* (1993).

## Immunohistochemistry

Immunohistochemistry offers the advantage of directly demonstrating cells in the affected tissue (Pedro *et al.*, 2005). The advent of specific antibodies developed for immunohistochemical reactions, together with the standardization of a specific method to meet the objectives of the present study, permitted analysis of the production of various biochemical markers in the paraffin-embedded intestine samples for measurement of the colonic cytokines (IL-6 and TNF- $\alpha$ ), oxidative stress markers (myeloperoxidase (MPO) and malondialdehyde (MDA)), and adhesion molecules (CD62 and ICAM-1). Quantification of IHC was performed according to the following semiquantitative scores (Hernández-Rodríguez *et al.*, 2004) based on the percentage of positively stained cells as following: 0, no staining; 1,  $\leq$  25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%.

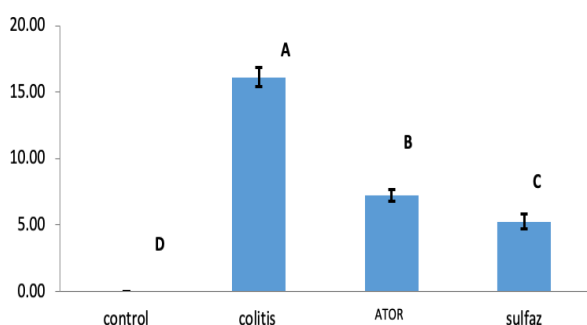
## Statistical analysis

Results were collected, summarized, analyzed, and presented using three statistical software programs: the statistical package for social science (SPSS version 22), Microsoft Office Excel 2013, and Med Calc 2014. Numeric variables were presented as mean and standard deviation. The results of Kolmogorov Smirnov test of normality distribution for numeric variables were significant, and comparison of mean values among groups was carried out using Kruskal Wallis Test, and then a comparison between any two groups was made using Mann Whitney U test. Spearman correlation test was used to evaluate correlations between histological scores and immune histochemical expression scores. P-value was significantly considered when it was equal to or less than 0.05 (Daniel, 2009).

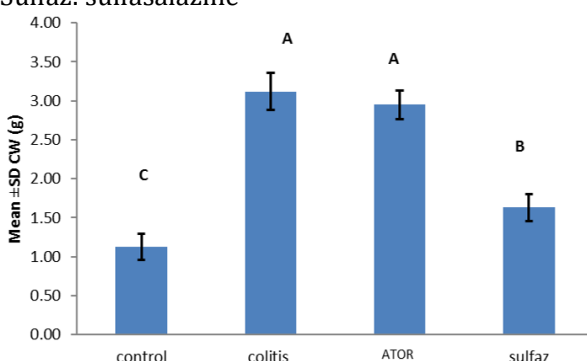
## RESULTS

Rectal administration of acetic acid was applied in this study is one of the modalities that has been used to produce macroscopical colonic mucosal injury in rats. Acetic acid elicited an intense inflammatory reaction on the 7<sup>th</sup> day of colitis; the distal colon showed severe edematous inflammatory injury. The colonic mucosa was inflamed, hyperemic, and hemorrhagic compared to the normal animals. However, oral administration of atorvastatin and sulfasalazine after the induction of colitis significantly ( $p < 0.01$ ) attenuate the colonic damage scores, as shown in figure 1. Nevertheless, atorvastatin failed to produce a significant reduction in the colonic weight as compared with sulfasalazine group that demonstrated significant ( $p < 0.05$ ) reduction effect comparable to the normal group, as shown in figure 2. However, both atorvastatin and sulfasalazine showed a significant decrease ( $p < 0.01$ ) in DAI, as shown in figure 3. Furthermore,

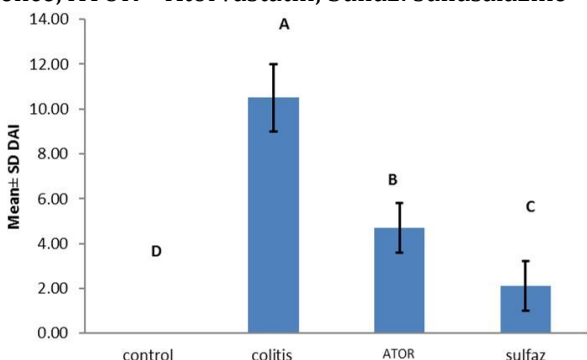
both drugs elicit significant ( $P < 0.01$ ) decrease in the macroscopic score, as shown in figure 4.



**Figure 1: Mean area of mucosal damage in control and study groups;** Capital letters for comparison; different letters indicate significant difference; similar letters indicate insignificant difference; MD: Mucosal damage; ATOR = Atorvastatin; Sulfaz: sulfasalazine



**Figure 2: Mean colonic weight (CW) in gram in control and study groups;** Capital letters for comparison; different letters indicate significant difference; similar letters indicate insignificant difference; ATOR = Atorvastatin; Sulfaz: sulfasalazine

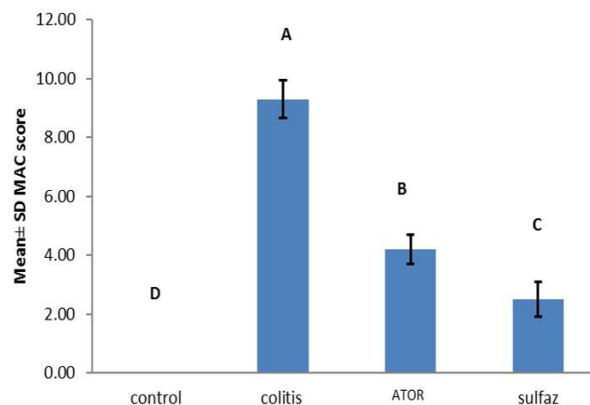


**Figure 3: Mean disease activity index (DAI) in control and study groups;** Capital letters for comparison; different letters indicate significant difference; similar letters indicate insignificant difference; ATOR = Atorvastatin; Sulfaz: sulfasalazine

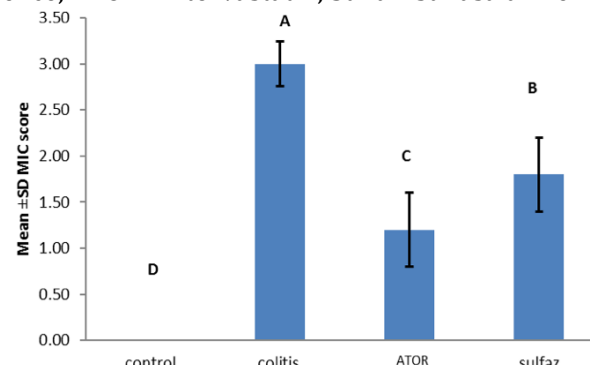
**Effect of atorvastatin on histopathological features**

The present study demonstrated characteristic histological features in untreated colitis, essentially the loss of intestinal crypt architecture and sloughing of intestinal cells, reduced goblet cell number and presence of different inflammatory

cell compared to normal as demonstrated in figure 6 and 7. On the other hand, atorvastatin and sulfasalazine treated groups; the histopathological changes were significantly ( $P < 0.05$ ) attenuated as judged by epithelization of colonic mucosa, reduction of oedema and neutrophil infiltration as shown in figure 8. Both of these drugs revealed a significant decrease in the pathological scores as compared with the colitis group, figure 5.



**Figure 4: Mean macroscopic score (MAC) in control and study groups;** Capital letters for comparison; different letters indicate significant difference; similar letters indicate insignificant difference; ATOR = Atorvastatin; Sulfaz: sulfasalazine



**Figure 5: Mean histopathological score (MIC) in control and study groups;** Capital letters for comparison; different letters indicate significant difference; similar letters indicates insignificant difference; ATOR = Atorvastatin; Sulfaz: sulfasalazine

**Effect of atorvastatin on adhesion molecules (ICAM-1 and CD62)**

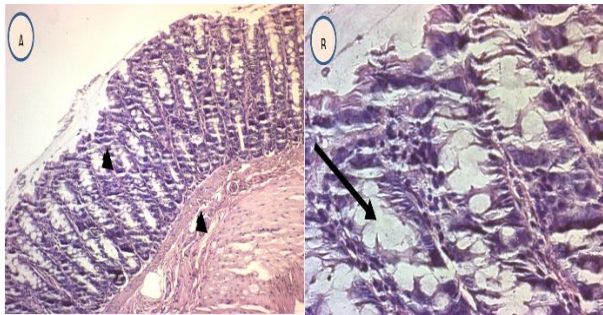
The increased colonic ICAM-1 in the colitis group was found to be decreased significantly ( $p < 0.05$ ) after atorvastatin and sulfasalazine treatment, as shown in figure 9. Also, both tested drugs cause significant ( $p < 0.01$ ) decrease in CD62 compared to colitis group, as shown in figure 10.

**Effect of atorvastatin on proinflammatory cytokines (TNF-  $\alpha$  and IL-6).**

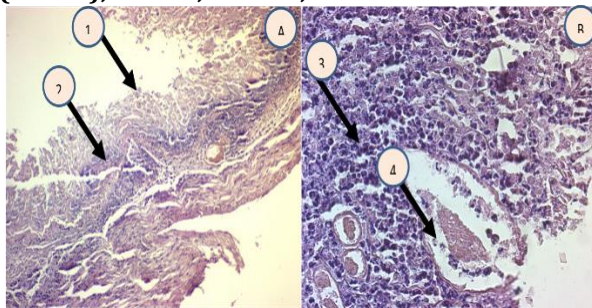
As shown in figures 11 and 12, colonic levels of TNF-  $\alpha$  and IL-6 showed a drastic raise after acetic acid introduction compared to those of the control group. In contrast, these values were significantly



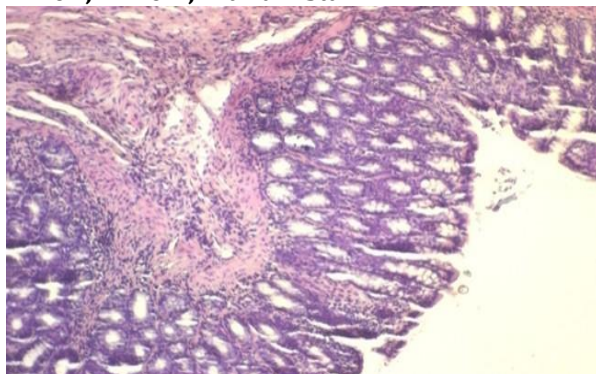
( $p < 0.05$ ) lower in rats treated with atorvastatin and sulfasalazine. However, atorvastatin significantly ( $p < 0.05$ ) decrease pro-inflammatory cytokines as compared with sulfasalazine treated group.



**Figure 6: Histological section through colonic wall showing a normal mucosal and submucosal pattern with no evidence of inflammation (arrowhead) and preservation of goblet cells (arrow); A: 10X; B: 40 X; H and E stain**



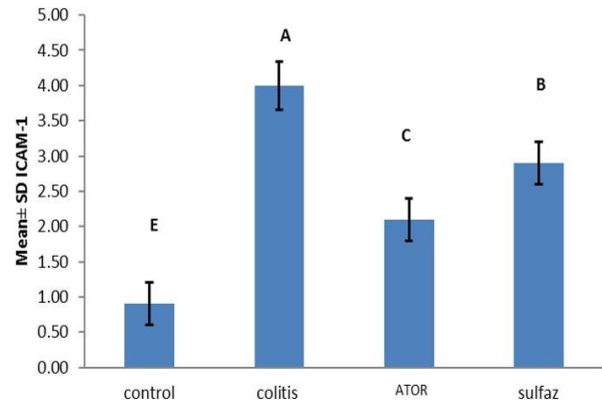
**Figure 7: Histological section through colonic wall showing mucosal ulceration (1); superficial inflammation (2); mononuclear inflammatory infiltrate (3) and crypt abscess (4) in experimentally induced colitis in the rat; A: 10X; B: 40 X; H and E stain**



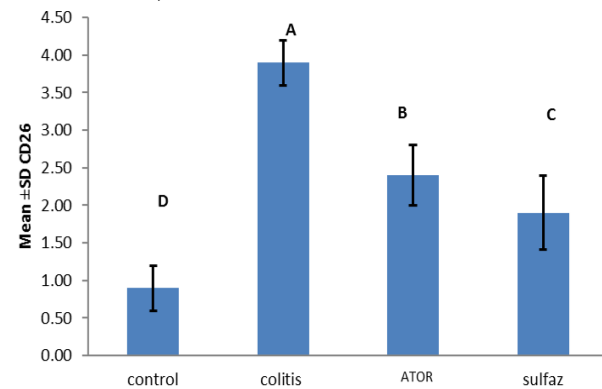
**Figure 8: Histological section through colonic wall showing atorvastatin effect in which there is evidence of mucosal regeneration and glandular formation, less severe inflammation and goblet cells regeneration; A: 10X; B: 40 X; H and E stain**

**Effect of atorvastatin on oxidative stress markers (MDA and MPO):** Administration of atorvastatin or sulfasalazine to acetic acid treated rats significantly ( $p < 0.05$ ) reduced MDA compared to the colitis group, as shown in figure 13. On the other

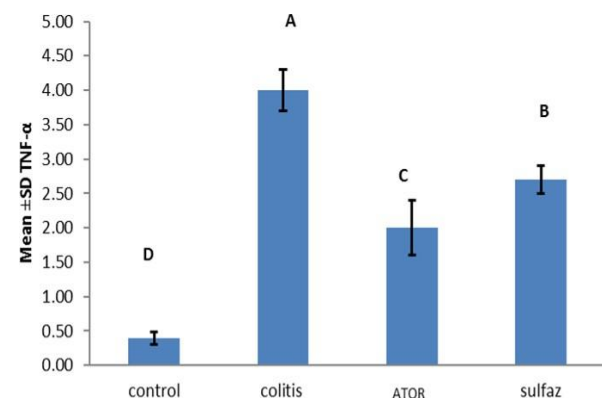
hand, treatment with either atorvastatin or sulfasalazine significantly ( $p < 0.01$ ) decreased acetic acid-induced myeloperoxidase production in tissue as depicted in figure 14.



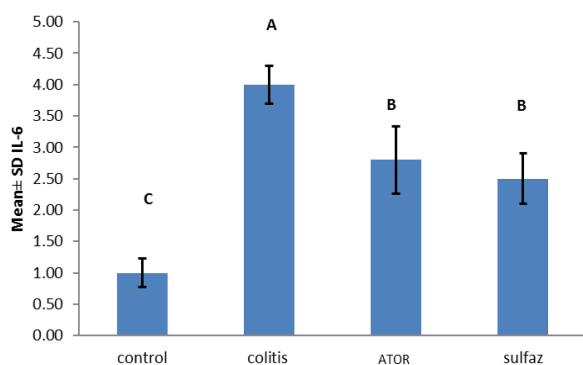
**Figure 9: Mean intracellular adhesion molecule-1 (ICAM-1) score in control and study groups; Capital letters for comparison; different letters indicate significant difference; similar letters indicate insignificant difference; ATOR = Atorvastatin; Sulfa: sulfasalazine**



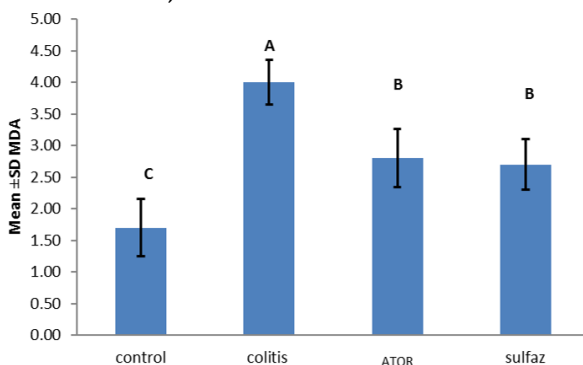
**Figure 10: Mean CD62 score in control and study groups; Capital letters for comparison; different letters indicate significant difference; similar letters indicate insignificant difference; ATOR = Atorvastatin; Sulfa: sulfasalazine**



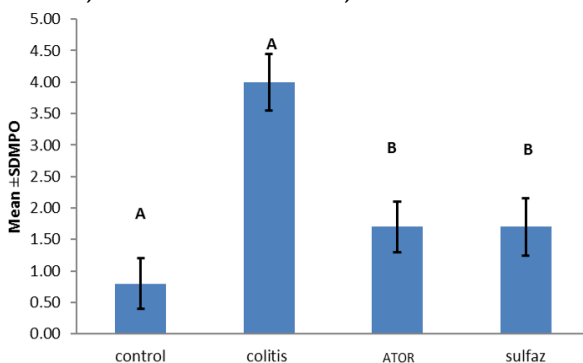
**Figure 11: Mean TNF-α score in control and study groups; Capital letters for comparison; different letters indicate significant difference; similar letters indicate insignificant difference; ATOR = Atorvastatin; Sulfa: sulfasalazine**



**Figure 12: Mean IL-6 score in control and study groups;** Capital letters for comparison; different letters indicate significant difference; similar letters indicate insignificant difference; ATOR = Atorvastatin; Sulfaz: sulfasalazine



**Figure 13: Mean malondialdehyde (MDA) score in control and study groups;** Capital letters for comparison; different letters indicate significant difference; similar letters indicate insignificant difference; ATOR = Atorvastatin; Sulfaz: sulfasalazine



**Figure 14: Mean myeloperoxidase (MPO) score in control and study groups;** Capital letters for comparison; different letters indicate significant difference; similar letters indicate insignificant difference; ATOR = Atorvastatin; Sulfaz: sulfasalazine

## DISCUSSION

The present study demonstrated that the effect of atorvastatin on experimentally induced colitis in rats and concluded that atorvastatin significantly improved gross changes in rat colon. These results are correlated with the finding of Maheshwari *et al.* (2015). Additionally, Mohsen *et al.* (2015) also noticed a marked improvement in rat colonic mucosa, gross appearance, following administration of

statin. However, the current study showed that atorvastatin had been failed to decrease the colonic weight in experimentally induced colitis in rats and this finding was comparable to Jahovic *et al.*, (2006) study that found the administration of statins did not significantly reduce colonic weight. Atorvastatin resulted in marked reduction on DIA in experimentally induced colitis, and this result is in accordance with the findings of Maheshwari *et al.*, (2015). Moreover, atorvastatin in the present study reduced macroscopic score and a histopathological score of the colon in experimentally induced colitis, and this finding is correlated with Jahovic *et al.*, (2006). One proposed protective mechanism of atorvastatin is through reduced expression of intercellular adhesion molecules such as ICAM-1 and E-selectin. The present study also showed, in accordance with Sasaki *et al.*, (2003) that administration of atorvastatin had significantly reduced the immunohistochemical expression of adhesion molecules (ICAM-1 and E-selectin) in the mucosa of rats undergoing experimentally induced colitis. The reduced level of adhesion molecule by a statin is thought to be mediated through upregulation of NO production by endothelial cells (Naito *et al.*, 2006). The present study has also shown that administration of atorvastatin causes a significant reduction in immunohistochemical expression of IL-6 and TNF- $\alpha$  in the colonic mucosa of experimentally induced colitis in rats. This finding is in accordance with Maheshwari *et al.*, (2015); Mohsen *et al.*, (2015); Iseri *et al.*, (2009). Another proposed mechanism for statin is its anti-oxidant potential mediated by fluorophenyl moiety, which acts as a free radical scavenger (Dai and Mumper, 2010). Inconsistency with the anti-oxidant effect of atorvastatin, it has been shown in the present study that the addition of atorvastatin caused a marked reduction in immunohistochemical expression of both MPO and MDA (Maheshwari *et al.*, 2015, Matloub *et al.*, 2012).

## CONCLUSION

Atorvastatin has potent anti-inflammatory and anti-oxidant effects that can be used successfully in the treatment of experimentally acetic acid-induced colitis in rats.

**Conflict of Interest:** The authors declare no conflict of interests.

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