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Reserch Article

EFFECT OF ATORVASTATIN (HMG-COA REDUCTASE INHIBITOR) ON EXPERIMENTALLY INDUCED COLITIS IN MICE

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ABSTRACT

Objective: Statins show pleiotropic effects that can make them act as new therapeutic agents to a variety of chronic inflammatory and autoimmune diseases, suggesting that even modest efficacy might provide a beneficial therapeutic action.

Methods: Adult mice of both sexes weighing 25-30g were used. The animals were classified into five groups. Group (I) served as control. In group (II) mice received carboxymethylcellulose 0.45mL (CMC). In group (III) mice received atorvastatin in a dose of 20mg/kg. In group (IV) Insult of the bowel was done by daily oral administration of indomethacin (1mg/mouse). In group (V) the mice received atorvastatin in addition to indomethacin. The parameters investigated include: disease activity index (DAI) that includes: body weight, colon length, consistency of colon content and macroscopic mucosal damage score. Serum level of C-reactive protein and tumor necrosis alpha TNF-alpha were measured in addition to histopathological studies.

Results: The body weights of mice received indomethacin alone or with atorvastatin showed significant decrease. Indomethacin administration induced high damage score, the total macroscopic score and DAI were high as well as significant elevation of the mean serum levels of C-reactive protein and TNF-alpha. The histopathological examination revealed severe loss of epithelium, mucosal ulceration, crypt disruption and inflammatory cellular infiltration in the colonic wall. Atorvastatin administration ameliorated significantly all the previous parameters.

Conclusion: Atorvastatin have the potential to ameliorate the colonic inflammatory response induced by indomethacin, suggesting that it could have important future applications in the treatment of IBD.

Keywords: Statins, colitis, Atorvastatin, Inflammatory bowel diseses, Disease activity index .

INTRODUCTION

Inflammatory bowel diseases (IBD) are considered chronic disorders. They are heterogeneous groups of diseases with unknown etiology, unspecific pathogenesis producing sever inflammation of the gastrointestinal tract. Inflammatory bowel diseases include two forms of intestinal inflammation: crohn's disease and ulcerative colitis. Ulcerative colitis causes mucosal inflammation which is limited to the large bowel (the colon) whereas crohn's disease virtually causes transmural chronic inflammation to any segment in digestive tract. [1 - 3].

IBD is characterized by tissue edema, increased gut epithelial permeability and extensive leukocyte infiltration of the gut [4, 5].

The literature suggests that multiple immune, genetic and environmental factors influence both the initiation and progression of colitis [4,5]. The IBDs are characterized by Pathological invasion of Inflammatory cells into Colonic mucosa and increased the concentration of soluble mediators of inflammation which may activate one another or have parallel effects.[6] The geographical studies showed differences in the rate of appearance of IBD in age, group, affected, onset, race and geographical areas [7].

Statins, the most widely prescribed cholesterol-lowering drugs, are considered to be first-line therapeutics for the prevention of coronary heart disease and atherosclerosis. Statins act by inhibiting the enzyme 3-hydroxy-3-methylglutaryl- COA (HMG-CoA) reductase, the rate-limiting enzyme in endogenous cholesterol biosynthesis, which catalyzes the reduction of HMG-CoA to mevalonic acid. Inhibition of this enzyme has proven to be effective for lowering plasma total cholesterol low-density lipoprotein-cholesterol, and triglyceride levels in humans and can therefore be useful to treat atherosclerotic and dyslipidemic disorders. [8] However, the clinical benefits of statins appear to extend beyond their lipid lowering effects. Besides reducing cholesterol biosynthesis, inhibition of mevalonate by statins also leads to a reduction in the synthesis of important intermediates, such as the isoprenoids farnesyl pyrophosphate and geranylgeranyl pyrophosphate. These intermediates are involved in the posttranslational prenylation of several proteins (e.g., Ras, Rho, Rac) that modulate a variety of cellular processes including cellular signaling, differentiation, and proliferation. Given the central role of these isoprenylated proteins in endothelial function, atherosclerotic plaque stability, platelet activity, coagulation, oxidation, and inflammatory and immunologic responses, it could be anticipated that statins may exert multiple beneficial effects in a broad spectrum of disorders including cardiovascular disease, osteoporosis, Alzheimer's disease and related vascular dementia, viral and bacterial infection. [9]

In the present study was designed to investigate the effect of atorvastatin on indomethacin-induced colitis in mice as an experimental model of inflammatory bowel disease.

The parameters investigated include disease activity index score (DAI): bodyweight, bloody stools, colon length and macroscopic mucosal damage serum level of C-reactive protein and tumor necrosis factor alpha (TNF- alpha). Histopathological study of the colons was studied in different groups of animals receiving drugs.

MATERIALS AND METHODS

Drugs Used: Atorvastatin calcium (Lipitor, Pfizer, Egypt, Company).

- Indomethacin (Liometacen, the Nile Company for pharma ceuticals and chemical Industries).

Chemical Used: Carboxy methyl cellulose 0.05% obtained from the laboratory of pharmaceutical department faculty of pharmacy. It was used to dissolve atorvastatin calcium.

Kits Used: Kit used for determination of serum level of c-reactive protein (CRP) that were obtained from Abazyme, LLC, USA. Reagents used for determination of serum tumour necrosis factor (TNF-α) that were obtained from Ray Biotech, Inc. USA.

Animals and Experimental Design:

This Study was conducted on mice of both sexes weighing 25- 30 gm. They bred in the animal house of Cairo University. They were caged in fully ventilated room, exposed to natural daily light/ dark cycle. They were allowed food and water ad-libitum. They were acclimatized for 4 days before randomly allocated to the following groups. The animals were handled according to the guidelines of Helsinki declaration rights (1975) of using laboratory animals.

The mice were classified into five groups: Group I: 12 normal mice received distilled water orally daily and served as control. Group II: 12 Normal mice received carboxymethyl cellulose (CMC) (0.45 ml) orally daily for 5 days. Group III: 12 normal mice received atorvastatin (20 mg / kg / day) orally for 5 days. Group IV: 20 normal mice received indomethacin (1mg/ mouse) daily orally for 5 days and served as a model of IBD. Group V: 20 mice received atorvastatin (20 mg/ kg/ day) on day one of induction of colitis by indomethacin. It was given orally for 5 days.

The animals of all groups were observed daily for the presence of any mortality. The disease activity index score (DAI), was used as an evaluation method for colitis. It include: measurement of body weight daily for monitoring any loss of body weight observed, diarrhea and bloody stool.

Blood samples were obtained from all groups of animals at the end of the duration of the experiment. Venous blood samples were obtained from retro-orbital venous plexus in mice of all groups by capillary glass tube at end of 5 days. The blood samples will be collected in the centrifuge tubes and incubated at 37C until blood clotted and then centrifuged to separate sera.

Serum samples were analyzed for measurement of: 1- Serum level of C-reactive protein (CRP). 2- Serum level of tumor necrosis factor (TNFα).

At the end of the experiment on day 6, and after collection of the blood samples, the animals of all were scarified The distal colons were isolated .There lengths were measured Then these colons were excised longitudinally from the antimesenteric border & observed for any hemorrhagic contents & ulceration Also the evaluation method included scores that monitored filled colon and stool consistency as measures of bowel dysfunction. Colon length shrinkage score was considered as an indicator of inflammation and functional changes. Macroscopic damage evaluation was based on gross observation of inflammation, erythema, hyperemia and ulcerations. This scoring method is routinely used and has a potential to offer mechanistic insights into the efficacy of experimental compounds. [10,11].

Histopathological Study

At the end of the experiment, after the macroscopic scoring of the collected colons, distal colon segments about four centimeters each, were fixed in 10% neutral buffered formalin and embedded in paraffin for light microscopic study. They were prepared for histopathological examination Sections were prepared and stained with Hematoxylin and eosin (H&E).

For each case, there was an assessment of histological changes in the: 1- Surface epithelium loss. 2- Crypts destruction 3- Inflammatory cell infiltration. 4- Smooth muscle architecture.

Statistical Analysis Methods:

Data were collected, checked, revised and entered the computer. Data were analyzed by SPSS statistical package version 17. Excel computer program was used to tabulate the results, and represent it graphically. Qualitative variables were expressed as percentages.

Qualitative variables were expressed as count and %. The significant difference in the distribution of the qualitative variables were tested by the Chi square test of distribution at $p<0.05$.

Quantitative variables from normal distribution were expressed as mean and S.D. One Way ANOVA used to declare the significant difference between groups at $p<0.05$. Duncan multiple comparison test at $p<0.05$ was used to declare the significant between each group and the control group.[12]

RESULTS

The body weights of mice received indomethacin alone or with atorvastatin showed significant decrease. Indomethacin administration induced marked shrinkage of colons and nearly devoid of fecal contents, indicating colonic hyper motility. High damage score was observed. The total macroscopic score and DAI were high. Moreover, indomethacin produced significant elevation of the mean serum levels of C-reactive protein and TNF-alpha. The histopathological examination of colon revealed severe loss of epithelium, mucosal ulceration, crypt disruption and inflammatory cellular infiltration in the colonic wall. Atorvastatin administration ameliorated significantly the DAI and the total macroscopic score. The serum levels of C-reactive protein and TNF-alpha were significantly lowered compared to colitis group. Moreover, the marked histological damage was also improved in atorvastatin treated mice.

Table 1: Table shows the changes in morality of mice in all groups

Groups	Total.no	No. of alive mice	No. of dead mice	% mortality
Control	12	10	-----	No mortality
CMC	12	9	2	25%
Ator	12	10	2	16.7%
Indo-colitis	20	9	11	55%
Indo+Ator	20	10	10	50%
chi-square			10.00	
P-value			0.019*	

*= there is a significant difference in distribution by using Chi-square test at $p<0.05$

Table 2: Table shows the mean body weight of mice (g) of different groups at the end of the experiment.

Groups	Minimum	Maximum	Mean + SD	% Reduction
Normal (group 1)	24.00	32.00	28.13± 3.40	-
CMC (group II)	22.00	32.00	27.13± 3.80	3.55%
Atorvastatin (group III)	23.00	30.00	26.63± 2.92	5.33%
Indomethacin induced colitis (Model) (Group IV)	17.00	25.00	20.50± 2.98	27.12%
Indomethacin + Atorvastatin (Group V)	17.00	25.00	21.38± 2.97	24%
F-ratio			9.576	
P-Value			0.000*	

S.D = Standard Deviation.

* = Significant difference between groups by using one way ANOVA at $p < 0.05$ the same letter means that no significant difference between the two groups by using Bonferroni multiple comparison test at $P < 0.05$.

Table 3: Table shows the mean ± SD) macroscopic changes observed in mice of different groups; the disease activity index (DAI), {n = 8}.

Groups	Colon length (cm)	The score of consistency of colon content	Colon damage score	Total macroscopic score
Control (group 1)	9.56 ± 0.94 8- 11 cm	0.00	0.00	0.00
CMC (group II)	10.13 ± 0.92 9-11.5 cm	0.75 ± 0.89 0.00 - 2.00	0.00	0.75 ± 0.89 0.00 - 2.00
Atorvastatin (group III)	9.75 ± 0.80 8.50 -11 cm	0.00	0.00	0.00
Indomethacin induced colitis (Model) (Group IV)	6.56 ± 1.02 5.50-8.00 cm	3.63 ± 1.19 2.00-5.00	2.88 ± 0.83 2.00 - 4.00	9.25 ± 1.75 6.00 - 11.00
Indomethacin + Atorvastatin (Group V)	8.75 ± 1.00 7-10 cm	1.63 ± 0.74 1.00 - 3.00	1.88 ± 0.64 1.00 - 3.00	4.63 ± 1.77 3.00 - 8.00
P-Value	F- ratio 18.483 One way ANOVA 0.000*	Value: 32.646 Kruskal-Wallis Test 0.000*	Value: 37.188 Kruskal Wallis Test 0.000*	Value: 35.592 Kruskal-Wallis Test 0.000*

* = significant $p \leq 0.05$.

Table 4: Table shows the mean serum level of C-reactive protein and TNF-alpha (ng / ml) in different groups.

Groups	C-reactive protein	TNF-alpha
	Mean + SD	Mean + SD
Control (group 1)	$22.75^{a} \pm 1.12$	$185.88^{a} \pm 11.80$
CMC (group II)	$26.38^{b} \pm 1.30$	$190.38^{a} \pm 8.80$
Atorvastatin (group III)	$24.13^{a} \pm 0.83$	$199.13^{b} \pm 6.15$
Indomethacin induced colitis (Model) (Group IV)	$51.38^{c} \pm 4.63$	$305.88^{c} \pm 7.81$
Indomethacin + Atorvastatin Group V)	$29.75^{b} \pm 1.49$	$200.25^{b} \pm 2.82$
F-ratio	181.31	314.569
P-Value	0.000*	0.000*

S.D = Standard Deviation.

* = Significant difference between groups by using one way ANOVA at $p \leq 0.05$ the same letter means that no significant difference between the two groups by using Bonferroni multiple comparison test at $P < 0.05$.

DISCUSSION

The results of the present study showed that the body weights of mice received indomethacin were significantly decreased with mortality about 55%. Colons showed marked shrinkage and nearly devoid of fecal contents, indicating colonic hyper motility. High damage score was observed. The total macroscopic score and disease activity index were high. Moreover, indomethacin produced significant elevation of the mean serum levels of C-reactive protein and TNF-alpha. The histopathological examination of colon revealed sever loss of epithelium and sever ulceration, damage of the all layers of the wall of the colon with the presence of inflammatory cellular infiltration.

These results were in accordance with a study. Yamagiwa et al., (2001) [13]. The mice received oral indomethacin (1mg/ mouse) daily for five days. They found severe gastroenteropathy (i.e. paralytic stomach and necrotic intestine) was induced on the sixth day. Ulcer formation was also seen at many sites in the digestive tract, especially in the colon. In parallel with the increase in the number of leukocytes in the digestive tract, the proportion of granulocytes increased at various sites, for example, in the intraepithelium and lamina propria of the colon and the lamina propria of the appendix.

Yamada et al., (1993) [14]. Found that a single injection of indomethacin (Indo) produces acute intestinal mucosal injury and inflammation that resolve completely within three to seven days, whereas two daily injections of Indomethacin produce both acute and chronic injury and inflammation. The enterohepatic circulation of indomethacin is important in promoting the acute phases of injury and inflammation.

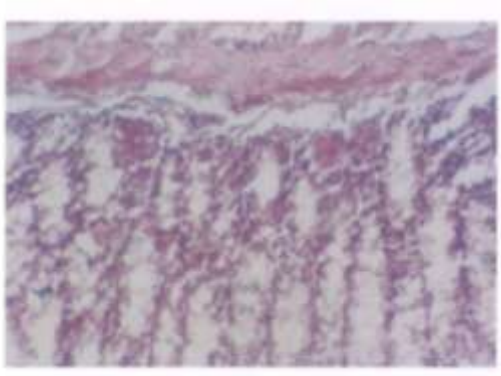

Fig. 1: It shows light micrograph of the distal colon demonstrating the glandular epithelium with plenty of globet cells. The connective tissue corium is evident. The mucalris mucosa is seen. (H & E, x200)

Moreover, Yamato et al., (2009) [15] found that single subcutaneous administration of indomethacin (10 mg/ kg) suspended in saline with a drop of polysorbate 80. Caused small-intestine ulceration on the side of the mesenteric attachment 24 h after administration, Intestinal ulcers were most severe at day 1 (24 h after indomethacin administration), after which gradual healing was observed. Macrosopically, ulcers were almost healed in 7 days. Histologic observation revealed denuded epithelium and severe edema in the

submucosa on day 1. Granulation tissue was clearly observed on the ulcer bed on day 4, and neoepithelial cells covered the damaged areas on day 7. These macro- and microscopic observations are consistent with a report by Hatazawa et al., (2006). [16]

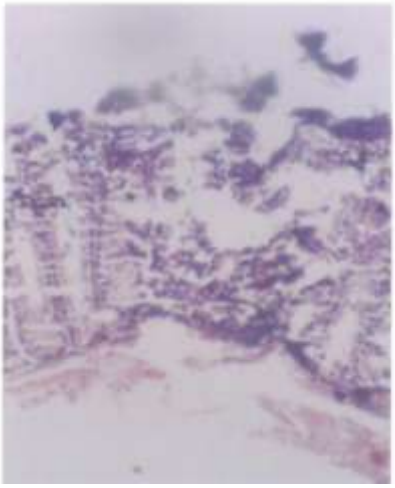

Fig. 2: It shows light micrograph of the distal colon of mice with indomethacin-induced colitis. There is severe ulceration of mucosa. Muocularis mucosa is disrupted. Inflammatory reaction is present underneath the ulcer. (H & E, x200)

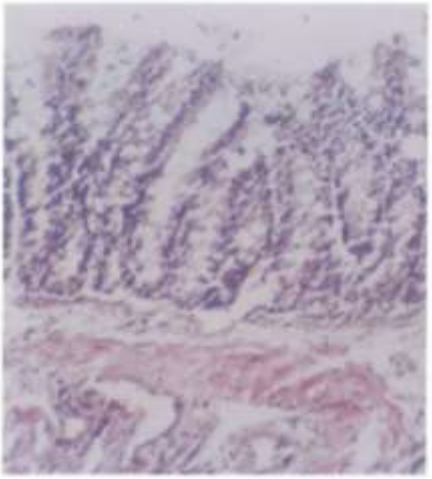

Fig. 3: It shows light micrograph of distal colon of mice received atorvastatin on day one of induction of colitis by indomethacin. Exhibiting mild to moderate shedding of tips of the glandular mucosa. Mild inflammatory reaction is seen in the corium. The muscolosa is unaffected. (H & E, x200)

In the present work, the mice received atorvastatin (20 mg / kg orally daily for 5 days) at the same time with indomethacin produced significant loss-of body weight with morality of 50% in comparison to normal. They showed mild improvement in these two parameters in compared to colitis group. On the other hand, atorvastatin attenuated significantly the DAI and the total macroscopic score. The shrinkage of the colons were significantly inhibited. The colons were moderately filled with faecal contents showing normal appearance. The mucosal damage score was significantly improved compared to colitis group. The serum levels of C- reactive protein and TNF-alpha were significantly lowered compared to colitis group but still elevated than normal. The histopathological examiniation revealed marked improvement of the epithelium with normalize smooth muscle architecture and minimal inflammatory cell infilammatory cell infiltration. These results were in agreement with other studies.

Park et al., (2004) [17]. Observed that atorvastatin treatment abrogated the TNBS-induced inflammation and restored a normal histologic appearance of the colon. Mild lymphoid hypertrophy and mild inflammatory cell infiltration in mucosa and sub mucosa were noted, but severe mucosal inflammation including crypt distruption or ulceration was no longer detected. They suggested that oral statin treatment suppresses TNBS induced colitis.

Hyo et al.,(2004)[18] tested atorvastatin in experimental colitis, a disease model of inflammatory bowel disease. To induce colitis, dextran sodium sulfate (DSS) or trinitrobenzene sulfonic acid (TNBS) were administrated to C57BL/6 or BALB/c mice respectively. DSS-induced colitis was not affected by atrovastatin treatment, but in contrast, the administration of atorvastatin relieved TNBS-induced colitis with a resultant rapid recovery of weight loss and a reduction in colonic length shortening. Histologically, inflammatory cell infiltration in the colonic wall, mucosal ulceration and crypt disruption were also suppressed in atorvastatin treated mice. They suggested that atorvastatin preserves intestinal integrity in colitis, probably via the modulation of Th cell-mediated immune response, in a manner independent of innate immunity.

In addition acute colitis which occurred during administration of DSS was considered to be induced by innate immunity but not acquired immunity. [19] Atorvastatin did not relieve acute colonic inflammation induced by DSS but it leads to disease progression.

Statins in addition to their lipid-lowering effects, have anti-inflammatory properties, which suggest that they are able to regulate molecules important for immunomodulation. Statins may have multiple targets with respect to immune modulation. They exert their anti-inflammatory effects in a cholesterol dependent and nondependent manner and that at least in part, by interfering with endothelial adhesion and transendothelial migration of leukocytes to sites of inflammation. Certain statins bind LFA-1 (lymphocyte function associated antigen-1) and inhibit its interaction with ICAM-l (intracellular adhesion moleculel) in T-cell adhesion co-stimulation and inhibit its interaction with ICAM-1 in T-cell adhesion/co-stimulation. [20]

Other recognized effects of statins are mediated via the inhibition of the mevalonate pathway. Mevalonate is a substrate in cholesterol biosynthesis, but it also participates in the post-translational modification of proteins involved in cell division and maturation. Statins were found to inhibit the production of pro- inflammatory cytokines and chemokines, and these effects were reversed by mevalonate. [21, 22]

The effects and the potencies of statins varied widely depending on the form of statins, However, Sasaki et al., (2003) [23]. Reported that intraperitoneally injected pravastatin relieved DSS-induced colitis by preventing leukocyte infiltration and gut injury, probably by increasing eNOS (constitutive NO synthetase) expression and activity. Atorvastatin is known to be the most powerful MHC class II repressor. [20]

Atorvastatin decreased resistin mRNA expression in a dose- and time-dependent manner. The study of Shyu et al., (2009) [24], study confirmed the previous findings that TNF-α could induce resistin expression in human macrophages and atorvastatin could inhibit the resistin expression induced by TNF-α. The inhibitory effect of atorvastatin on TNF-α induced resistin expression was mediated by rac and resistin promoter. These findings provided another evidence of pleiotropic effect of statin. Statin therapy may become another therapeutic strategy for controlling resistin- associated pathologic cardiovascular disease in humans.

In other model of inflammation Garjani et al., (2008) [25], showed that oral administration of atorvastatin, simvastatin, and lovastatin have lipidlowering-independent anti-inflammatory and anti-leukocyte accumulation activities in carrageenan induced rat paw edema model. The potency and effectiveness of statins in this model was comparable to their inhibitory potency on HMG-CoA rducatase. Where, atorvastatin had the strongest and lovastatin the weakest anti-inflammatory effect. Grip et al., (2008) [26] investigated the effect of atorvastatin as anti-inflammatory treatment in crohn's

disease. Atovastatin was used for 13 weeks (80 mg daily) combined with existing anti-inflammatory therapy. In addition to the lowering of cholesterol levels, atorvastatin led to significant decrease of CRP. CRP is useful as a laboratory marker to predict prognosis and relapse in patients with CD [27] Hence, it has been shown that strong anti-inflammatory agents work particularly well in patients with active gut inflammation and elevated levels of CRP [28].

There was a significant lowering of CRP levels by statin therapy in patients with CD.

Anti-inflammatory properties of statins probably include various mechanisms that may or may not involve the HMG-CoA reductase/mevalonate pathway [29]. Atorvastatin has a biphasic pro- and anti- inflammatory effects that are mainly independent of its effects on blood cholesterol and the anti-inflammatory effects are related to mevalonic acid pathway. [30,31]

CONCLUSION

The results of the present study revealed that atorvastatin, one of the potent and commonly used statins, have the potential to ameliorate the colonic inflammatory response induced by indomethacin. As statins have modes of action that differ from currently approved IBD treatment modalities. Moreover, statins have relatively safe and well tolerated profile. The findings of the present work suggested that atorvastatin possesses a colonic protective effect. It could be useful and could have important future applications in the treatment of IBD and other inflammatory and tissue injury conditions.

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