Ameliorative Effects of Sildenafil in Acetic Acid-Induced Chronic Colitis in Rats

Osama M. Ahmed[1*], Alaa Afifi[2], Tarek M. Ali[3], Shimaa A. Ramadan[3] and Ayman M. Mahmoud[1]

[1]Physiology Division, Zoology Department, Faculty of Science, Beni-Suef University, Egypt
[2]Physiology Department, Faculty of Medicine, Cairo University, Egypt
[3]Physiology Department, Faculty of Medicine, Beni-Suef University, Egypt
[*]Corresponding author: osamamoha@yahoo.com

Abstract: This study aims to assess the effect of sildenafil citrate, a potent inhibitor of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase (PDE) 5, on the colon histological integrity, oxidant-antioxidant status and pro-inflammatory cytokines in a rat model of acetic acid-induced colitis. Chronic colitis was induced under light ether anesthesia by intrarectal administration of 1 ml of 5% (v/v) acetic acid (AA) in male albino rats, with a second intrarectal administration of the same dose after 16 days. Control rats received an equal volume of saline intrarectally. Experimental rats were treated orally with either sildenafil citrate (5 and 10 mg/kg/day) or saline for 3 days. Tissue samples were used for the measurement of malondialdehyde (MDA) and glutathione (GSH) levels, and glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities. Blood was collected for the assessment of serum AST, ALT, creatinine, urea, tumor necrosis factor (TNF)-α and interleukin (IL)-1β levels. Obtained results revealed that in colitis group, the colonic tissue was characterized by lesions such as cystic dilatation, hemorrhage, and leukocytic infiltrations, increased lipid peroxidation with a concomitant reduction in GSH content, and decreased GPx and SOD activities. Serum hepatic and renal function parameters, TNF-α and IL-1β levels were higher in the colitis group compared to control values. Sildenafil, in a dose dependent manner, reversed these parameters nearly back to control values. In conclusion, sildenafil citrate administration to rats with chronic AA-induced colitis seems to be beneficial *via* prevention of inflammatory processes, lipid peroxidation, cytokine production and alleviation of the anti-oxidant defense system.
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1. Introduction

Inflammatory bowel diseases (IBD), namely ulcerative colitis and Crohn's disease are two chronic idiopathic diseases characterized by prominent intestinal inflammation **(Carter *et al*., 2004)**. Although the pathophysiology of IBD is not known with certainty, immunological processes and reactive oxygen species (ROS), such as peroxide anion, hydrogen peroxide (H_2O_2), and hypochloric acid have been proposed to contribute considerably in development of tissue injury **(Fiocchi, 1998)**. Many other inflammatory mediators have been also related; tumour necrosis factor-α (TNF-α) plays a prominent role and the neutralization of this cytokine is accompanied by a remarkable clinical response in patients with IBD **(Colon *et al*., 2001)**.

In addition, toxic oxidants are capable of destroying tissue if their rate of production exceeds the capacity of endogenous antioxidant defense mechanisms **(Kruidener *et al*., 2003)**. Under normal physiological conditions, antioxidant defense mechanisms protect tissues from ROS. Defense mechanisms consist of several radical scavenger and enzymes, including superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and peroxidases. However, the gut is potentially vulnerable to oxidant injury due to a low concentration of antioxidant enzymes, which are mainly localized in epithelial cells **(Grisham *et al*., 1990)**.

Sildenafil is a selective and potent inhibitor of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase (PDE5), which catalyzes the hydrolysis of cGMP and has a relaxant effect on the smooth muscle cells of the arterioles supplying the human corpus cavernosum **(Gibson, 2001; Rosalmeida *et al*., 2003)**. The data of a recent study have shown that sildenafil, acting *via* nitric oxide (NO)-dependent mechanism, prevented indomethacin-induced gastropathy, possibly through a reduction of leukocyte adhesion and maintenance of gastric blood flow **(Santos *et al*., 2005)**.

In the light of the above mentioned findings, this study is designed to assess the effect of two different doses of sildenafil on the extent of colon histological deteriorations, oxidant-antioxidant status and the pro-inflammatory cytokines, TNF-α and IL-1β, in a rat model of AA-induced colitis.

2. Materials and Methods:
Chemicals

Sildenafil citrate was supplied from Pfizer Inc. (Pfizer, Egypt), stored at 2-4 °C and protected from

sunlight. All other chemicals were of analytical grade and were obtained from standard commercial supplies.

Experimental animals

Male albino rats (*Rattus rattus*) weighting about 150-180 g were used as experimental animals in the present investigation. The animals were housed in standard polypropylene cages with stainless steel good aerated covers and maintained under controlled room temperature (22±2 °C) with 12 h light - dark cycle and were fed a standard diet of known composition, and water *ad libitum*. The animals used in the present study were maintained in accordance with the principles and guidelines of the Canadian Council on Animal Care as outlined in "Guide for the Care and Use of Laboratory Animals" (1993).

Induction of colitis:

After an overnight fasting, colonic inflammation was induced under light ether anesthesia by intrarectal administration of 1 ml of 5% (v/v) acetic acid in 0.9% NaCl with a 8 cm long cannula **(MacPherson and Pfeiffer, 1978)**, with a second intrarectal administration of the same dose after 16 days. The rats in the control group were subjected to the same procedure with the exception that isotonic saline was substituted for acetic acid.

Experimental design

The experimental animals were divided into six equal groups, each group comprising six rats designated as follows. **Group 1** served as control rats; **Group 2** was administered sildenafil at dose level of 5 mg/kg b. wt.; **Group 3** received sildenafil at dose level of 10 mg/kg b. wt.; **Group 4** served as colitis control group; Group 5 received sildenafil at dose level of 5 mg/kg b. wt., starting 5 minutes after intrarectal administration of the second dose of acetic acid and continued for 3 days and Group 6 received sildenafil at dose level of 10 mg/kg b. wt., starting 5 minutes after intrarectal administration of the second dose of acetic acid and continued for 3 days. At the end of the treatment period, rats were sacrificed under diethyl ether anesthesia and blood samples were collected from jugular vein. After coagulation, blood samples were centrifuged. The supernatant sera were fractioned and kept in deep freezer at – 30 ºC until used.

Biochemical studies:

Serum levels of the proinflammatory cytokines, TNF- α and IL-6, were determined by specific ELISA kits according to the manufacturer's instructions (R&D Systems, USA). The concentration of proinflammatory cytokines was determined spectrophotometrically at 450 nm. Standard plots were constructed by using standard cytokines and the concentrations for unknown samples were calculated from the standard plot.

Lipid peroxidation, reduced glutathione content, and superoside dismutase (SOD) and glutathione peroxidase (GPx) activities were also measured in colon homogenate according to the methods of **Preuss et al. (1998)**, **Beutler et al. (1963)**, **Marklund and Marklund (1974)** and **Kar and Mishra (1976)**, respectively. Serum AST activity **(Murray, 1984)**, ALT activity **(Murray, 1984)**, creatinine concentration **(Young, 1995)** and urea level **(Kaplan, 1984)** were determined using reagent kits purchased from Spinreact (Spain).

Histopathological studies:

After scarification, decapitation and dissection, colon from each rat was rapidly excised and then perfused in saline solution. Pieces from the colon and rectum of rats of different groups were taken and fixed in 10% neutral buffered formalin for twenty four hours. Fixed organs were sent to Histopathology laboratory in Faculty of Veterinary Medicine, Beni-Suef University for further processing, blocking in wax, sectioning and staining with hematoxylin and eosin (H&E).

Statistical analysis:

The data were analyzed using the one-way analysis of variance (ANOVA) (PC-STAT, University of Georgia, 1985) followed by LSD test to compare various groups with each other. Results were expressed as mean $\pm$ SE and values of $P > 0.05$ were considered non-significantly different, while those of $P < 0.05$ and $P < 0.01$ were considered significant and highly significant, respectively.

3. Results:

The effect of sildenafil on serum TNF-α and IL-1β of normal and AA-induced colitic rats was illustrated in figures (1) and (2) respectively. In normal rats, only the higher dose (10 mg/kg b.wt.) produced a highly significant ($P < 0.01$) effect on serum TNF-α and IL-1β levels. In AA-induced colitic rats, both doses of sildenafil induced a highly significant ($P < 0.01$) decrease of TNF-α and IL-1β as compared to AA-induced colitic control rats; both doses have more or less similar effects.

Colon GSH content was highly significantly ($P < 0.01$) decreased in AA-induced colitis as compared to normal. The treatment of AA-induced colitic rats with the higher dose of sildenafil produced a significant ($P < 0.05$) improvement of the decreased GSH level while the administration of the lower dose had a non-significant effect (Fig. 3).

The administration of AA to normal rats produced a highly significant ($P < 0.01$) increase in colon lipid peroxidation (Fig. 4). The treatment of AA-induced colitis in rats with sildenafil citrate prevent this elevation of lipid peroxidation to great extent ($P < 0.01$).

SOD activity in colon exhibited a different behavioral pattern, it showed a highly significant decrease (P<0.01) in AA-administered rats as compared with normal (Fig. 5). This deficiency was significantly (P<0.05) improved in colitic rats treated with sildenafil citrate at dose level of 5 mg/kg and highly significantly (P<0.01) increased at the higher dose.

Glutathione peroxidase activity was highly significantly (P<0.01) decreased in AA-induced colitis groups when compared with normal group, as illustrated in figure (6). The treatment of colitis in rats with sildenafil citrate only at its higher dose induced a highly significant (P<0.01) increase in the enzyme activity as compared with the chronic induced colitis control rats. However, the lower dose of sildenafil citrate had a non-significant effect (P>0.05) in chronic colitic rats. Concerning one-way ANOVA, it was found that the effect between groups on colon MDA, GSH, SOD and GPx was very highly significant (P<0.001) throughout the experiment.

Data showing the effect on liver function parameters in serum were represented in table (1). AA administration produced a highly significant (P<0.01) elevation of serum AST and ALT as compared with normal rats. The treatment of chronic colitis groups with sildenafil produced a pronounced amelioration of the elevated serum AST and ALT activities. Both doses produced a highly significant (P<0.01) decrease of the elevated ALT activity. On the other hand, while the low dose of sildenafil produced a significant (P<0.05) decrease of the elevated AST activity, the higher dose induced a highly significant (P<0.01) decrease. The higher dose of sildenafil citrate appeared to be more potent in reducing the elevated AST and ALT activities. One-way ANOVA showed that the

effect between groups on serum AST and ALT was very highly significant (P<0.001) throughout the experiment.

The changes in kidney function parameters in serum as a result of treatment of acetic acid-induced colitis with sildenafil were represented in table (2). There was a highly significant (P<0.01) increase in serum urea concentration of AA-administered rats as compared with normal. This elevation highly significantly (P<0.01) decreased following sildenafil-treatment.

Data represented in this study also illustrate that serum creatinine concentration exhibited a highly significant increase (P<0.01) in AA-administered rats as compared with normal control group. This elevation is significantly reduced in sildenafil-treated colitic rats as compared with the colitis control group; the higher dose of sildenafil citrate seemed to be more effective in decreasing the elevated serum creatinine level in AA-induced colitic rats since its effect is highly significant (P<0.01).

Histopathological examination of colon sections of normal group illustrated no histopathological alterations and the normal histological structure of the mucosa, muscularis mucosa and lamina propria (Fig. 7). Colon of the chronic colitis groups showed submucosal proliferated fibrous tissue, some patches of surface erosion and loss of the superficial epithelium of mucosa and cystic dilations. In addition, there were also a leukoctic infiltrations as well as hemorrhage (Figs. 8 & 9). Sildenafil, dose-dependently, could significantly improve the inflammatory response induced by AA, however, there are still slight alterations represented by mild lymphocytic infiltration and hyperemia

Table 1: Effect of sildenafil citrate on serum AST and ALT activities of normal and AA-induced colitic rats.

Group \ Parameter	AST (U/L)	ALT (U/L)
Normal Control	32.59 ± 1.11[bc]	25.11 ± 1.32[c]
Normal + Sildenafil (5 mg/kg)	30.26 ± 3.43[c]	32.01 ± 1.95[bc]
Normal + Sildenafil (10 mg/kg)	33.59 ± 2.69[bc]	32.25 ± 0.41[bc]
Chronic Colitis	69.99 ± 5.61[a]	62.00 ± 4.32[a]
Chronic Colitis + Sildenafil (5 mg/kg)	49.76 ± 5.64[b]	38.70 ± 1.89[b]
Chronic Colitis + Sildenafil (10 mg/kg)	40.25 ± 4.02[bc]	31.42 ± 2.60[bc]
F-Probability	P<0.001	P<0.001
LSD at 5%	17.59	10.45
LSD at 1%	24.81	14.65

- Data are expressed as Mean ± SE. Number of animals in each group is six.
- Means, which share the same superscript symbol(s), are not significantly different.
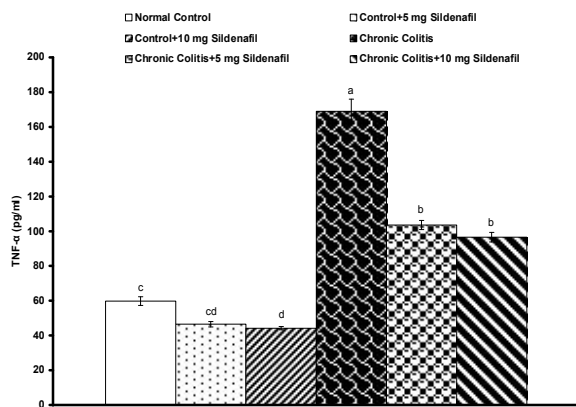
Table 2: Effect of sildenafil citrate on serum urea and creatinine levels of normal and AA-induced colitic rats

Group Parameter	Urea (mg/dl)	Creatinine (mg/dl)
Normal Control	23.71 ± 1.70^b	0.62 ± 0.03^b
Normal + Sildenafil (5 mg/kg)	23.30 ± 1.34^b	0.64 ± 0.03^b
Normal + Sildenafil (10 mg/kg)	23.27 ± 1.41^b	0.57 ± 0.01^b
Chronic Colitis	61.64 ± 2.85^a	0.84 ± 0.03^a
Chronic Colitis + Sildenafil (5 mg/kg)	22.98 ± 2.31^b	0.68 ± 0.04^b
Chronic Colitis + Sildenafil (10 mg/kg)	23.86 ± 2.18^b	0.62 ± 0.03^b
F-Probability	$P<0.001$	$P<0.01$
LSD at 5%	8.48	0.13
LSD at 1%	12.39	0.18

- Data are expressed as Mean ± SE. Number of animals in each group is six.
- Means, which share the same superscript symbol(s), are not significantly different.

Figure 1: Effect of sildenafil citrate on serum TNF-α of normal and AA-induced colitic rats. Means, which share the same superscript symbol(s), are not significantly different. F-prob.: P<0.001; LSD at 5% level: 15.23; LSD at 1% level: 21.35.
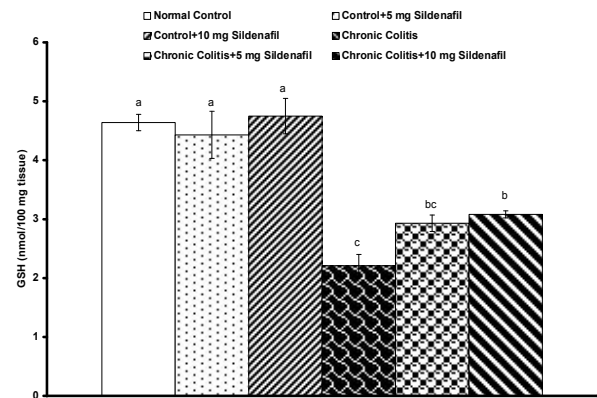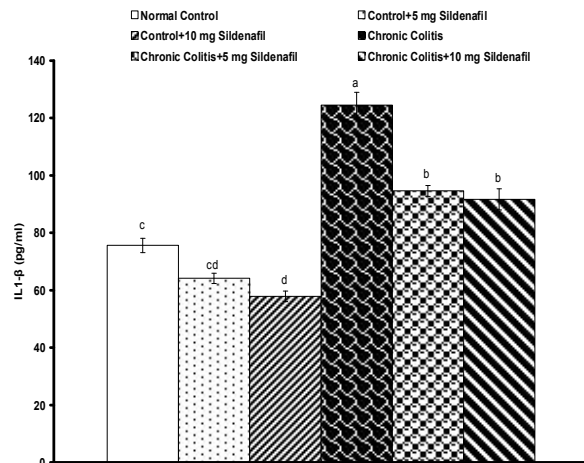

Figure 3: Effect of sildenafil citrate on GSH content in colon of normal and AA-induced colitic rats. Means, which share the same superscript symbol(s), are not significantly different. F-prob.: P<0.001; LSD at 5% level: 1.12; LSD at 1% level: 1.58.

Figure 2: Effect of sildenafil citrate on serum IL-1β of normal and AA-induced colitic rats. Means, which share the same superscript symbol(s), are not significantly different. F-prob.: P<0.001; LSD at 5% level: 12.53; LSD at 1% level = 17.57.
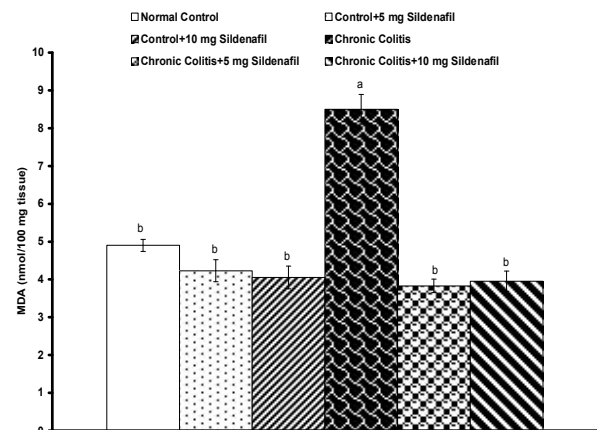

Figure 4: Effect of sildenafil citrate on MDA level in colon of normal and AA-induced colitic rats. Means, which share the same superscript symbol(s), are not significantly different. F-prob.: P<0.001; LSD at 5% level: 1.10; LSD at 1% level: 1.68.
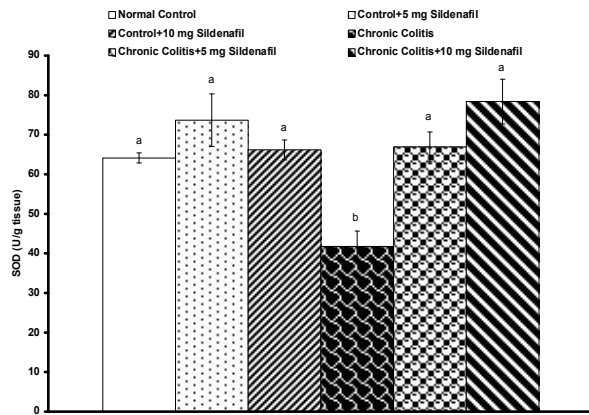
Figure 5: Effect of sildenafil citrate on SOD activity in colon of normal and AA-induced colitic rats. Means, which share the same superscript symbol(s), are not significantly different. F-prob.: $P<0.01$; LSD at 5% level: 18.82; LSD at 1% level: 26.38.
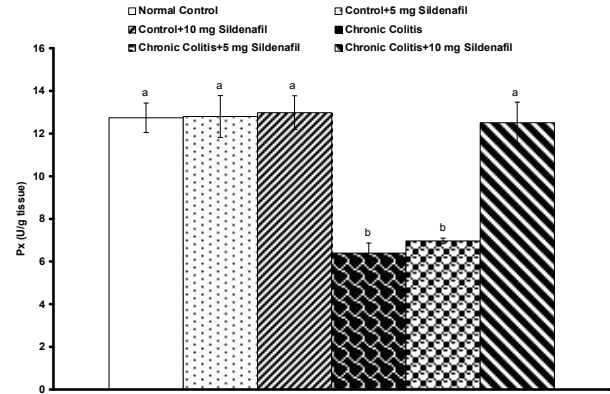

Figure 6: Effect of sildenafil citrate on GPx activity in colon of normal and AA-induced colitic rats. Means, which share the same superscript symbol(s), are not significantly different. F-prob.: $P<0.001$; LSD at 5% level: 3.18; LSD at 1% level: 4.46.
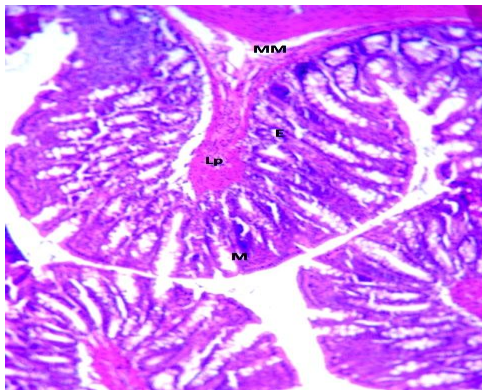

Figure 7: Photomicrograph of colon section of normal rats showing normal histological architecture. M: mucosa; E: mucosal epithelial cells; MM: musculais mucosa; Lp: lamina propria.
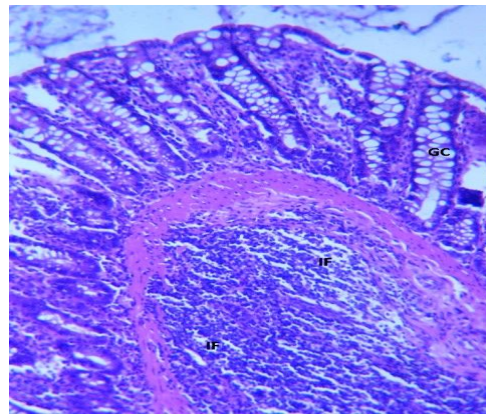

Fig. 9: Photomicrograph of colon section of chronic acetic AA-colitis in rats treated with 5 mg/kg b. wt. sildenafil citrate showing potential increase in the number of goblet cells (GC) in mucosa and hemorrhage and leukocytic infiltration (IF) in the submucosa. H&E X40
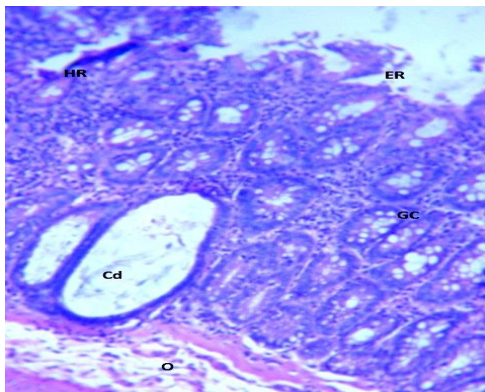

Fig. 8: Photomicrograph of colon section of chronic AA-induced colitis in rats showing sumbucosal proliferated fibrous tissue, some patches of surface erosion (ER) and loss of the superficial epithelium of mucosa, cystic dilation (Cd) of mucosal glands, hemorrhage (HR) and submucosal oedema (O). H&E *X*40
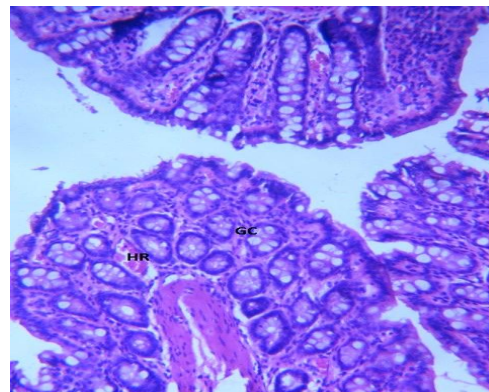

Fig. 10: Photomicrograph of colon section of chronic AA-induced colitis in rats treated with 10 mg/kg b. wt. sildenafil citrate showing hemorrhage (HR) and goblet cells (GC). H&E X40

4. Discussion:

The pathogenesis of IBD involves an interaction between genetic and environmental factors (Rampton and Shanhan, 2008). Although the pathophysiology of IBD is not known with certainty, immunological processes and reactive oxygen species (ROS), such as peroxide anion, hydrogen peroxide (H_2O_2), and hypochloric acid have been proposed to contribute considerably in development of tissue injury (Grisham, 1994 and Fiocchi, 1998).

Under normal physiological conditions, chemical and antioxidant defenses protect tissues from the damaging effects of ROS. The toxic oxidants can cause tissue injury if the rate of their production exceeds the capacity of endogenous antioxidant defense mechanisms (Williams *et al*., 1990 and Kruidener *et al*., 2003). The gut is potentially vulnerable to oxidant injury due to a low concentration of antioxidant enzymes, which are mainly localized in epithelial cells (Grisham *et al*., 1990). This suggests that colonic inflammation may produce high levels of oxidant products that probably exceed this relatively low antioxidant capacity and lead to oxidative stress and epithelial cell disruption (Lih Brody *et al*., 1996 and Yunus *et al*., 1999).

Oxidative stress and its consequent lipid peroxidation are able to aggravate free radical chain reactions, disrupt the integrity of intestinal mucosa barrier and activate inflammatory mediators, resulting in increased colonic MDA contents, as shown in both human and experimental animal studies (Girgin *et al*., 2000 and Ek *et al*., 2007). The present results showed that the levels of colonic MDA in colitis group were higher than the normal control group. Rats treated with both 5 and 10 mg/kg sildenafil have significantly reduced levels of MDA compared to the rats with AA-induced colitis. Therapy with sildenafil resulted in a marked decrease in MDA levels in colon, suggesting that sildenafil successfully inhibited lipid peroxidation induced by acetic acid. These results are in accordance with the findings of Iseri *et al*. (2009) who found that a subcutaneous dose of 5 mg/kg sildenafil ameliorated the elevated colonic MDA levels.

Furthermore, the status of antioxidant enzymes e.g., superoxide dismutase (SOD) decides the systemic protection against inflammation. SOD restrains the lipid peroxidation in colon by eliminating free-radicals, converting superoxide into peroxide (H_2O_2). A significant body of research has indicated that decreasing SOD activity in the local colon tissue leads to mucosal injury because of reduced ability of oxidative radicals scavenging (Barazzone & White, 2000 and Krieglstein *et al*., 2001). Our study showed that SOD activity significantly decreased in the colitis control group and these data were in agreement with several observations demonstrating the decrease in SOD activity under the effect of different sorts of stress

(Deliconstantinos and Villiotou, 2000). In contrast to the present study, Kruidenier *et al*. (2003) demonstrated that colonic mucosa Cu/Zn-SOD and Mn-SOD levels were higher than the control levels in patients with inflammatory bowel disease. Also, Kuralay *et al*. (2003) showed that tissue SOD levels were elevated in response to oxidative stress in AA-induced colitis model. Our data demonstrated that administration of sildenafil ameliorated alterations induced by AA in SOD, to reach the normal range of control group.

GSH is an important nonenzymatic antioxidant and, similar to other sulfhydryl-containing products, it also has regulatory and protective roles in the body. Our findings indicate a slightly lower GSH activity in the colitis group than control. Moreover GSH activity in sildenafil treated groups was significantly increased than control and colitis groups. These results are in agreement with those of Iseri *et al*. (2009) and Erarslan *et al*. (2010) who reported diminished colonic GSH levels in acetic acid-induced colitis in rats. In addition, Nieto *et al*. (2000) found that the level of GSH was lower in TNBS induced ulcerative colitis. Furthermore, Ek *et al*. (2007) found a significant decrease in the activity of GSH in the colonic tissue of the acetic acid induced-colitis.

Regarding glutathione peroxidase, the current data revealed a significant depletion of colon GPx activity and treatment of AA-induced colitis groups with sildenafil potentially alleviated the GPx activity. Because GPx is responsible for most of H_2O_2 neutralization (Pastor *et al*., 1997), which upon diffusion to the extracellular space result in oxidative disruption of apical intercellular tight junctions and colonic epithelial basement membranes (Pravda, 2005), sildenafil might be contributing to the integrity of the gastrointestinal barrier function.

Another important finding of the present study was that sildenafil, in dose dependent manner, attenuated production of the pro-inflammatory cytokines, TNF-α and IL-1β, which are believed to play a significant role in the pathogenesis of IBD (El-Medany *et al*., 2005; Mahgoub *et al*., 2005). They possess overlapping and synergetic activities inducing the production of other cytokines, adhesion molecules, arachidonic acid metabolites, as well as activating immune and non-immune cells. An *in vitro* study demonstrated that sildenafil was unable to decrease hypoxia-induced upregulation of TNF-α and IL-1β mRNA in pulmonary artery (Tsai *et al*., 2006). Similarly, sildenafil did not significantly inhibit any markers of inflammation including TNF-α, IL-4 and IL-5 levels in a murine model of allergic asthma (Clayton *et al*., 2004). In accordance with our findings, another study demonstrated that pretreatment with PDE5 inhibitor zaprinast at a dose of 10 mg/kg blocked lipopolysaccharide (LPS)-induced increase of TNF-α

level in serum of mouse (Iric *et al.*, 2001). However, the origin of these cytokines detected in serum in our study needs to be clarified.

AST and ALT estimation in serum is a useful quantitative marker to indicate hepatocellular damage (Hwang & Wang, 2001; Singh *et al.*, 2001). The increased activities of these serum markers observed in our study correspond to considerable liver damage induced in AA-induced colitis in rats. Administration of sildenafil significantly decreased the levels of AST and ALT, suggesting that it offers protection by preserving the structural integrity of the hepatocellular membrane. On the other hand, there was a significant elevation in creatinine and urea in the serum of the chronic induced colitis groups. Sildenafil administration significantly ameliorated the deteriorated renal function and this suggests that sildenafil may have a renal protective effect in AA-induced colitis.

Histopathological investigation of colon section taken from AA-treated rats showed superficial erosion of mucosal epithelial cells, diffuse goblet cells formation in mucosal epithelium with oedema and diffuse inflammatory cells infiltration in lamina propria and hypertrophy in the muscularis. These data are in accordance with Iseri *et al.* (2009) who revealed that AA induced massive epithelial loss, destruction of Lieberkuhn crypts, severe inflammatory cell infiltration, vasculitis and submucosal edema. On the other hand, treatment with both doses of sildenefil produced amelioration of histopathological changes induced by acetic acid confirming protection by such agent.

In conclusion, sildenafil citrate is beneficial in AA-induced colitis in rats and the mechanism of its anti-inflammatory action may involve the maintenance of oxidant-antioxidant status, prevention of lipid peroxidation and cytokine release.

Corresponding author:
Osama M. Ahmed
Physiology Division, Zoology Department, Faculty of Science, Beni-Suef University.
Salah Salim St., P.O. 62511, Beni-Suef, Egypt.
E-mail: osamamoha@yahoo.com
Mobile: +2-0100+1084893

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