Inhibitory Effect of Sildenafil (Viagra®) on Duodenal Motor Activity in Mice

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Abstract: Introduction: Sildenafil citrate (Viagra®) is known to relax visceral smooth muscle through the release of neurotransmitter nitric oxide (NO). However, the effect of sildenafil on the structure and motor function of the small intestine has not been fully investigated. **Methods:** Male Swiss mice were divided into three groups. In the first and second group, animals were treated orally with low- and high- dose of sildenafil (2, 4 mg/kg, b.wt., respectively), for 2 weeks. In the third group, sildenafil (30μM) was applied exogenously to the isolated duodenal segment. Physiological and histopathological characteristics were studied and compared to untreated controls. Duodenal motility was assessed using a Trendelenburg preparation to study aboral directed peristaltic motor complexes (PMCs). The contractile activity of the duodenum segment was recorded as changes in intraluminal pressure under isovolumetric conditions. The mean amplitude of PMCs and the frequency (interval) of phasic contractions were determined. Histopathological evaluation was determined using light and scanning electron microscope (SEM). **Results:** no change in behavior was observed in mice during treatment with low- or high- dose of sildenafil (2, 4 mg/kg) compared to control. However, macroscopic analysis showed the finger-shaped of villi in control group which became blunt and flatten in the first group. In the second group, the tip of villi was blunter and broader compared to the first group and control. Microscopical analysis showed minor changes in the first group when compared to control. However, in the second group, there was an increase in eosinophils and worsen intestinal tissue lesions. In submucosal layer, there was an increase in the number of blood vessels and the amount of connective tissue. Hypertrophy and hyperplasia of smooth muscle cells were also observed. Treatment of sildenafil for 2 weeks had no significant changes on motility. Exogenous sildenafil (30μM), significantly decreased PMCs amplitude and increased the interval (P<0.05) compared to control. **Conclusion:** treatment of male mice with sildenafil for 2 weeks induced structural damage, while exogenous sildenafil produced motor dysfunction of mouse duodenum.

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Key Words: Sildenafil, duodenum, peristaltic motor complexes.

1. Introduction:

Sildenafil citrate (Viagra®) is a systemic vasodilator due to its selectivity as a cGMP-specific phosphodiesterase type 5 (PDE5) inhibitor (Alvarez *et al.*, 2013). It is widely used to treat human erectile dysfunction and pulmonary hypertension by dilating small vessels and improving blood flow (Gibson, 2001).

Sildenafil plays a critical role in the modulation of the cGMP pathway (Yuan *et al.*, 2008). It acts by enhancing NO-mediated smooth muscles relaxation by blocking PDE5 which hydrolyzes cGMP, this results in maintaining elevated levels of cGMP and causes relaxation of smooth muscles in various organs (Graça *et al.*, 2008).

Enteric nervous system neurons produce NO, which is recognized as an important inhibitory neurotransmitter (Costa *et al.*, 2000). In the gastrointestinal (GI) tract, NO is released from inhibitory motor neurons during peristaltic reflex leading to descending relaxation of circular muscles (Grider, 2003). NO acts *via* activating soluble guanylate cyclase, improving production of guanosine cyclic monophosphate (cGMP). This lead to activate cGMP dependent protein kinase, which mediates the postjunctional responses (Williams *et al.*, 2005).

The concept of sildenafil citrate relaxing smooth muscles triggers the investigations of its beneficial effect to relive the symptoms in irritable bowel syndrome (IBS) (Gibson, 2001), which is characterized by disturbances of gastrointestinal motor activity. It has been reported that sildenafil decrease rectal tone in normal and IBS conditions (Fritz *et al.*, 2003). Sildenafil also normalizes the esophageal motility in patient with oesophageal motor disorders (Fox *et al.,* 2007).

Little physiological and histological studies were conducted about the influence of sildenafil on contractile activity in GI tract. In the present study, the rat duodenal segment was chosen to assess the effects of sildenafil and evaluates the efficacy and safety of such drug on histological and physiological motor function of GI tract. Therefore, the aim of this study was to investigate the effect of low- and high-

dose of sildenafil (2, 4 mg/kg respectively) on histopathological characteristics of the small intestine as well as to examine the effect of endogenous and exogenous sildenafil on motor activity of isolated mouse duodenum induced by intraluminal distention.

2. Material and methods

Animal Preparation

Experiments were performed on 15 male Swiss mice with body weights ranging from 25-30g divided randomly into three groups of 5 each. The first and second groups were treated orally with low- and high- dose of sildenafil (2, 4 mg/kg respectively) for two weeks. In the third group, sildenafil (30μM) was applied exogenously to the isolated duodenal segment. The maintenance of the animals was in full compliance with the standard laboratory animals care protocols approved by Institutional Animal Care and Use Committee (IACUC). Water and *ad libitum* were provided at the Animal House Laboratory, King Fahd Medical Research Center, King Abdulaziz University.

Drug

Sildenafil citrate (Viagra®; Pfizer, New York, NY) was dissolved in distilled water and stored at 5°C. Freshly diluted aliquot was given orally to the first and second group of animals in milliliter volumes while added to the organ bath in microliter volumes in the third group.

Tissue Preparation

Animals were stunned by a blow on the head and scarified by cervical dislocation two hours after last drug administration and various studies takes place. A mid-line laparotomy was performed and the complete small intestine was rapidly excised and placed in gassed (95% O_2 and 5%CO_2) Krebs bicarbonate buffer solution (composition in mM: NaC1 117, KC1 4.7, $NaHCO_3$ 25, CaC1 2 2.5, MgCl 2 1.2, $NaH_2PO_4.2H_2O$ 1.2 and D-glucose 11) cleared of any mesenteric connective tissue and the lumen flushed with Krebs solution for the further preparations. Histopathological characteristics were studied on the first and second groups, while physiological examination has been investigated on all groups. The most proximal ~5 cm of the mice duodenum was used for physiological studies and ~2 cm was used for histopathological examination.

Physiological studies

Tissues were prepared as described by (Abdu *et al.*, 2002). One duodenal segment approximately 5 cm in length was prepared from each animal and two in total were mounted horizontally in separate 20 ml perfusion chambers. Tissues were maintained at 37°C, perfused with Krebs solution at a rate of 5 ml/min, and allowed to equilibrate for at least 30 min before experiments started. Peristaltic motor complexes (PMCs) of mice duodenum were monitored and analyzed by using (Neurolog\NL 900D, Digitimer Ltd, Hertfordshire, England) to record contractile activity as changes in intraluminal pressure under isovolumetric conditions to compare their sensitivity to sildenafil. Exogenous sildenafil was added to the chambers 20 min after stopping perfusion and recording continued for a further 20 min before washing out.

Histopathological studies

Histopathological evaluation was determined on the first and second groups using light and scanning electron microscope (SEM). Small pieces of fresh specimen of duodenum were removed and immediately fixed in aqueous Bauin's solution (24hrs). Tissues were dehydrated cleared and embedded in paraffin, and sections were stained with hematoxylin and eosin for routine light microscopic study. For electron microscope study, duodenum was fixed in 3% gluteraldhyde for (2hrs), stained and examined by SEM at the department of Biological Sciences, Faculty of Science, King Abdul Aziz University, Jeddah, Saudi Arabia.

Data Analysis

The PMCs of duodenal contractility were measured in terms of their peak amplitude above baseline (cmH_2O) and interval (second) between them. In the first and second group of animals, the effect of sildenafil was quantified by calculating the intervals between PMCs in 20 min period. In the third group, the effect of sildenafil was quantified by calculating the intervals between PMC in 20 min period before drug application and the response effect in the 20 min following application. Responses are expressed as absolute values ± SE (n = 5), with n being the number of animals. Data were compared using Student's *t*-test and *Mann Whitney U*- test as appropriate. In all cases, probability values of $P < 0.05$ was regarded significant.

3. Results

Physiological studies

No change in agonistic behavior was observed in mice during treatment with sildenafil citrate.

The effect of sildenafil on duodenal contractility in orally treated mice Sildenafil did not produce any significant change in duodenal contractile activity in animals that received low-dose (2 mg/Kg) or high dose (4 mg/Kg) for two weeks. The intervals were 31.32± 5 *vs.* 41.30±4 s, n = 5, while the amplitude was 4.94± 2 *vs.* 3.88±3 cmH_2O, n = 5. The same result was observed in high-dose (4

mg/Kg) treated animals, the PMC intervals were 31.32± 2 *vs.* 25.25±2 s and the amplitudes were 4.94±2 *vs.* 5.27±1 cmH_2O, n = 5, (Figure 1).

The effect of exogenous sildenafil on mice duodenal contractility Exogenous application of sildenafil (30µM) produced a significant inhibition of PMCs frequency and amplitude in isolated mice duodenum. The intervals increased from 30.93±3 to 66.24±11s, $P < 0.05$, n = 5 while the amplitude decreased from 7.16±2 to 5.23±1 cmH_2O $P < 0.05$, n = 5, (Figure 2). The inhibitory effect of sildenafil (30µM) remained for 20 min and was reversible after washing the segment.

Histological studies

The specimens of Duodenum in control animals were similar and revealed normal histological pattern of chosen specimen (Figure 3, 6 and 9).

Treatment with low dose of sildenafil (2 mg/kg) The mice duodenum appeared more or less normal but revealed villus with blunt, flattened tip (Figure 4), vacuolation of some columnar cells with detached lamina propria (LP, Figure 7). A marked congestion indicated by the dilation and congestion of the vessels engorged with large number of red blood cells, sub mucosal odema were observed. On the other hand, the muscular layer had nearly normal structure with a little thickening of the circular smooth muscle layer and nearly free of inflammatory cells, mitotic figures were encountered within some crypts and disturbance of the architectural structure of the myenteric plexus. Disturbed myenteric plexus were detected (Figure 10).

Treatment with high dose of sildenafil (4 mg/kg) A significant morphological change in duodenum tissue was detected. The tips of villi were blunter and broader compared to the first group and control (Figure 5). There were also LP shedding of the surface, pyknosis and vacuolation of many columnar cells (Figure 8). Treatment with high dose of sildenafil (4 mg/kg) showed highly thickened (CM), hemorrhagic areas (BV) and the myenteric plexus with many cells have dark stained nuclei (Figure 11). Also, mononuclear cellular infiltration particularly esinophils, with increased connective tissue between muscle fibers was noticed. Hemorrhagic areas and aggregations of fibroblasts were detected in the sub mucosa.

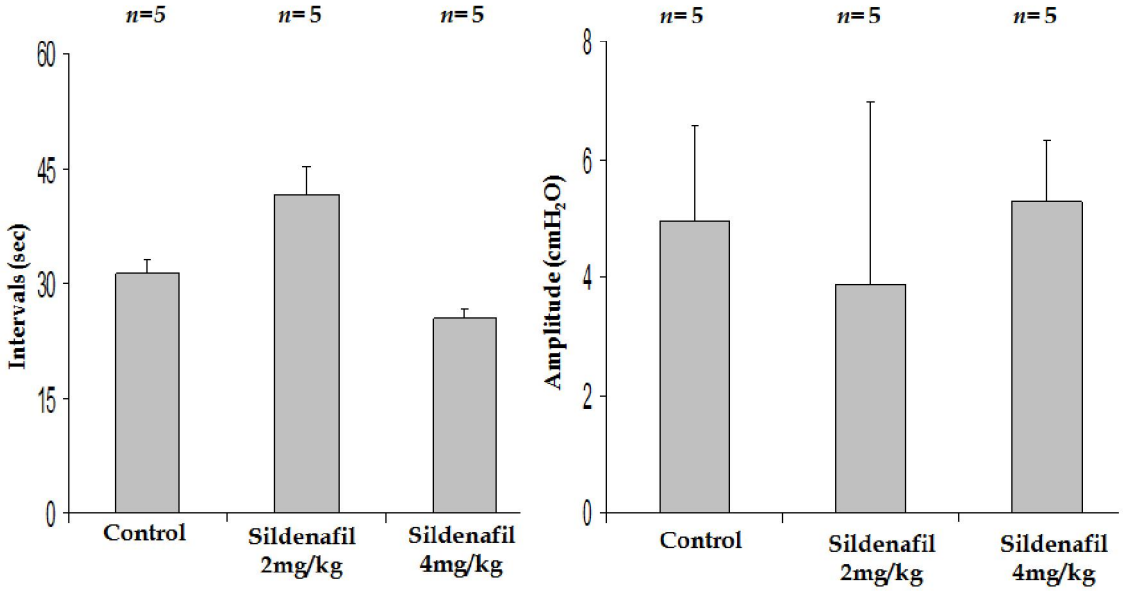

Figure 1. Effect of sildenafil on control and orally treated mice duodenum Histograms showing PMCs interval (left) and amplitude (right) in control and animals treated with low-and high-dose of sildenafil (2 mg/kg, 4 mg/kg respectively).

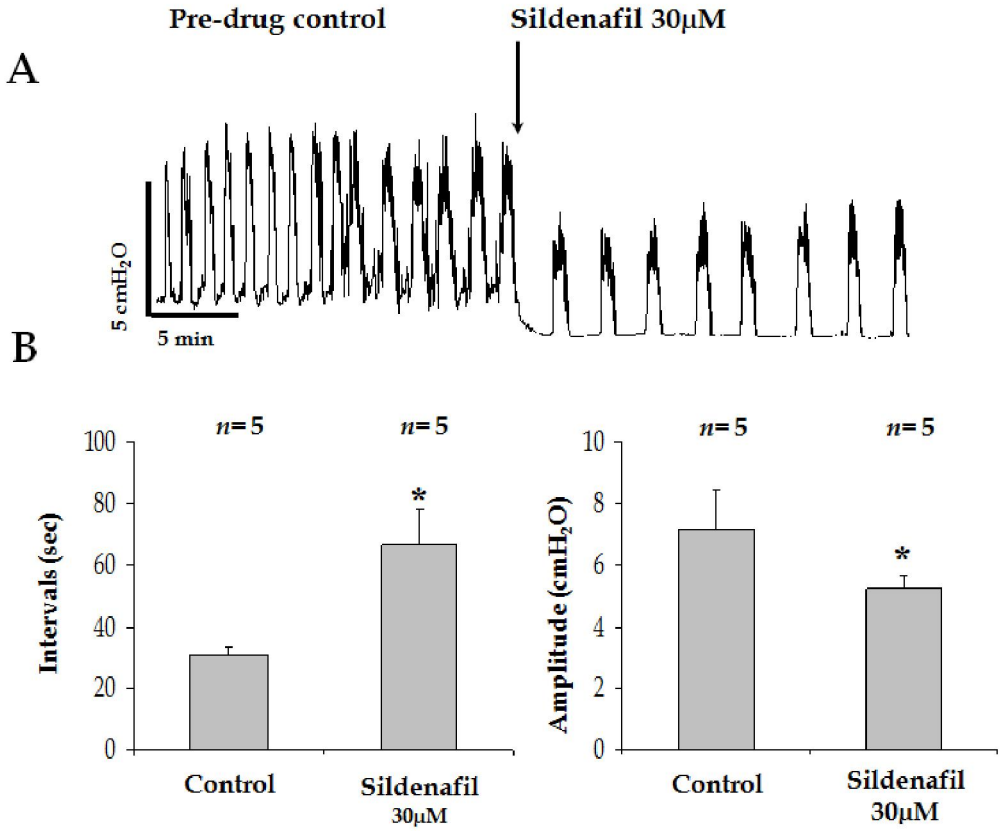

Figure 2. Effect of sildenafil on duodenal contractile activity (A) Representative traces showing the decrease in PMCs amplitude and the increase in intervals produced by sildenafil (30μM). (B) Histograms illustrating the interval between PMCs and the amplitude of PMCs before and after addition of sildenafil (30μM). $^{*}P< 0.05$ compared to pre-drug control.

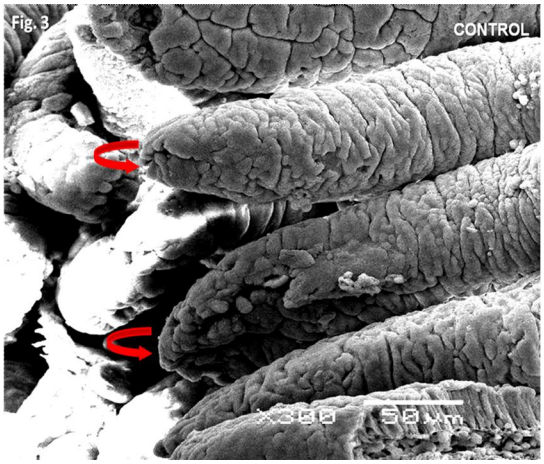

Figure 3. Ultra micrograph of control duodenum showing straight and finger shape intestinal villi (arrow) with leaf-like surface appearance.

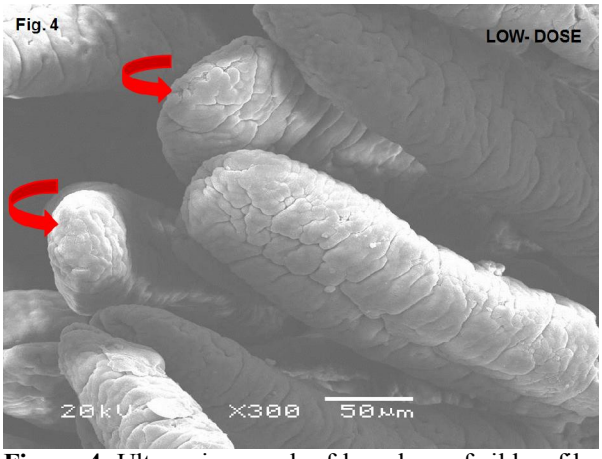

Figure 4. Ultra micrograph of low-dose of sildenafil (2 mg/kg) treated duodenum showing blunt and flattened tip intestinal villi (arrow).

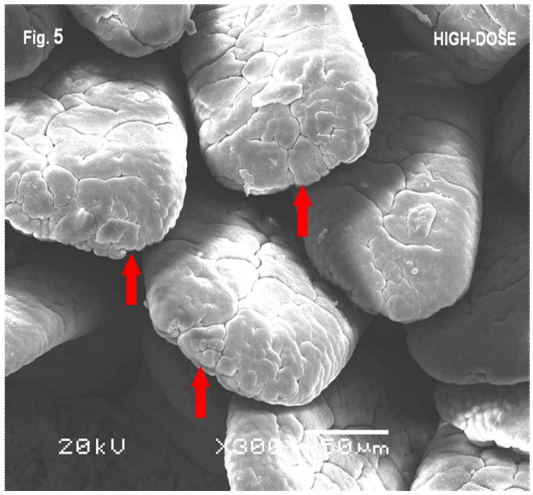

Figure 5. Ultra micrograph of high-dose of sildenafil (4 mg/kg) treated duodenum showing, the tips of villi were blunter and broader compared to the low-dose and control.

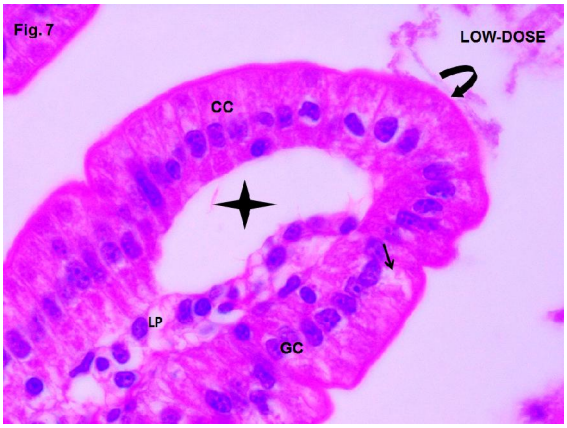

Figure 7. Cross section from duodenal low-dose (2 mg/kg) of sildenafil treated mice. Note the blunt, flattened tip villi (arrow) with vacuolated columnar cell (straight-arrow) and detached lamina propria (star). GC: goblet cell, CC: columnar cell and LP: lamina propria. (x 1000).

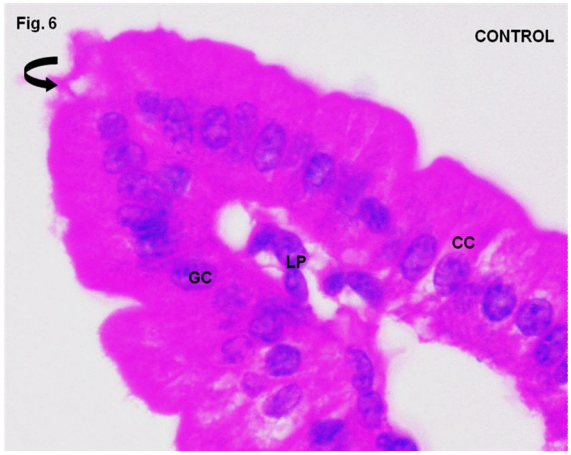

Figure 6. Cross section from duodenal control mice. Note the finger shape intestinal villi (arrow) with goblet cell (GC), columnar cell (CC) and lamina propria (LP). (x 1000).

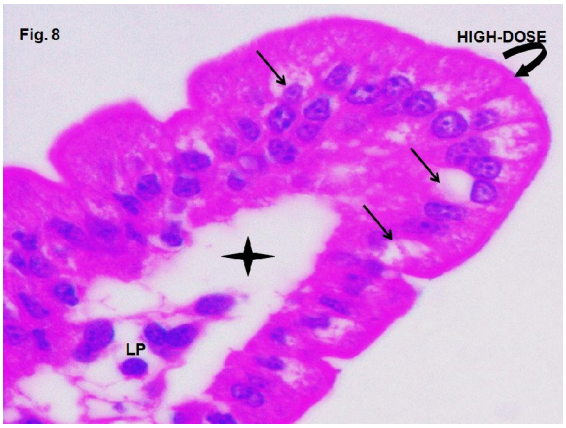

Figure 8. Cross section from duodenal of high-dose (4 mg/kg) of sildenafil treated mice. Note the blunter and flattener tip villi (arrow) with many vacuolated columnar cells (straight-arrow) and detached lamina propria (star). LP: lamina propria. (x 1000).

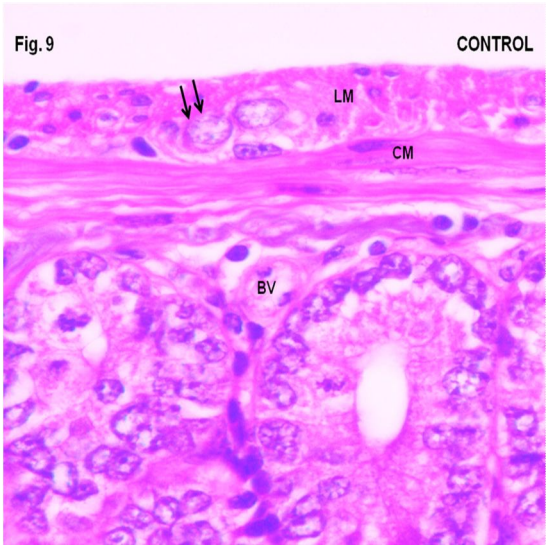

Figure 9. Cross section from duodenal control mice. Note the regular myenteric plexus (double-arrows) with large clusters of Parasympathetic ganglion cells are found between circular muscle (CM) and longitudinal muscle (LM). Blood vessel (BV). (x 1000).

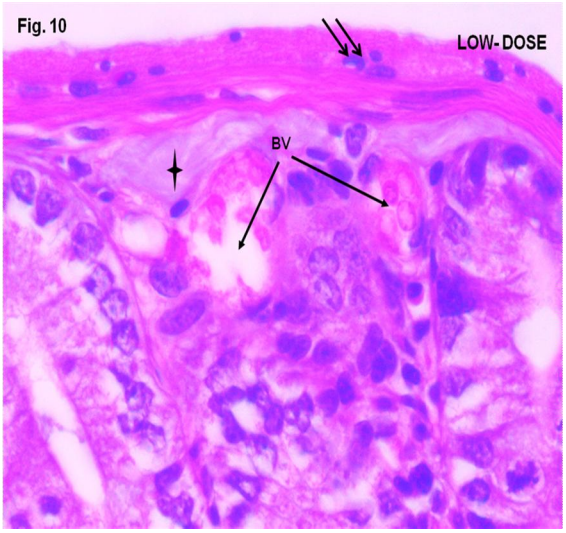

Figure 10. Cross section from duodenal low-dose (2 mg/kg) treated mice. Note dilated and congested blood vessels (BV), sub mucosal odema (stare), mitotic figure (arrow) and disrupted myenteric plexus (double-arrows). (x 1000).

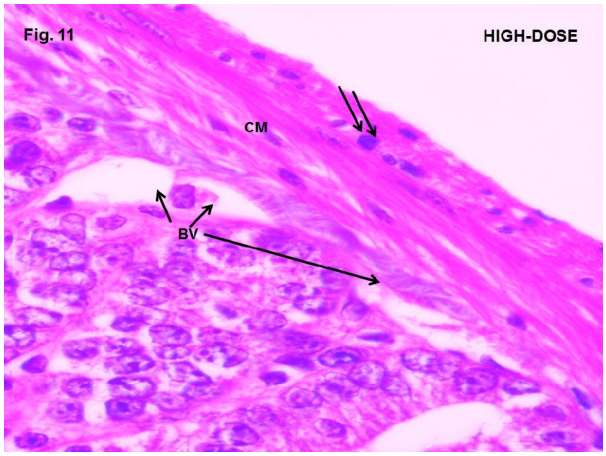

Figure 11. Cross section from duodenal high-dose (4 mg/kg) treated mice. Note the highly thickened (CM), hemorrhagic areas (BV) and the myenteric plexus with many cells have dark stained nuclei (double-arrow). (x 1000).

4. Discussion

This study was aimed to evaluate the *in vivo* effect of sildenafil on structure of the small intestine as well as to examine the *in vitro* effect of sildenafil on motor activity of isolated mouse duodenum by intraluminal distention. In addition, the histopathological alterations due to sildenafil treatment were assessed. Our results showed that exogenous sildenafil produced a significant inhibition in motor activity in isolated mice duodenum. In agreement with our results, it has been proven that sildenafil inhibits smooth muscle contraction in normal and pathological conditions (Fritz *et al*., 2003; Fox *et al*., 2007). Moreover, Bortolotti *et al*. (2001) reported that sildenafil inhibits interdigestive motor activity of the antrum and duodenum as it decreases the number and amplitude of antral and duodenal contractions, increases the duration of phase I and prevents the appearance of gastroduodenal phases III. They found that the variability in the appearance of phase III could be attributed to the duration of the inhibitory effect of sildenafil on oesophageal musculature which has been seen to be less than 1h. However, our study in disagreement to this suggestion that after longer time treatment (2 weeks) we found that sildenafil nearly

inhibited the muscle contraction activity in treated mice in particular with the low dose treatment. Therefore, these findings suggested that further studies with longer duration are needed before we can observe the effect of this drug on duodenal motor activity. On the other hand, the present study revealed that *in vitro* treatment of sildenafil reduced contractile activity in the isolated mice duodenum. These results are line with previous observations in animals and humans (Zhu *et al*., 2007; Clemente *et al*., 2008; Yuan *et al*., 2008)

There are several attempts to explain the mechanism of sildenafil on the duodenum muscle contraction. Numerous studies suggested that the inhibitory effect on gastroduodenal motility shown by sildenafil agrees well with the fact that NO inhibits gastrointestinal motility (Meile *et al*., 2006; Wittmeyer *et al*., 2010). The fact that the increase in NO-dependent cGMP activity after sildenafil induces a longer than normal phase I and prevents the occurrence of phase III fits well with the finding of Russo *et al*. (1999) that the decrease of NO due to the inhibition of NO synthesis initiates a gastroduodenal premature phase III and shortens phase I and strongly suggests that NO is involved in the regulation of migrating motor complex. In another study, Clemente *et al.* (2008) indicated that sildenafil acts on intestinal smooth muscle throughout activation of the NO–cGMP pathway. In addition, they observed that K^+ channels are important cellular components which are involved in the sildenafil induced myorelaxation in rat duodenum. Based on these findings the results of the present study suggest that the inhibitory effect of low dose of sildenafil on the motor activity of the duodenum could be attributed to the increase in NO synthesis and K^+ channels.

The histopathological studies on the effect of sildenafil on the animal tissues are still very rare. The histopathological findings in the current results indicated that low dose of sildenafil was not able to induce changes in mouse duodenum tissues. However, several changes such as increase in eosinophils and worsen intestinal tissue lesions were observed with high dose treatment of sildenafil. In agreement with our findings, Soydan *et al.* (2009) reported that histopathological examination on the intestine tissues of rats exposed to sildenafil observed no lesions were found. Moreover, sildenafil expressed protective effect against ischemia–reperfusion exposure, where, they reported that sildenafil pretreatment to ischemia–reperfusion significantly reduced ischemic injury. This protective action of sildenafil may attributed to the concentration used in the study of Soydan *et al.* (2009), who used a lower (1 mg/kg) dose than ours (2 and 4 mg/kg). In addition, Kukreja *et al.* (2005) reported a 0.5 mg/kg (i.v.) dose of sildenafil to be protective against ischemia– reperfusion injury in rabbits. In the present study, the results revealed no histopathological lesions on the in mouse duodenum tissues caused by low dose of sildenafil. However, several histopathological lesions were observed with high dose treatment of sildenafil. Therefore, the negative effect of sildenafil caused histopathological changes in mouse duodenum tissues is dose depending manner.

An attempt to understand the mechanism of sildenafil protection (with low dose) in ischemia–reperfusion, Kukreja *et al.* (2005) proposed that the vasodilatory effect of sildenafil could release endogenous mediators such as adenosine and/or bradykinin and may trigger a signaling pathway by activation of kinases (protein kinase C), which results in NO synthase phosphorylation and eventually NO production. NO may potentially activate guanylate cyclase, resulting in elevated cyclic GMP levels. cGMP may activate protein kinase G (PKG), resulting in histopathological protective effects. Sildenafil might be effective with the low dose in promoting normal smooth muscle contractility by producing NO. On the other hand, the histopathological lesions observed with the high dose of sildenafil might be attributed to decrease of NO production and subsequently suppression in of guanylate cyclase, resulting in decrease of cyclic GMP levels.

Conclusion

Oral treatment with high dose of sildenafil for 2 weeks induced structural damage of duodenal wall. Exogenous sildenafil produced motor dysfunction of mouse duodenum. These results may lead to more investigations of sildenafil beneficial effect to relive the symptoms in IBS.

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