The cytoprotective effect of different doses of Sildenafil on indomethacin-induced gastric mucosal damage in rats:

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Summary:

Background: Sildenafil, a selective phosphodiesterase -5 (PDE-5) inhibitor increases cyclic guanosine monophosphate (cGMP) and amplifies many biological actions of nitric oxide (NO) which is an important mediator of gastric mucosal defense ; was evaluated in this study using different doses for its cytoprotective effect on gastric mucosal damage induced by indomethacin . We also evaluated the effect of this drug on NO production, Prostaglandin E2 (PGE2) synthesis, Myeloperoxidase (MPO) activity, and Interlukin-4 (IL-4) expression.

Methods: The study was performed between April and July 2008 in the Department of Pharmacology / College of Medicine /Baghdad University .It was conducted on 80 adult male albino rats, divided into 8 groups, the first served as a control received the vehicle, the second received indomethacin orally of 60mg/kg .The third and fourth groups were pretreated 30 minutes prior indomethacin with oral sildenafil 10 and 20mg/kg respectively. The other groups were pretreated 30 minutes prior to sildenafil (10and20mg/kg) with intraperitoneal NG-L-Arginine Methyl Ester (L-NAME) 20mg/kg with or without L-Arginine. The rats were then sacrificed after 4 hours and their stomachs were isolated and submitted to macroscopical assessment and for the measurement of the gastric PGE2, MPO, and IL-4.

Results: Sildenafil (10 and 20 mg/kg) pretreatment resulted in a significant ($p<0.01$) decrease in the gastric damage score. The MPO activity was significantly ($p<0.01$) decreased, while the level of IL-4 was significantly ($p<0.01$) increased. On the other hand PGE2 level was not changed. L-NAME given 30 min before 10 and 20 mg/kg sildenafil, significantly ($p<0.01$) abolished the protective effects of sildenafil and also reversed the effects of sildenafil on MPO activity and IL-4 levels. On the other hand addition of L-Arginine resulted in the restoration of the protective effects of sildenafil which was also reflected on the gastric damage score, MPO activity and IL-4 levels.

Conclusions: The results demonstrate that the injurious effect of indomethacin can be reduced or ameliorated by sildenafil pretreatment, and that the protective effect of sildenafil against indomethacin was through an NO dependent pathway.

Keywords: sildenafil , cytoprotection , NSAIDs gastropathy.

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Introduction:

NSAIDs are a widely used group of medications with many clinical applications in different areas of modern medicine (1) ,however NSAIDs-induced gastropathy is the major problem of this group of drugs (2). Although the damaging effect of these drugs is generally ascribed to their ability to inhibit gastric prostaglandins (PGs) (3) .Other protective mechanisms which are partially or totally independent of PGs inhibition may also be important (4,5).The role of nitric oxide (NO) in the maintenance of the integrity of the gastric mucosa has been demonstrated in recent years (6) .This mediator through activation of the cyclic Guanosine Monophosphate (cGMP) pathway seems to modulate important functions involved in the mechanisms of gastric mucosal injury by NSAIDs including mucus gel secretion , mucosal blood flow , leukocyte adherence and release of oxidants by activated neutrophils and influencing the expression anti-inflammatory cytokines (7,8) .Sildenafil (9) , a selective Phosphodiesterase-5 (PDE5) inhibitor increases cGMP and amplifies many biological actions of nitric oxide was evaluated in this study using different doses for its cytoprotective effect on gastric mucosal damage induced by indomethacin . We also evaluated the effect of this drug on NO production, prostaglandin E2 (PGE2) synthesis, myeloperoxidase (MPO) activity, and interleukin-4 (IL-4) expression.

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Methods:

This study was conducted with 80 adult male albino-Wister rats weighing (150-200 g). The study was initiated after seeking approval from the ethical and scientific committee in the Department of Pharmacology / College of Medicine /Baghdad University on February 2008. Rats were starved for at least 24 hours before indomethacin administration. On the day of the experiment, water was held two hours before the procedure. Indomethacin was used for induction of gastric damage in a dose 60 mg/kg at a concentration of 15mg/ml , sildenafil was dissolved in the vehicle of (0.9% NaCl contain tween 80 and 1% CMC) and its concentrations was adjusted to 2.5mg/ml. N^G-L-

Arginine Methyl Ester (L-NAME) a NOS (nitric oxide synthase) inhibitor was dissolved in phosphate buffer saline (PH 7.2) at a concentration of 32.5 mg/ml according to the method of Griffith and Kilbourn (1996)[10] for intraperitoneal (I.P) administration. L-arginine (NOS substrate) was dissolved in distilled water, according to instructions provided by Sigma–Aldrich Company, at a concentration of 100 mg/ml, for (I.P) administration. All drugs were freshly prepared immediately before the use. The animals were divided into 8 groups the first group served as a control received the vehicle , the second group received indomethacin orally of 60mg/kg .The third group was pretreated 30 minutes prior indomethacin with sildenafil 10mg/kg orally. The fourth group received sildenafil 20mg/kg orally. In order to investigate the role of NO in the protective mechanism of sildenafil the other groups were pretreated 30 minutes prior to sildenafil (10and20mg/kg) with intraperitoneal L-NAME (20mg/kg) with or without L-Arginine (200mg/kg).The rats were sacrificed after 4 hours following indomethacin administration and their stomachs were isolated .The lengths of ulcerative lesions were measured with a digital caliper and the stomach quickly divided into three parts and each part was kept in suitable and special buffer and stored at -20^{o}C for biological assay .In addition full thickness pieces of the gastric corpus were stored in 10% formalin for histopathological study. Assessment of gastric mucosal damage: Gastric damage score: was calculated by the summation of the lengths of all linear erosions according to Santucci, et al. (1994) (11). Biological assays: Gastric mucosal samples were collected each in specific buffer and stored in freeze until evaluation of biological parameters: A :prostaglandin E2 assay: The samples used for assay of PGE2 were kept in sodium phosphate buffer (10 mmol/l ; pH 7.4).At the time of the procedure, tissue was minced with scissors, placed in a shaking water bath at (37^{o}C) for 20 min, then samples were centrifuged at (9000 x g) for 1 min the concentration of PGE2 in the supernatant was determined by enzyme linked immunosorbent system (ELISA) using commercially available kit according to Wallace, et a l.(2000) (12) . B: Gastric MPO activity assay : The samples used to assay gastric MPO were kept in phosphate buffer saline (50 mmol/l ; pH 6) .One hundred milligram of gastric tissue was homogenized in 2 ml of PBS (50 mm) containing 0.5% hexadecyl trimethyl ammonium bromide (HTAB) (pH 6). Each sample was homogenized on ice bath for 2 minutes using a polytron homogenizer and then centrifuged at 2000 x g for 5 min at 4^{o}C. MPO activity of supernatant was determined by adding 0.1 ml of the supernatant to 2.9 ml of 50 mm phosphate buffer containing 0.167 mg/ml of O-diansidine HCl and 50 µl of 1% H2O2, the change in absorbance at 460 nm over a 3 minutes period was measured spectrophotometrically. One unit of MPO activity was defined as that which would convert 1 Mmol of H2O2 to water in 1 min at 22^{o}C. The results were reported as the MPO unit /mg of tissue according to Bradley, et al. (1982) (13). C: IL-4 expression assay: Quantitative measurement of IL-4 was conducted using a solid phase ELISA. The samples that were used to assay gastric IL-4 were kept in phosphate buffer Saline (pH 7.4). At the time of the procedure specimens of gastric mucosal scrapings were homogenized with sample buffer and centrifuged at (1000 x g) for 15 min and the resulting supernatant diluted. Samples and standards were pipetted into the microtiter wells precoated with antibody specific for rat IL-4 and after incubation for 2hrs at 37 ^{o}C the complex was then probed with 100 ML biotinylated antibody, and washed with 350 ML wash buffer .After being washed, the retained complex was reacted with 100 ML streptavidine peroxide and incubated with 90 ML tetramethyl benzidine (TMB) reagent for spectrophotometric IL-4 quantifications according to Slomiany , et al.(1998) (14) .

Statistical analyses: Statistical analyses were done using SPSS version 15. All data were expressed as mean + standard error of mean (SEM). One-way analysis of variance (ANOVA–test) was used for comparison between several experimental groups. A probability value P <0.01 was considered statistically significant.

Results:

Intragastric instillation of 60 mg/Kg indomethacin on empty stomach, caused extensive multiple hemorrhagic lesions affecting mostly the glandular portion of the stomach in all animals (100% induction). A) I- At sildenafil (10mg/kg): Indomethacin-induced gastric mucosal damage was reduced by sildenafil pretreatment (10 mg/Kg) 30 min. prior to indomethacin intragastric instillation. The gastric damage score was significantly reduced by 90% ($p<0.01$), the gastric PGE2 level was not significantly changed, the gastric MPO activity was significantly reduced ($p<0.01$) and gastric IL-4 was significantly increased compared to the indomethacin treated group, figures (1), (3), (4) and (6). B) I- At sildenafil (20mg/kg): On higher doses, 20 mg/Kg of sildenafil, the protective effect was further increased .Gastric damage score was significantly reduced by 98% ($p<0.01$). However gastric PGE2 levels, MPO activity, and the gastric IL-4 were not significantly different from sildenafil (10mg/kg) treated group, figures (1), (3), (4) and (6).Effect of L-NAME on the sildenafil protective action : A)-I- At sildenafil (10mg/kg) :The mucosal protective effect of sildenafil of 10 mg/Kg was reversed by I.P. administration of L-NAME 30 min. prior to sildenafil instillation .The gastric damage score was significantly increased($p<0.01$) ,gastric PGE2 level was not significantly changed ,gastric MPO activity was significantly($p<0.01$) increased and gastric IL-4 was significantly reduced ($p<0.01$) compared to sildenafil (10mg/kg) treated group, figures (2) ,(3), and , (7) . B) -I- At sildenafil (20mg/kg): On higher dose, 20 mg/Kg sildenafil; the protective effect was also reversed by L-NAME

The gastric damage score was significantly increased (p<0.01) , gastric PGE2 level was not significantly changed ,There was a significant increase (P<0.01) in MPO activity and the gastric IL-4 was significantly decreased (p<0.01) compared to sildenafil (20mg/kg) treated group, figures (2) , (3), (5), and (7) Effect of L-arginine on the L-NAME reversal of sildenafil protective effect : A)-I-At sildenafil (10mg/kg): Administration of L-arginine reduced the lesion parameters .This means that L-arginine restored the protective effect of sildenafil at (10mg/Kg) that was reversed by L-NAME .The gastric damage score was significantly reduced(p<0.01) ,gastric PGE2 level was not significantly changed , gastric MPO activity was significantly suppressed(P<0.01) and gastric IL-4 was significantly restored compared to sildenafil(10mg/kg) group that received L-NAME without L-arginine , figures (2) , (3), (5), and (7) B)-I-At sildenafil 20mg/kg): L-arginine also restored the protective effect of sildenafil at 20mg/kg that was reversed by L-NAME. The gastric damage score was significantly reduced (P<0.01), gastric PGE2 was not significantly affected, gastric MPO activity was significantly suppressed (p<0.01) and gastric IL-4 was significantly restored (p<0.01) compared to the sildenafil (20mg/kg) group that received L-NAME without L-arginine, figures (2), (3), (5), and (7)

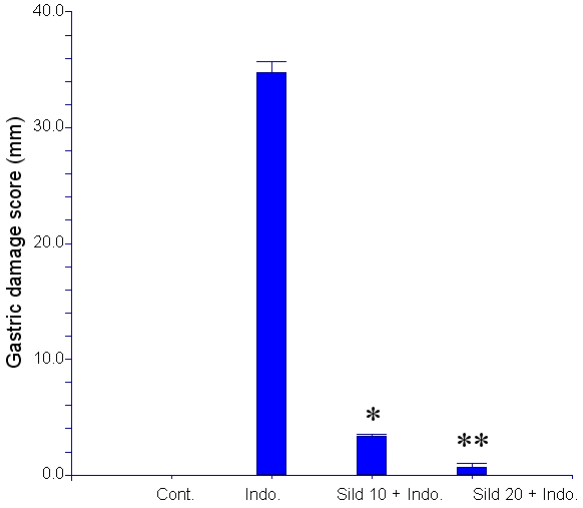


Figure (1): The protective effect of sildenafil on the gastric damage score induced by indomethacin. * P < 0.01 when compared with indomethacin group. ** P < 0.01 when compared with indomethacin group. Cont: control, indo: indomethacin. Sild -10: sildenafil 10mg/kg, sild -20: sildenafil 20mg/kg.

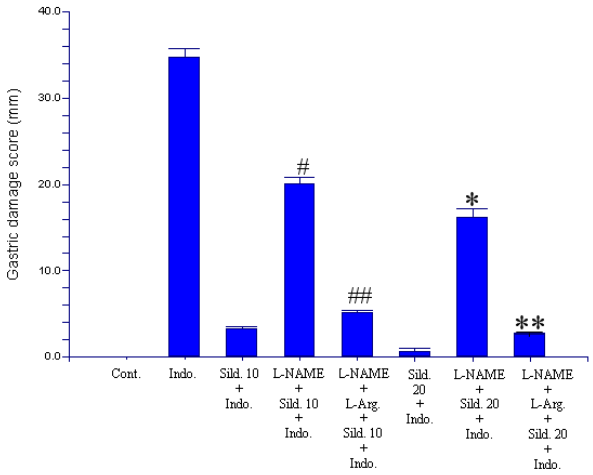


Figure (2) the loss of the protective effect of sildenafil on indomethacin induced gastric damage score by L-NAME administration and its restoration by the addition of L-arginine. # P< 0.01 when compared with 10 mg/kg sildenafil alone treated group. ## P<0.01 when compared with 10 mg/kg sildenafil + L-NAME treated group. * P < 0.01 compared with20mg/kg sildenafil group. ** P < 0.01 compared with 20mg/kg sildenafil + L-NAME treated group. Cont: control, indo: indomethacin. Sild -10: sildenafil 10mg/kg. Sild -20: sildenafil 20mg/kg, L-Arg: L-Arginine.

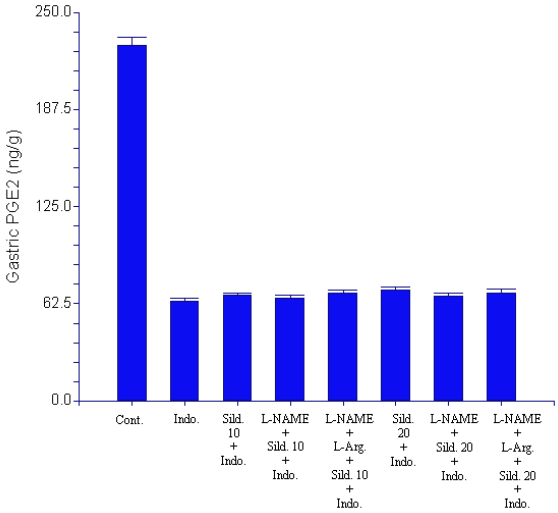
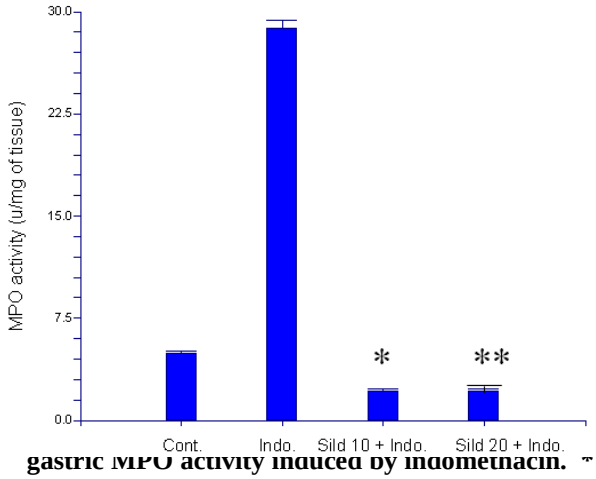


Figure (3): Gastric PGE2 levels following sildenafil pretreatment with or without L-NAME (with or without L- Arginine) compared with Indomethacin alone showing no significant alterations. Cont: control, indo: indomethacin. Sild -10: sildenafil 10mg/kg. sild -20: sildenafil 20mg/kg, L-Arg: L-Arginine



gastric MPO activity induced by indomethacin. * P < 0.01 when compared with indomethacin group. ** P < 0.01 when compared with indomethacin group. Cont: control, indo: indomethacin. Sild -10: sildenafil 10mg/kg sild -20: sildenafil 20mg/kg.

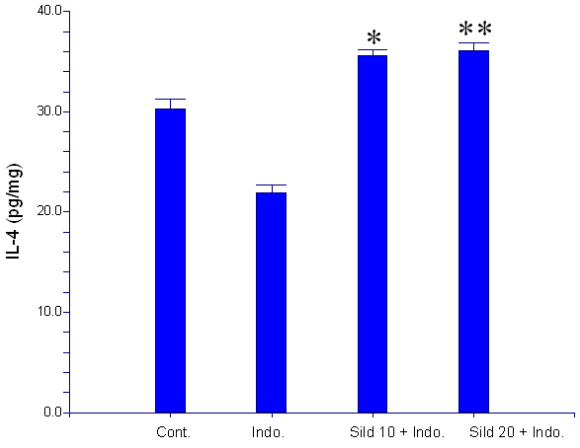


Figure (6): The protective effect of sildenafil on the expression of gastric IL-4 during indomethacin induced mucosal injury. * P < 0.01 compared with Indomethacin group. ** P < 0.01 compared with Indomethacin group. Cont: control, indo: indomethacin. Sild -10: sildenafil 10mg/kg, sild -20: sildenafil 20mg/kg.

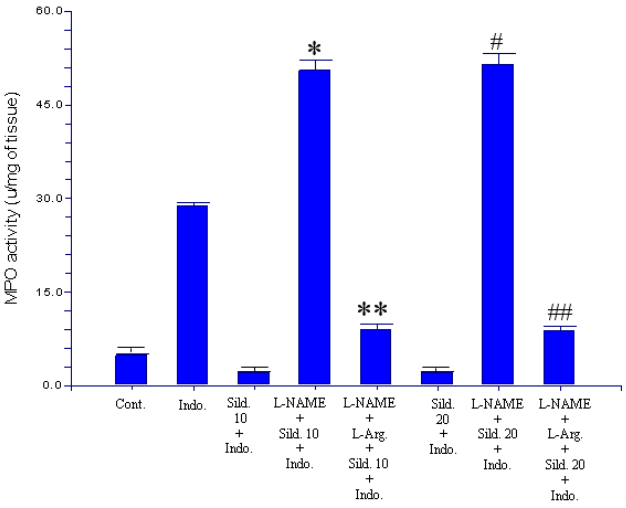


Figure (5): The loss of the effect of sildenafil on the indomethacin induced increase in the MPO activity by L-NAME administration and its restoration by the addition of L-arginine. * P < 0.01 compared with 10mg/kg sildenafil alone treated group. ** P < 0.01 compared with 10mg/kg sildenafil + L-NAME treated group. # P< 0.01 when compared with 20 mg/kg sildenafil alone treated group. ## P<0.01 when compared with 20 mg/kg sildenafil + L-NAME treated group. cont: control , indo: indomethacin. Sild -10: sildenafil 10mg/kg, sild -20: sildenafil 20mg/kg, L-Arg: L-Arginine.

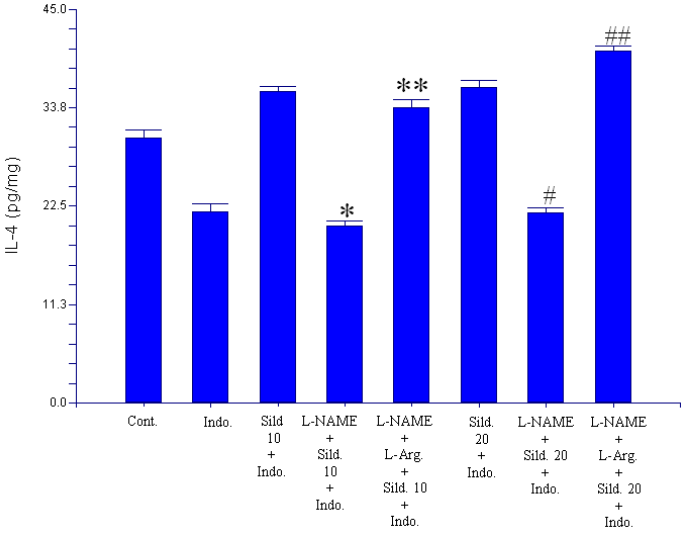


Figure (7): The loss of the effect of sildenafil on the indomethacin induced decrease in the expression of IL-4 by L-NAME administration and its restoration by the addition of L-arginine. * P < 0.01 compared with 10mg/kg sildenafil alone treated group. ** P < 0.01 compared with 10mg/kg sildenafil + L-NAME treated group. # P< 0.01 when compared with 20 mg/kg sildenafil alone treated group. ## P<0.01 when compared with 20 mg/kg sildenafil + L-NAME treated group. Cont: control, indo: indomethacin. Sild -10: sildenafil 10mg/kg. Sild -20: sildenafil 20mg/kg, L-Arg: L-Arginine

Discussion:

Sildenafil and other PDE5 inhibitors are increasingly recognized for their use in the treatment of male erectile dysfunction and more recently to reduce pulmonary artery hypertension and alleviate the symptoms associated with Reynaud's phenomenon (15). This study demonstrates the protective effect of sildenafil against indomethacin-induced gastric lesions. At 10 mg/Kg dose sildenafil elicited a 90% reduction in the extent of gastric damage score. These findings are similar to the study of Santos et al., (2005) (2), where the reduction of gastric damage score was 88%. Further increase in the dose of sildenafil to 20 mg/Kg, resulted in further decrease in the gastric damage score to 98% compared to indomethacin alone treated group. To investigate whether sildenafil cytoprotective effects could be through NO release ; L-NAME was used for this purpose , L-NAME in this study abrogated the protective effects of sildenafil on the gastric damage score which was restored back by L-arginine coadministration . This indicates that the protective action of sildenafil is mediated through NO.It has been recognized that one of the early events of gastric damage associated with the use of NSAIDs is free radical generation with neutrophil activation and its adherence to the vascular endothelium (16 , 17, 18, 19) . This neutrophil infiltration and oxyradical generation is reflected by the MPO activity and since this is specific to neutrophil it is often used an indicator of neutrophil infiltration in tissues (19) .This study also shows that sildenafil pretreatment in both doses (10 & 20 mg/kg) decreased indomethacin-induced granulocyte infiltration into gastric tissue as reflected by a significant ($p<0.01$) reduction in the MPO activity. This effect of sildenafil on leukocyte migration is in part dependant on the presence of NO, because treatment with L-NAME abolished this effect of sildenafil on MPO activity which was restored by the addition of L-Arginine .Gastric PGE2 levels were not affected by sildenafil, which clearly indicate that this protective mechanism offered by sildenafil is independent on PGs. It has been shown that extensive gastric mucosal damage as that produced by indomethacin resulted in reduction of IL-4 , which is one of the anti-inflammatory cytokines that suppress the secretion of proinflammatory cytokines IL-1, IL-2, IL-6 and block the synthesis of metalloproteinase which is important for apoptotic signal generation and TNF-α production (20) .This present study, shows that at both 10 and 20 mg/kg sildenafil significantly ($p<0.01$) increased gastric IL-4 by (62%, and 65%) respectively. This increment in IL-4 levels seems also to be NO dependent because the addition of L-NAME significantly ($p<0.01$) suppressed gastric IL-4 to levels similar to that seen with indomethacin alone and that effect of L-NAME was abolished by coadministration of L-arginine. In conclusion, these results demonstrate that the injurious effect of indomethacin can be reduced or ameliorated by sildenafil pretreatment in a dose dependent manner, and that the protective effect of this agent was not through the PGs pathway since inhibition of PGs synthesis by indomethacin was not altered by prior administration of sildenafil. Furthermore, the loss of the protective effects of sildenafil by prior administration of L-NAME , and its restoration by the addition of L-Arginine indicate that sildenafil acts via NO dependent mechanisms.

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