The cytoprotective effect of isosorbide dinitrate on indomethacin-induced gastric mucosal damage in rats

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Abstract

Background and aim:

NSAIDs have been associated with an increased risk of gastric mucosal damage which is mainly ascribed to inhibition prostaglandin synthesis involved in the protection of the mucous membrane .On the other hand , it was also demonstrated that nitric oxide (NO) plays an important role in the integrity and maintenance of the gastric mucous membrane . The present experimental study was carried out to investigate whether isosorbide dinitrate , a NO donor drug , would prevent gastric mucosal injury induc ed by indomethacin .

Materials and methods:

This study was conducted on 60 adult male albino rats , divided into 6 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 with isosorbide 5 and 10mg/kg orally respectively then given indomethacin . In order to investigate the protective mechanism of isosorbide dinitrate the fifth and sixth groups were pretreated 30 minutes prior to isosorbide 5mg/kg dose with intraperitoneal L-NAME 20mg/kg with or without L-Arginine ,then given indomethacin . 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 prostaglandinE2 (PGE2), myeloperoxidase (MPO) , and interlukin-4 (IL-4) .

Results :

Isosorbide dinitrate in a dose 5mg/kg pretreatment produced a significant reduction($P<0.01$) in gastric damage score, a significant ($P<0.01$) rise in gastric IL-4 levels ,and significant decrease ($P<0.01$) in MPO activity but did no interfere with the inhibitory effect of indomethacin on gastric PGE2 levels . On the other hand doubling the dose of isosorbide dinitrate to 10 mg/kg resulted in paradoxical effect reflected by significant increase ($P<0.01$) in the gastric damage score, significant increase ($P<0.01$) in MPO activity, and significant decrease ($P<0.01$) in IL-4 expression. L-NAME given 30 minutes before 5 mg/kg isosorbide, significantly ($P<0.01$) abrogated the protective effects of isosorbide dinitrate at 5 mg/kg on the gastric mucosa as well as its effects on the MPO activity, and IL-4 expression. This loss of protection how ever was restored by coadministration of L-Arginine, which was also reflected by a decrease in MPO activity, and an increase in the IL-4 levels.

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Iintroduction:

Organic nitrates including isosorbide dinitrate have been used for many years in the treatment of cardiovascular diseases(1) . It has been reported that patients who are NSAID users and are treated on a chronic regular basis with nitrates, either orally or transdermally exhibit less ulcerogenic effect on their stomachs and a significantly lower risk of GI bleeding(2) . Nitric oxide released from this medication stimulates guanylyl cyclase in smooth muscles and this lead to increase the cGMP which causes dephosphorylation of myosin light chain and relaxation(3) .It has also been demonstrated that release of NO by these drugs, contribute to the GI motility, tonus, permeability and blood flow to the vessels of gastric wall (4).Furthermore, isosorbide dinitrate and other organic nitrate may exert cytoprotective actions through the stimulation of mucous production (5) .NSAIDs are a widely used group of medications with many clinical applications in different areas of modern medicine [6],however NSAIDs-induced gastropathy is the major problem of this group of drugs [7]. The damaging effect of these drugs is generally ascribed to their ability to inhibit gastric prostaglandins (PGs) [8] However, other protective mechanisms which are partially or totally independent of PGs inhibition may be important .The role of NO in the maintenance of the integrity of the gastric mucosa has been demonstrated in recent years[9].This mediator through activation of the cGMP pathway seems to modulates important functions involved in the mechanisms of gastric mucosal injury by NSAIDs including mucus gel secretion , release of oxidants by activated neutrophils and influencing the expression anti-inflammatory cytokines [10] . In this study the protective role of NO in indomethacin induced gastric mucosal injury was evaluated using the NO donor drug isosorbide dinitrate .

Materials and methods

This study was conducted with 60 adult male albino-Wister rats weighing (150-200 g).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 , isosorbide dinitrate was dissolved in the vehicle of {0.9% NaCl contain tween 80(surfactant agent) and 1% carboxy methyl cellulose (CMC)}and its concentrations was adjusted to 1.25mg/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) [11] , L-arginine (NOS substrate) was dissolved in distilled Water according to instructions provided by SigmaAldrich company at a concentration of 100 mg/ml for intraperitoneal (I.P) administration. All drugs were freshly prepared immediately before use. The animals were divided into six groups the first group served as a control received the vehicle , the second group received indomethacin orally of 60mg/kg .The third and fourth groups were pretreated orally 30 minutes prior indomethacin with isosorbide 5 and 10mg/kg respectively . In order to study the role of NO in the protective effect of isosorbide 5 and 10mg/kg doses , intraperitoneal L-NAME

20mg/kg with or without L-Arginine 200mg/kg was administered 30 minutes before isosorbide and these served as the fifth and sixth groups respectively . 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° C for biological assay .**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) [12]

. **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°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)[13]

.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°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°C. The results were reported as the MPO unit /mg of tissue according to Bradley , *et al. (1982)* [14]**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°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) [15]. 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 (ANOVAtest) 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) .

Effect of different doses of isosorbide dinitrate pretreatment on indomethacin-induced gastric mucosal damage in rats:A) I- At isosorbide dinitrate(5mg/kg): macroscopically, Indomethacin-induced gastric mucosal lesions was significantly reduced by isosorbide dinitrate pre-treatment. At this dose, isosorbide dinitrate produced a significant reduction($p<0.01$) in the gastric damage score by 98% mean (0.41 ± 0.14mm) compared to(34.71 ± 0.96 mm) in the indomethacin alone treated group as shown in figure (1) .II- The effect on gastric PGE2,MPO,and IL-4: The gastric PGE2 level was not significantly changed mean (68.5± 1.9 ng/g) versus to (63.9 ± 2.1 ng/g) in the indomethacin treated group as shown in figure (3). While there was a significant decrease ($p<0.01$) in MPO activity by 92.8% mean (2.04 ± 0.18 u/mg) compared to (28.4± 0.55 u/mg) in indomethacin treated group, as shown in figure (4) . Gastric IL-4 was significantly increased ($p<0.01$) mean (37.26± 0.93 pg/mg) versus to (21.9± 0.84 pg/mg) in the indomethacin treated group , figure (6).B)-I) At isosorbide dinitrate (10mg/kg): Doubling the dose of isosorbide dinitrate to 10mg/kg resulted in significantly increased ($p<0.01$) gastric damage score (12.88± 1.2 mm) versus to (0.41± 0.14 mm) in the isosorbide 5mg/kg treated group, figure (1) .II) The effect on gastric PGE2,MPO,and IL-4: Gastric PGE2 level was not significantly affected (mean 67.1± 1.7ng/g) versus to (68.5 ± 1.9 ng/g) in the isosorbide 5mg/kg treated group figure (3) ,also there was no significant difference when compared with (63.9 ± 2.1 ng/g) in the indomethacin treated group, figure (3). The MPO activity was significantly increased ($p<0.01$) (29.39±1 u/mg) compared to (2.04±0.18 u/mg) in the isosorbide (5mg/kg) treated group , figure (4) , but with an insignificant difference when compared with (28.4± 0.55 u/mg) in the indomethacin treated group, figure (4) The gastric IL-4 was significantly reduced ($p<0.01$) mean(19.3±0.42 pg/mg) versus to (37.26 ± 0.93 pg/mg) in the isosorbide 5mg/kg treated group, as shown in figure (6), and no significant difference when compared with (21.9 ±0.84 pg/mg) in the indomethacin treated group, figure (6) 3. Effect of NOS inhibitors (L-NAME) on the isosorbide dinitrate 5mg/kg protective action :

I)Macroscopically : The mucosal protective action of isosorbide dinitrate (5 mg/Kg) was reversed by I.P. administration of L-NAME 30 min prior to isosorbide administration .Regarding to the gastric damage score was significantly increased($p<0.01$) (mean 18.16 ± 1.6 mm) versus to (0.41±0.14 mm) in the isosorbide (5mg/kg) group that did not receive L-NAME, figure (2).

II)The effect on gastric PGE2,MPO,and IL-4: Gastric PGE2 level was not significantly affected mean(67.7 ± 4.1 ng/g) compared to (68.5 ±1.9 ng/g) ($p<0.01$) in the isosorbide(5mg/kg) group that did not receive L-NAME, figure (3). There was a significant increase ($p<0.001$) in gastric MPO activity mean (45.03 ± 0.77 u/mg) versus to (2.04 ± 0.18 u/mg) in the isosorbide(5mg/kg) group that not received L-NAME, figure (5). While gastric IL-4 was significantly decreased($p<0.01$) mean(18.5± 0.58 pg/mg) versus to (37.26± 0.93 pg/mg) in isosorbide (5mg/kg) group that not received L-NAME , figure (7). 4. Effect of L-arginine on L-NAME reversal of 5mg/kg isosorbide dinitrate protective effect :When L-Arginine was coadministered with L-NAME, the gastric mucosal injury was reduced . This means that L-arginine restored the protective effect of isosorbide at 5 mg/Kg which was reversed by L-NAME . There was a significant reduction($p<0.01$) in gastric damage score mean (1.07 ± 0.3 mm) compared to (18.16± 1.16 mm) in the isosorbide (5mg/kg) that received L-NAME without L-arginine ,figure (2) While there was no significant difference when compared with (0.41± 0.14 mm) in the isosorbide (5mg/kg) treated group, figure (2) II) The effect on gastric PGE2,MPO,and IL-4: Gastric PGE2 level was not significantly affected mean (65.8 ±2.3 ng/g) compared to (67.7± 4.1 ng/g) in the isosorbide (5mg/kg) that received L-NAME without L- arginine ,figure (3), also there was no significant difference when compared with (68.5± 1.9 ng/g) in the isosorbide (5mg/kg) treated group, figure (3) .Regarding to the gastric MPO activity, there was a significant suppression ($p<0.01$) of mean (8.8± 0.6 u/mg) versus to (45.03± 0.77 u/mg)in isosorbide (5mg/kg) that received L-NAME without L-arginine , figure (5). While there was significant increase ($p<0.01$) in MPO activity compared with (2.04 ± 0.18 u/mg) in the isosorbide (5mg/kg) treated group, figure (5) . The gastric IL-4 was significantly ($p<0.01$) increased , mean(36.6± 0.85 pg/mg) compared to (18.5± 0.58 pg/mg) in the isosorbide (5mg/kg) that received L-NAME without L-arginine , figure (7). While there was no significant difference when compared with (37.26 ±0.93 pg/mg) in the isosorbide (5mg/kg) treated group, figure (7)

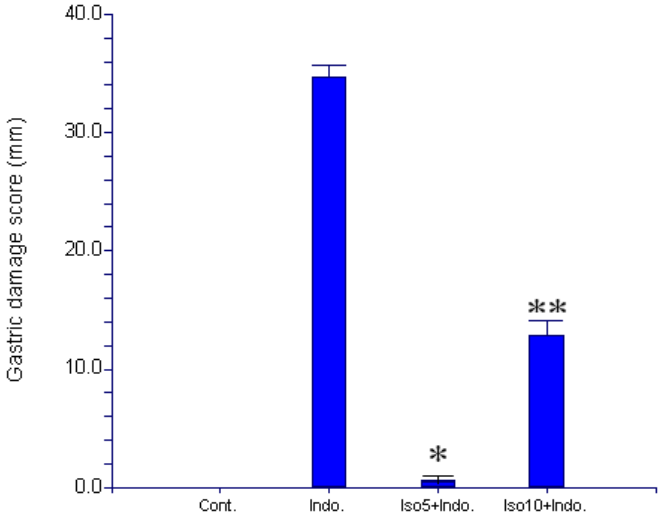

Figure (1): The protective effect of 5 mg/kg isosorbide dinitrate and the paradoxical effect of 10 mg/kg isosorbide dinitrate on the gastric damage score induced by indomethacin. The results are expressed as the mean ± SEM * P < 0.01 when compared with indomethacin group.
** P < 0.01 when compared with 5mg/kg isosorbide dinitrate group. cont: control , indo: indomethacin. iso-5:isosorbide 5mg/kg.1so-10: isosorbide 10mg/kg

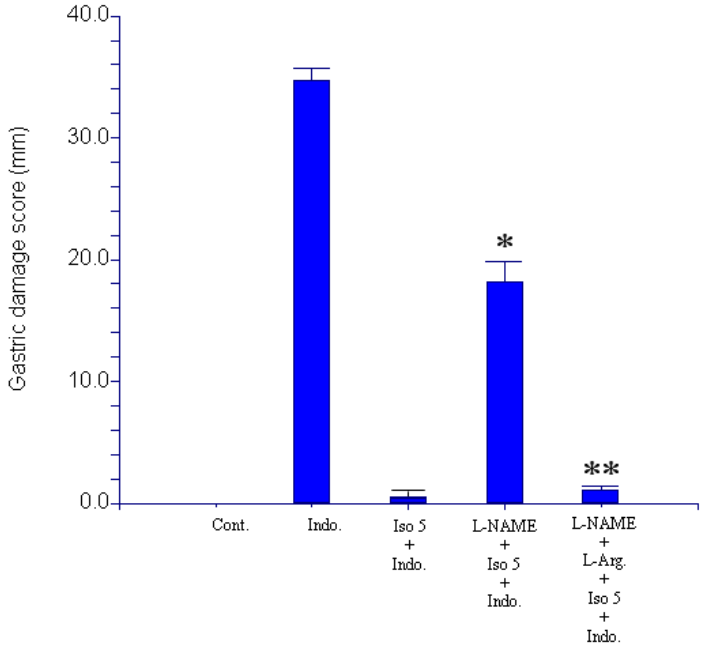

Figure (2): The loss of protective effect of 5 mg/kg isosorbide dinitrate by L-NAME and its restoration by addition of L-Arginine on the indomethacin induced gastric damage score gastropathy. The results are expressed as the mean ± SEM .
* P < 0.01 when compared with 5mg/kg isosorbide dinitrate group.
** P < 0.01 compared with 5mg/kg isosorbide dinitrate group that received L-NAME . cont: control , indo: indomethacin. iso-5:isosorbide 5mg/kg , L-Arg :L-Arginine

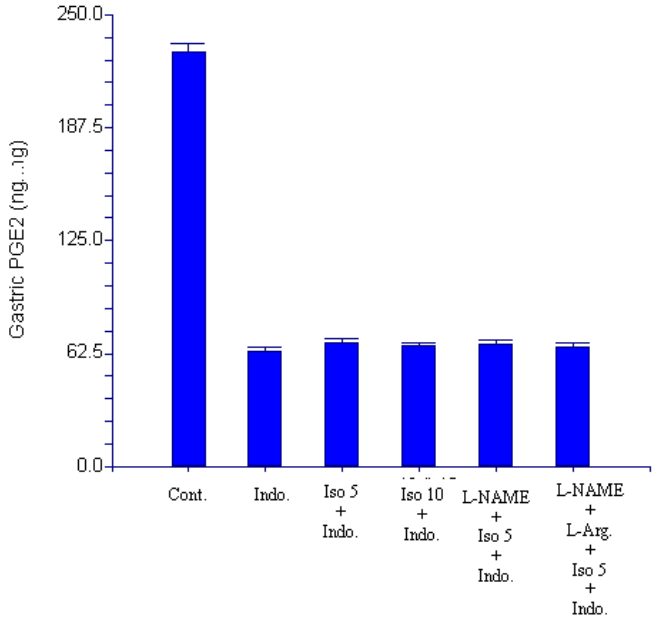

Figure (3): Gastric PGE2 levels following isosorbide dinitrate pretreatment with or without L-NAME (with or without L- Arginine) compared with Indomethacin alone showing no significant alterations. The results are expressed as the mean ±
SEM. cont: control , indo: indomethacin. iso-5:isosorbide 5mg/kg , L-Arg :L-Arginine .

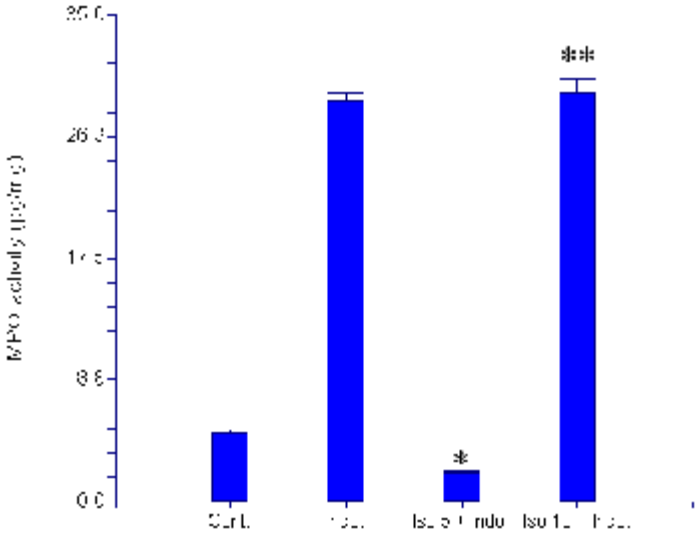

Figure (4): The effect of 5 mg/kg isosorbide dinitrate and the paradoxical effect of 10 mg/kg isosorbide dinitrate on the increase gastric MPO activity induced by indomethacin .The results are expressed as the mean ±SEM .
* P < 0.01 when compared with indomethacin group.
** P < 0.01 when compared with 5 mg/kg isosorbide dinitrate group.
cont: control , indo: indomethacin. iso-5:isosorbide 5mg/kg.1so-10:
isosorbide 10mg/kg

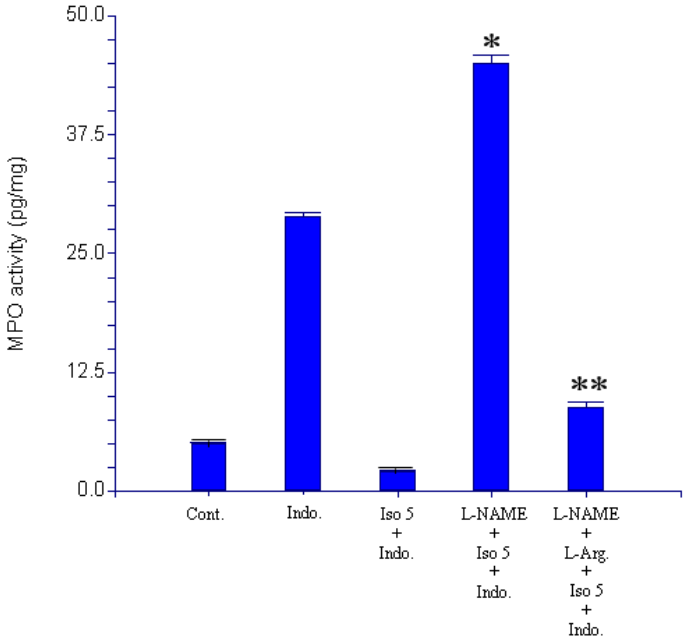

Figure (5): The loss of the effect of isosorbide dinitrate on indomethacin induced increase in MPO activity by L-NAME administration and its restoration by the addition of L-arginine .The results are expressed as the mean ±SEM

* P < 0.01 compared with 5mg/kg isosorbide dinitrate group.

** P < 0.01 compared with 5mg/kg isosorbide dinitrate group that received L-NAME.

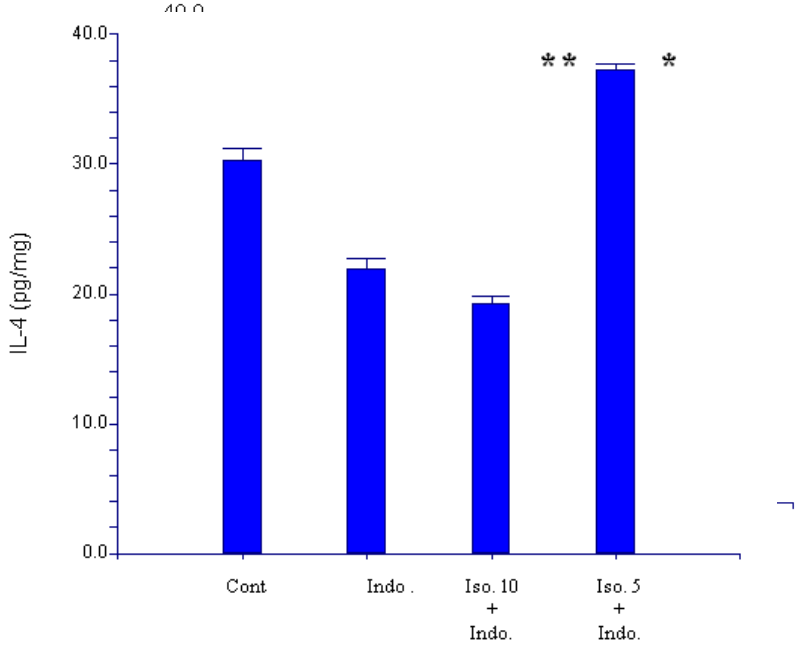

Figure (6): The effect of 5 and 10 mg/kg isosorbide dinitrate on the expression of gastric IL-4 during indomethacin induced mucosal injury . Values expressed as mean ±SEM

* P < 0.01 compared with Indomethacin group.

** P < 0.01 compared with 10mg/kg isosorbide dinitrate group.

cont: control , indo: indomethacin. iso-5:isosorbide 5mg/kg.1so-10: isosorbide 10mg/kg

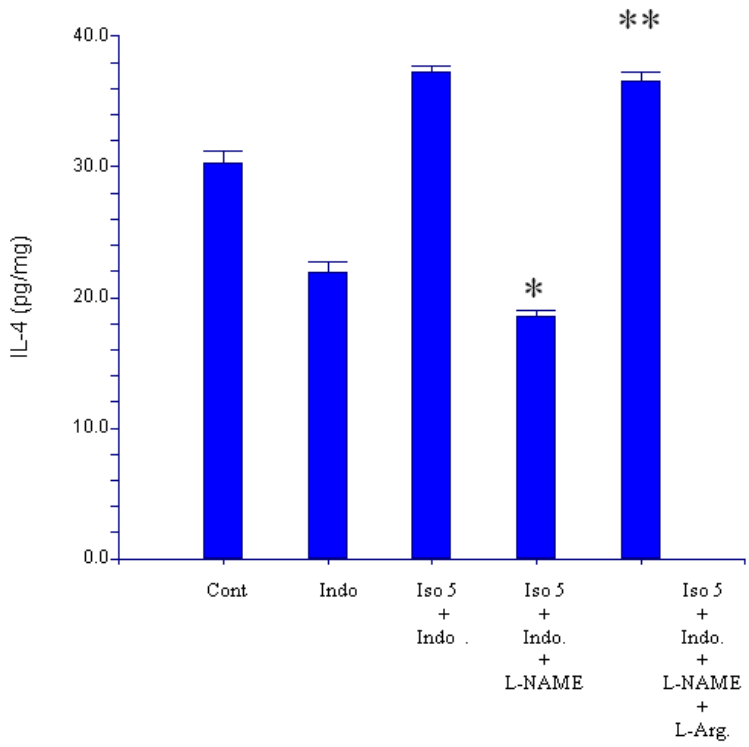

figure (7): The loss of the effect of isosorbide dinitrate on the indomethacin induced decrease in the expression of IL-4 by L-NAME administration and its restoration by the addition of L-arginine. The results are expressed as the mean + SEM.
* P < 0.01 when compared with 5mg/kg isosorbide dinitrate group.
** P < 0.01 compared with 5mg/kg isosorbide dinitrate group that received L-NAME. cont: control , indo: indomethacin. iso-5:isosorbide 5mg/kg , L-Arg :L-Ar

Discussion

Although NSAIDs-induced gastric mucosal injury was mainly attributed to the loss of the protective effects of PGs due to inhibition of its synthesis by these drugs ,which was demonstrated in this study by a significant reduction of 72% in PGE2 with indomethacin when compared to the control group .Nevertheless ,other mechanisms also seem to play important roles . A current proposal for gastropathy of NSAIDs based on the demonstrated capacity of these drugs in reducing the mucosal blood flow, with polymorphonuclear activation and its adherence to the vascular endothelium [16],this combined with oxyradical generation which are well recognized as one of the early events of gastric damage associated with the use of NSAIDs[17 ,18]. Neutrophil infiltration and free radicals generation are reflected by the MPO activity ,an enzyme that catalyses the oxidation reaction of H2O2 , and since this is specific to neutrophils it is often used as an indicator of neutrophil infiltration in tissues [19] . In this work indomethacin induced gastric damage was accompanied by significant ($p<0.01$) increase in this inflammatory marker (MPO) by 474%. This finding is comparable with observation of Santos *et al*., (2005) [7], where the increase in MPO was 440%. Furthermore ,it has been shown that extensive mucosal damage as that

produced by indomethacin resulted in reduction of IL-4. IL-4 is one of cytokines that suppress the secretion of proinflammatory cytokines IL-1,IL-2,& IL-6 and also block the synthesis and processing of metalloproteinase [20]. In this study, the results obtained showed that gastric mucosal response to indomethacin was accompanied by significant reduction ($p<0.01$) 27.6% in IL-4 level and this reduction was similar to that of 20.7% reported by Slomiany *et al.*, (1999) [21]

.In the present study , pretreatment with isosorbide dinitrate at a dose (5 mg/Kg) elicited a 98% significant reduction in the extent of gastric damage score caused by indomethacin, these finding confirm the protective effect of nitrates on the gastric mucosa . Owing to the fact that PGE2 level was not affected by isosorbide dinitrate , this means that the protective effect is unrelated to PGE2 . In order to prove that the protective effects of isosorbide dinitrate at 5 mg/Kg observed in this study is NO dependant pathway , pretreatment with L-NAME (a competitive, non-selective NOS inhibitor) abrogated the protective effects of isosorbide dinitrate, which was again restored by coadministration with L-arginine (NOS substrate).These finding clearly show that the protective effect of isosorbide is through NO dependent pathway .Also this study showed that gastric MPO activity was significantly reduced ($p<0.01$) by 92.8% by 5mg/kg isosorbide compared with indomethacin treated group, Which was reversed by the addition of L-NAME but restored back by L-arginine coadministration.

This reflects the potential anti-inflammatory effect of nitric oxide .Furthermore the same dose of isosorbide resulted in a significant rise ($p<0.01$) in gastric IL-4 level ,in fact the level of IL-4 obtained was even higher than in the control group , while the addition of L-NAME significantly suppressed IL-4 to a level just below that seen with indomethacin alone treated group , this effect of L-NAME on IL-4 was again reversed by the addition of L-arginine . This may confirm that isosorbide at 5mg/kg enhance IL-4 production possibly through NO. When the dose of isosorbide dinitrate was increased to 10mg/kg and by comparing it with 5mg/kg isosorbide dinitrate treated group , gastric damage score was significantly ($p<0.01$) increased , while the gastric PGE2 level was not significantly changed ,also there was a significant($p<0.01$) increase in MPO activity and significant ($p<0.01$) reduction in the gastric IL-4 level . All these finding point to a paradoxical effect of isosorbide dinitrate when its dose was increased to 10mg/kg . A suggested explanation for the paradoxical effect is that NO at physiological concentration is released through the cNOS enzymatic expression; this cNOS has maintenance activities on gastric mucous membrane function maintenance . On the other hand , NO overproduction (by increasing the dose) is released through the iNOS expression which would exhibit a cytotoxic activity, interacting with oxygen free radicals [5] .Other studies showed that high

concentration of NO exhibits a toxic actions mainly as a result of production of peroxynitrite ($ONOO^-$) the reaction product of NO with superoxide anion ($O2^-$) [22] .In addition peroxynitrite is capable of initiating lipid peroxidation [22] . Importantly low levels of NO offer protection against gastric injury , but high levels can induce gastric injury[23] .**Conclusion :**5mg/kg isosorbide dinitrate pretreatment demonstrated a cytoprotective effect against indomethacin induced gastric mucosal injury and this effect is lost by the addition of the
L-NAME , and restored again by L-arginine coadministration, which indicates that the cytoprotective effect of isosorbide dinitrate is through NO dependent pathway. However this cytoprotective effect of isosorbide is lost by doubling the dose to 10mg/kg.

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