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Investigation of the gastroprotective activity of some drugs in indomethacin-induced gastric lesion in rats

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Abstract

Long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) increases the risk of severe gastric events. To minimize these risks, patients often require concomitant acid-suppressive therapy. This study was conducted to investigate the putative gastroprotective effect of sildenafil, verapamil and propranolol and possible mechanisms underlying the effect of these drugs in experimentally-induced gastric lesions in rats. Rats were assigned to vehicle (saline), control (indomethacin, 30-mg/kg, p.o.), sildenafil (10-mg/kg, p.o.), verapamil (10 mg/kg, p.o.), propranolol (10 mg/kg, p.o.) and ranitidine (50-mg/kg, p.o); the drugs were administered 30-minute prior to indomethacin. After 4-hours, all rats were sacrificed, and stomach of each rat examined for gastric lesions either for biochemical or histopathological analysis. Indomethacin induced marked ulcerative lesions in form of strikes in the gastric mucosa. Furthermore, pre-treatment with sildenafil, verapamil, and propranolol significantly reduced gastric acid secretion, ulcer scores, and lipid peroxides. Moreover, they markedly protect the stomach against indomethacin effect. The results confirm that each drug has a gastroprotective effect but with different mechanisms in prevention.

1. Introduction

Peptic ulcers are multi-etiologic, frequently recurrent and widespread chronic disease (Süleyman et al., 2001). Peptic ulcers occur mainly in the stomach (gastric ulcer) or proximal duodenum (duodenal ulcer), and they continue to be a common disease that causes a substantial socioeconomic burden and negatively impacts on quality of life (Lee et al., 2010).

The pathogenesis of gastric ulcers is based on complex interactions between aggressive and protective factors (Prabha et al., 2009). Nonsteroidal anti-inflammatory drugs (NSAID) are known to be aggressive agents for gastric ulcer development (Kamel et al., 2009), they follow H. pylori in the ulcer etiopathogenesis (Bulent et al., 2007). As the prevalence of H. pylori, the infection has declined in worldwide; gastric ulcer has become more commonly associated with the use of nonsteroidal anti-inflammatory drugs (NSAIDs) (Makola et al., 2007). Indomethacin causes damage in the gastric mucosa and impairs ulcer healing as an adverse effect. Although the inhibition of cyclooxygenase (COX), which leads to depletion of endogenous prostaglandins (PGs), is a major pathogenic factor, it is unlikely that PG deficiency alone is sufficient to initiate the process that ultimately results in gastric ulceration (Takeuchi et al., 2001).

There are many anti-ulcerogenic drugs, among which the most efficient is omeprazole. However, these drugs do not always provide an effective treatment of ulcer (Bulent et al., 2007). Therefore, treatment of ulcers is still a major problem, and new drugs are urgently needed for the treatment of gastro-duodenal ulcer.

Sildenafil (SLD) is currently used in the treatment of functional impotence; it increases the efficiency of the guanosine cyclic 3', 5'- monophosphate (cGMP),

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which displays an inhibitory effect on the smooth muscle cells of the arterioles. Accumulating evidence from both animal and human studies indicates that NO plays critical roles in normal wound repair. The beneficial effects of NO on wound repair may be attributed to its functional influences on angiogenesis and inflammation (Pan et al., 2005).

Verapamil is a widely used for clinical treatment of cardiovascular diseases. It is accepted that calcium antagonists inhibit mobilization of calcium from intracellular stores with resultant inhibition of calcium effects on gastric function (Candido et al., 1993).

Consequently, the non-selective β-adrenoceptor antagonist, propranolol, was suggested that they are associated with gastroprotective and anti-secretory effects (Bhandare et al., 1990; Ray et al., 1993).

In the present study, we aimed to examine the putative gastroprotective effect of SLD, verapamil, and propranolol in indomethacin-induced gastric mucosal lesions. Further, the present study was extended to investigate possible mechanisms underlying their gastroprotective effect.

2. Materials and Methods

2.1. Animals

Sixty male albino rats weighing 150-200 g were used in the present study. Rats were housed in stainless steel cages with free access to food and water under controlled laboratory condition (temperature 25±3 and regular dark-light cycle). Rats were habituated to the experiment condition 10 days before conduction of the experiment.

All experiments were done under approval from institutional animal use & care committee.

2.2. Drugs

Indomethacin Powder was a gift from Nile Pharmaceutical Co (Cairo, Egypt). It was dissolved in 1% aqueous solution of Tween-80 to reach a final concentration of 2.5%. Ranitidine HCL: Powder was kindly provided by Medical Union Pharmaceuticals (MUP, Ismailia, Egypt) and it was prepared in water. Sildenafil Powder was a gift from MUP (Ismailia, Egypt). It was dissolved in 1% aqueous solution of Tween-80. Verapamil Powder was a gift from Sigma Pharmaceutical Co (Quesna, Egypt). It was dissolved in water. Propranolol Powder was a gift from Al-Qahira Pharmaceutical Co (Cairo, Egypt). It was dissolved in water

2.3. Induction of Experimental gastric lesion

Rats were fasted overnight before starting the experiments. Rats received an acute dose of indomethacin (30 mg/kg, p.o.). The indomethacin-treated rats were examined for gastric lesions four hours after indomethacin administration.

2.4. Experimental groups

Rats were randomly divided into 6 groups, 10 per each. Rats were assigned to vehicle (saline), control (indomethacin, 30 mg/kg, p.o.), ranitidine (50 mg/kg, p.o.), sildenafil (10 mg/kg, p.o.), verapamil (10 mg/kg, p.o.) and propranolol (10 mg/kg, p.o.). However, the control group received 0.5 ml distilled water. The drugs were administered 30 minutes before indomethacin.

2.5. Anesthesia:

After four hours, rats were anesthetized in a jar with a tight-fitting lid containing an appropriate amount of ether and sacrificed by cervical dislocation. After that, a laparotomy was performed and the stomach of each rat was dissected after pyloric ligation.

2.6. Acid determination

Gastric secretion was determined by the method of Aguwa et al., (1984). The gastric contents were collected by washing with 1 ml of saline and centrifuged at 3000 rpm for 10 min. The total acid concentration was determined in the supernatant by titration to pH 7.0 with 0.01N NaOH using phenolphthalein as indicator. After that, pH value of the gastric juice was calculated by the following formula: $pH = - \log [H +]$

2.7. Quantification of ulceration

The glandular portion comprising of the fundic and corpus region of each stomach was opened longitudinally along the greater curvature and examined microscopically. The number and severity of lesions in the glandular mucosa were scored from 0 to 5 according to the method of Clement et al., (1998).

0	No lesion
0.5	diffuse hyperemia
1	1 to 2 small ulcers
1.5	3 to 6 small ulcers
2	7 to 10 small ulcers
2.5	More than 10 small ulcers

3 1 marked ulcer plus 0 to 4 small ulcers

3.5 1 marked ulcer plus 5 or more small ulcers

4 2 marked ulcers plus 0 to 4 small ulcers

4.5 2 marked ulcers plus 5 or more small ulcers

5 3 or more marked ulcers

Then, the protective effect of the drugs was evaluated by calculating % inhibition of ulceration by the following formula: inhibition of ulceration = [(ulcer index in control - ulcer index in the test) / ulcer index in control] X 100 (Yinusa et al., 2000).

2.8. Mucin assay

Following a reported method, free mucin in the gastric tissue was estimated. Briefly, 0.5 g of the glandular segment of the stomach was added to 10 ml of 0.1% alcian blue solution (in 0.16 M sucrose buffered with 0.05 M sodium acetate; final pH was adjusted to 5.5). The stomach tissue was left to be stained for 2 hrs in the alcian blue solution. After that, the uncomplexed dye was removed by two washes with 0.25 M sucrose. The Comlexed dye was then eluted by immersion in 5 ml of 0.5 M magnesium chloride for 2 hrs. Dye extract was shaken briefly with an equal volume of diethyl ether and then centrifuged at 3600 rpm for 10 minutes. The optical density of the aqueous layer was read at 598 nm. A standard curve of alcian blue was plotted using different concentrations of alcian blue dye. Results were obtained as microgram per gram tissue (Kitagawa et al., 1986).

2.9. Preparation of tissue homogenate

0.5 g of stomach tissues were homogenized in phosphate-buffered saline (PBS) pH 7.4 using a glass-Teflon homogenizing tube (Glas Col homogenizer system, Vernon hills, USA). The homogenate was centrifuged at 2500 rpm for 10 minutes, and the supernatant was carefully removed from the pellet and used for biochemical analyses.

2.10. Biochemical analysis

2.10.1. Determination of nitric oxide in tissues

Nitrite was determined as an oxidation product and indicator of NO synthesis as described previously by Moshage et al., (1995).

The method is based on the addition of Griess reagent which converts nitrite into deep purple azo chromophore. No levels was expressed as μmol/g tissue.

2.10.2. Determination of lipid peroxides

Lipid peroxides (LPs) were assessed according to the method of Ohkawa et al. (40) based on the reaction with thiobarbituric acid using 1,1,3,3, tetra methoxy propane as a standard.

2.10.3. Estimation of GSH concentration in tissue:

Table 1: Total acidity and pH value in the gastric juice in indomethacin-treated rats and the effect of pretreatment with different drugs. The control group were treated with indomethacin (30 mg/kg, p.o.) and sacrificed 4 hrs later.

Groups	Total acidity (g/l)	pH
Saline	1.3±0.12	1.45±0.05
Indomethacin	3.38±0.16#	1.0±0.03#
Ranitidine ***(50 mg/kg, p.o.)***	0.57±0.06*	1.85±0.05*
SILD ***(10 mg/kg, p.o.)***	1.43±0.08*	1.46±0.03*
VPM ***(10 mg/kg, p.o.)***	0.8±0.48*	1.68±0.03*
Propranolol ***(10 mg/kg, p.o.)***	1.6±0.14*	1.59±0.14*

SILD: Sildenafil, VPM: verapamil

Rats were pretreated with ranitidine, SILD, VPM or propranolol 30 min. before indomethacin administration. The stomachs were dissected and gastric juices were collected for acid determination. Data are expressed as mean±SEM and analyzed using ANOVA followed by Bonfferroni's multiple comparisons test ($n=8$).

#: significantly different from saline group at $p \leq 0.05$.

*: significantly different from indomethacin group at $p \leq 0.05$.

Table 2: Ulcer scores and % inhibition of ulceration in indomethacin-treated rats and the effect of pretreatment with different drugs. The control group were treated with indomethacin (30 mg/kg, p.o.) and sacrificed 4 hrs later.

Groups	Ulcer score	% Inhibition of ulceration
Saline	0	100
Indomethacin	5±0#	0#
Ranitidine ***(50 mg/kg, p.o.)***	0.13±0.08*	99*
SILD ***(10 mg/kg, p.o.)***	0.38±0.18*	91*
VPM ***(10 mg/kg, p.o.)***	1.19±0.23*	80.6*
Propranolol ***(10 mg/kg, p.o.)***	2±0.2*	60*

SILD: Sildenafil, VPM: verapamil
Rats were pretreated with ranitidine, SILD, VPM or propranolol 30 min. before indomethacin administration. The stomachs were dissected and opened along the greater curvature and examined for the ulcer score.
% Inhibition of ulceration = [(ulcer index in control - ulcer index in test)/ulcer index in control] X 100.
Data are expressed as mean±SEM and analyzed using ANOVA followed by Bonfferroni's multiple comparisons test ($n=8$).
#: significantly different from saline group at $p \leq 0.05$.
*: significantly different from indomethacin group at $p \leq 0.05$.

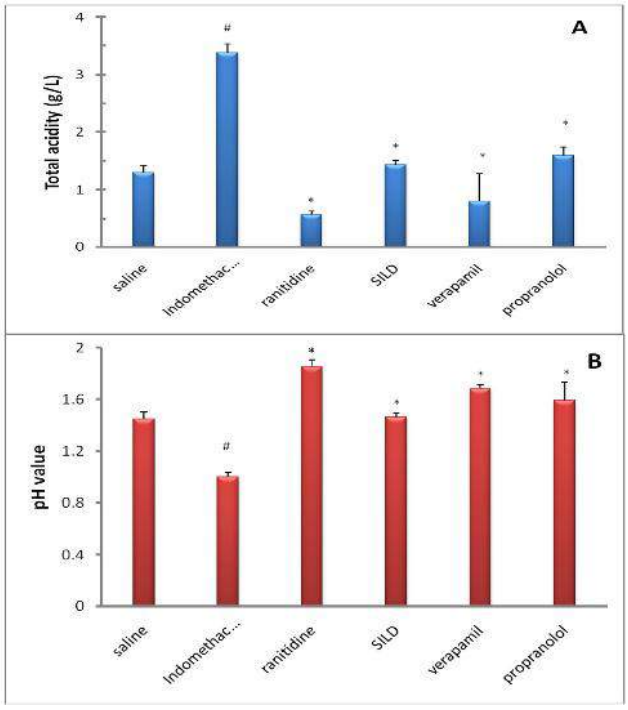


Figure 1: Total acidity (panel-A) and pH value (panel-B) in the gastric juice of indomethacin-treated rats (30 mg/kg, p.o.) and the effect of pretreatment with different drugs.

Administration of indomethacin (30 mg/kg, p.o.) induced a significant increase in the total acidity and pH values as compared to saline group (p≤0.05). Pretreatment with ranitidine (50 mg/kg, p.o.), SILD (10 mg/kg, p.o.), verapamil (10 mg/kg, p.o.) or propranolol (10 mg/kg, p.o.) suppressed acid production and pH value of the gastric juice as compared to indomethacin group (p≤0.05).
Data are expressed as mean±SEM and analyzed using ANOVA followed by Bonfferroni's multiple comparisons test (n=8).
#: significantly different from saline group at p≤0.05.
***: significantly different from indomethacin group at p≤0.05.**

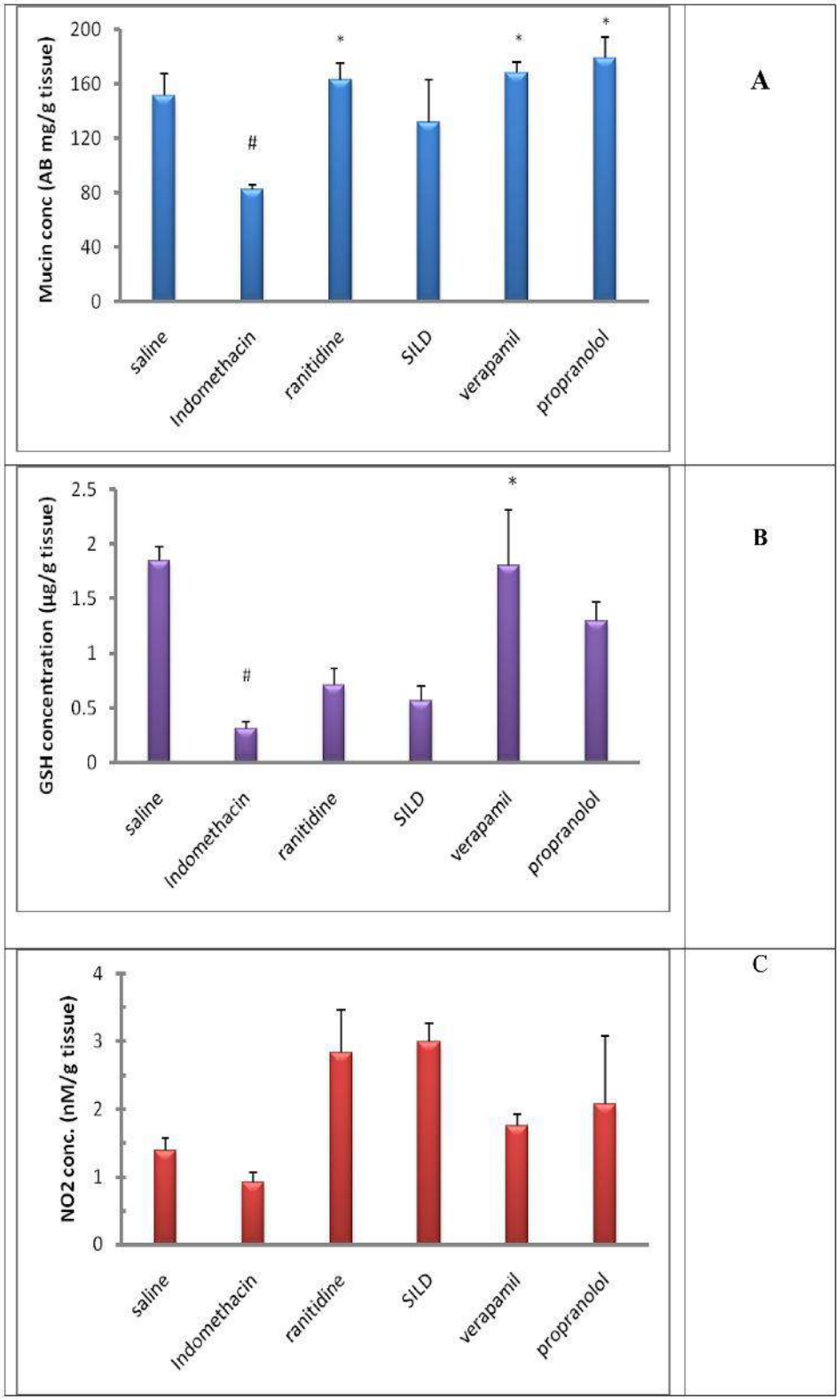


Figure 2: Ulcer scores (panel-A) and % inhibition of ulceration (panel-B) in the gastric juice of indomethacin-treated rats (30 mg/kg, p.o.) and the effect of pretreatment with different drugs.

Administration of indomethacin (30 mg/kg, p.o.) induced a significant increase in the ulcer score as compared to saline group ($p \leq 0.05$). Pretreatment with ranitidine (50 mg/kg, p.o.), SILD (10 mg/kg, p.o.), verapamil (10 mg/kg, p.o.) or propranolol (10 mg/kg, p.o.) suppressed ulcer score and improved the % inhibition of ulceration as compared to indomethacin group ($p \leq 0.05$). % inhibition = [(ulcer index in control - ulcer index in test) / ulcer index in control] X 100.
Data are expressed as mean±SEM and analyzed using ANOVA followed by Bonfferroni's multiple comparisons test (n=8).
#: significantly different from saline group at $p \leq 0.05$.
***: significantly different from indomethacin group at $p \leq 0.05$.**
***: significantly different from indomethacin group at $p \leq 0.05$.**

Glutathione in the stomach homogenate was measured according to the colorimetric method of Sedlak and Lindsay (1968). The glutathione level in the gastric mucosa was expressed as nano mole per milligram tissue.

2.10.4. Determination of TNF-α

TNF-α was determined according to Mysliwska et al. (1998), using Biosource International Kits (Camarillo, California, USA).

2.11. Histopathological examination

For histological assessment, the glandular area of stomach was fixed in 10% phosphate-buffered paraformaldehyde solution and prepared for staining with, hematoxylin and eosin and then examined under a light microscope. The specimens were assessed according to the criteria of Laine and Weinstein (1988). In brief, a 1 cm length of each histological section was assessed for epithelial cell loss (a score of 0-3), edema in the upper mucosa (a score of 0-4), hemorrhagic damage (a score of 0-4) and presence of inflammatory cells (a score of 0-3). The sections the sections were assessed by an experienced pathologist without the knowledge of treatments.

2.12. Statistical Analysis

Data were expressed as mean±SEM and statistically analyzed using SPSS program version 16. One-way analysis of variance, ANOVA, followed by Bonferroni's multiple comparisons test are used for the statistical analysis. For all comparisons, differences were considered significant at $p \leq 0.05$.

3. Results and Discussion

In the present study, oral administration of indomethacin (30 mg/kg, p.o.) induced a significant increase in the total acid concentration (3.38 ± 0.16 g/L) as compared with the saline group (1.3 ±0.12 g/L, $P \leq 0.05$, **Table 3**). Accordingly, the pH value of the gastric juice in indomethacin group was significantly lower than saline group (1.01 ± 0.03 vs. 1.45 ± 0.05, $p \leq 0.05$, **Table 1, Figure 1**).

Additionally, indomethacin treatment induced a marked increase in the ulcer score as compared to saline group (5 ± 0 vs. 0). Meanwhile, the percent of inhibition was significantly lower than the saline group (0% vs. 100%, $p \leq 0.05$, **Table 2, Figure 2**).

Pretreatment with Ranitidine suppressed the acid production and subsequently elevated the pH value as compared to indomethacin group (0.57±0.06 and 1.85±0.05 vs. 3.38±0.16 and 1±0.03, respectively, $p \leq 0.05$, **Table 3**). Furthermore, Ranitidine attenuated the ulcer score and increased % inhibition of ulceration as compared to indomethacin group.

Similar to ranitidine, pretreatment with Sildenafil, Verapamil or Propranolol markedly suppressed acid production and increased pH value as compared to indomethacin group ($p \leq 0.05$, **Table 3**).

Table 3: Gastric mucin contents and GSH in stomach homogenate in the experimental groups and the effect of pretreatment with different drugs. The control group were treated with indomethacin (30 mg/kg, p.o.) and sacrificed 4 hrs later.

Group	Mucin (AB mg/g tissue)	GSH (µg/g tissue)
Saline	151±16.4	1.85±0.13
Indomethacin	82±6[#]	0.31±0.06[a]
Ranitidine ***(50 mg/kg, p.o.)***	163±12.7[*]	0.71±0.15
SILD ***(10 mg/kg, p.o.)***	132±30.8	0.56±0.14
VPM ***(10 mg/kg, p.o.)***	168 ± 8.4[*]	1.8±0.51[*]
Propranolol ***(10 mg/kg, p.o.)***	179±14.93[*]	1.3±0.17

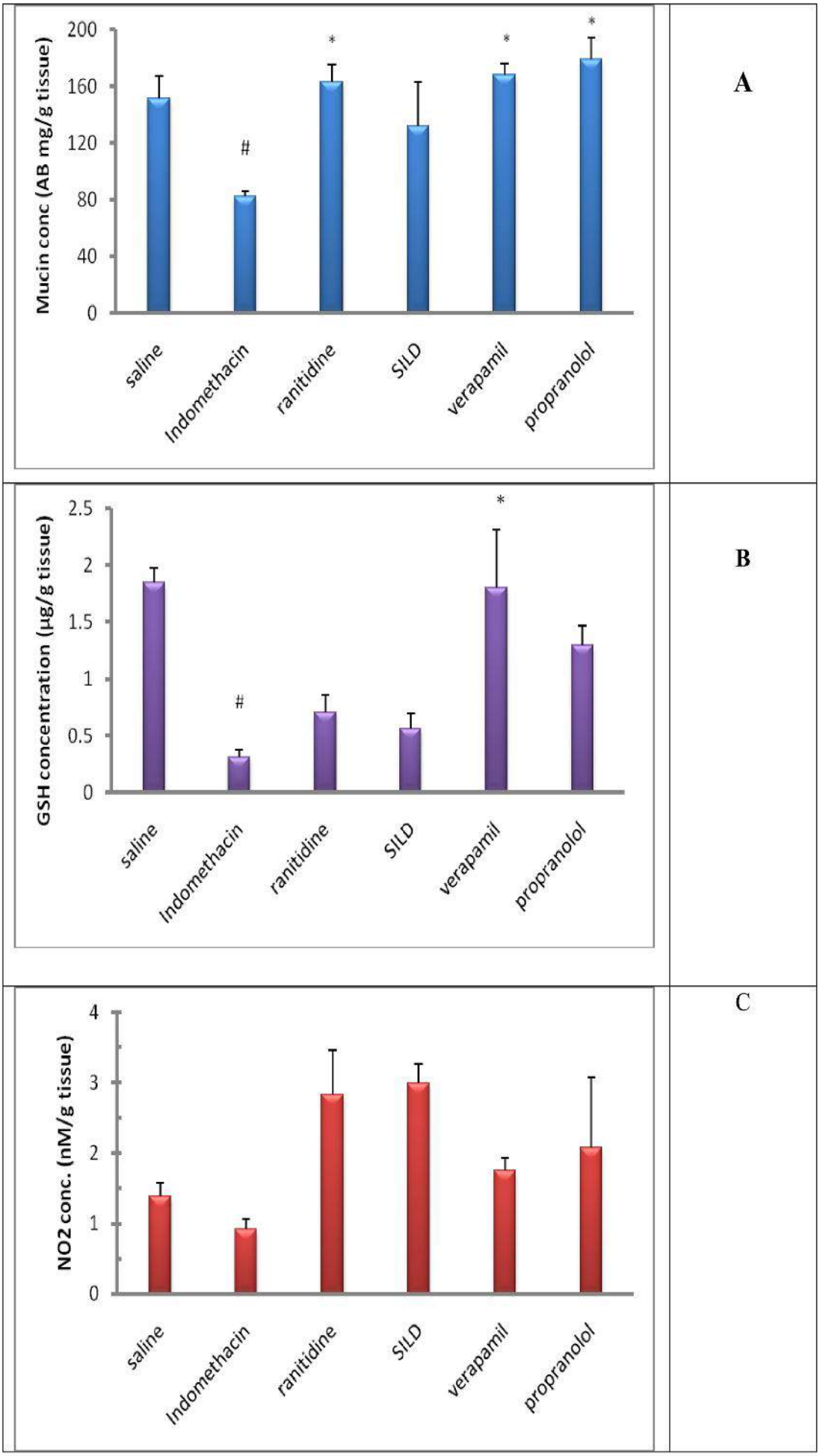
SILD: Sildenafil, VPM: verapamil, GSH: reduced glutathione

Rats were pretreated with ranitidine, SILD, VPM or propranolol 30 min. before indomethacin administration. The stomachs were dissected and 0.5 g of the tissue was frozen and subsequently homogenized for the determination of the measured parameters

Data are expressed as mean±SEM and analyzed using ANOVA followed by Bonfferroni's multiple comparisons test ($n=8$).

#: significantly different from saline group at $p \leq 0.05$.

*: significantly different from indomethacin group at $p \leq 0.05$.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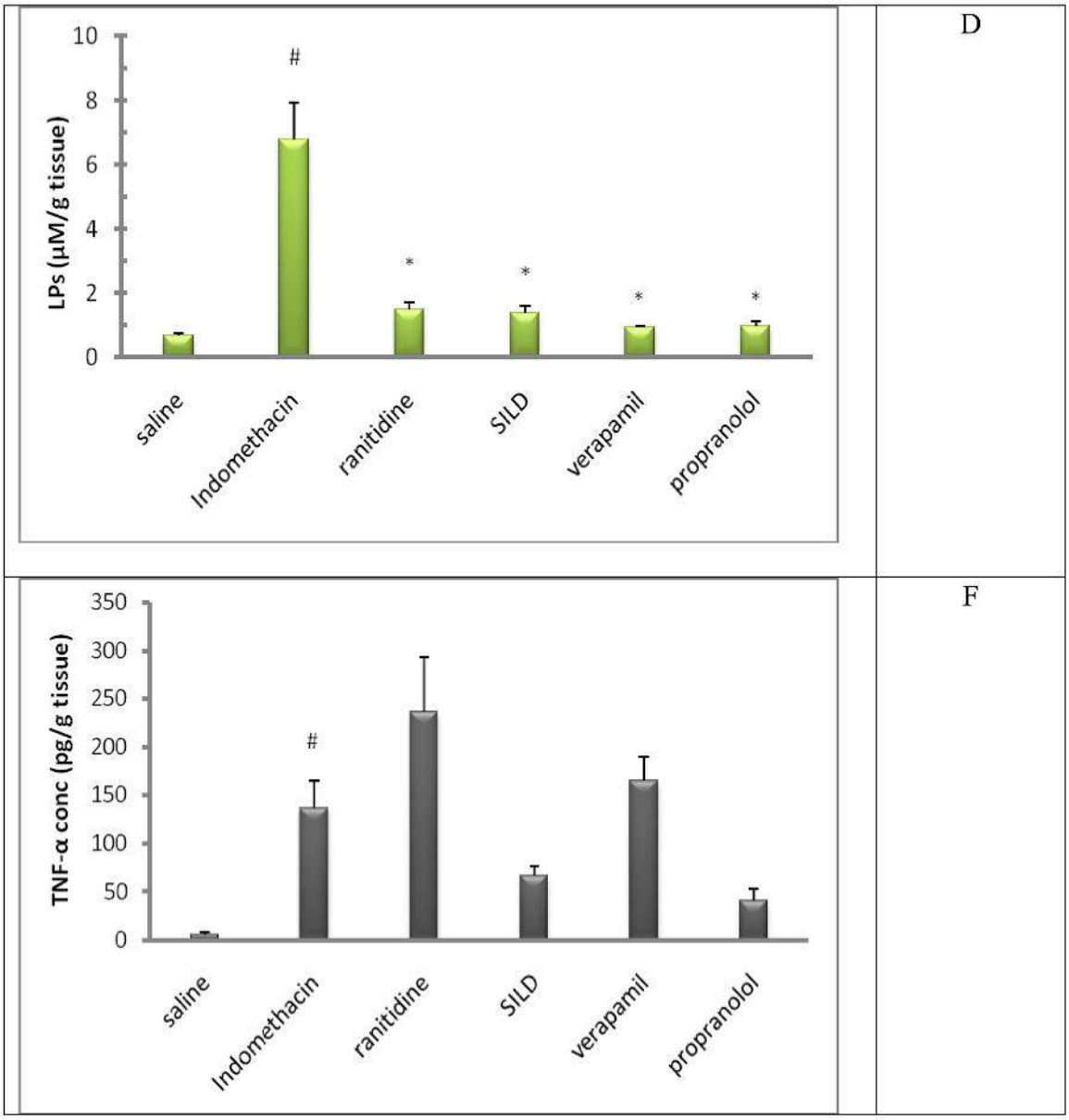

Figure 3: The antioxidant and anti-inflammatory action of the different drugs in the gastric juice of indomethacin-treated rats (30 mg/kg, p.o.).

Administration of indomethacin (30 mg/kg, p.o.) induced a significant increase in the oxidant and inflammatory markers as compared to saline group (p≤0.05). Pretreatment with ranitidine (50 mg/kg, p.o.), SILD (10 mg/kg, p.o.), verapamil (10 mg/kg, p.o.) or propranolol (10 mg/kg, p.o.) suppressed the inflammatory and oxidant markers as compared to indomethacin group (p≤0.05). Data are expressed as mean±SEM and analyzed using ANOVA followed by Bonfferroni's multiple comparisons test (n=8).
#: significantly different from saline group at p≤0.05.
***: significantly different from indomethacin group at p≤0.05.**

Moreover, the mean ulcer score was attenuated by pretreatment with these drugs as compared to indomethacin group (p≤ 0.05, **Table 3**).

However, the inhibition % of ulceration was significantly enhanced by these drugs as compared to indomethacin group (91%, 80.6%, 60% vs. 0%, respectively, p≤0.05, **Table 3**). On the other hand, the biochemical parameters measured in the stomach tissue differed significantly in Indomethacin-treated rats as compared with saline-treated rats; this with the exception of NO. Mucin and GSH were significantly decreased. However, LPs and TNF-α were significantly elevated in the stomach tissue of indomethacin group (p≤0.05, **Figure 3**).

Pretreatment with Ranitidine significantly restored tissue mucin concentration and suppressed the production of LPs in the stomach as compared to indomethacin-group. Ranitidine group showed higher TNF-α level as compared to Indomethacin-group (p≤0.05, **Figure 3**). Pretreatment with Sildenafil

Table 4: Gastric NO, LPs and TNF-α in stomach homogenate in the experimental groups. The control group were treated with indomethacin (30 mg/kg, p.o.) and sacrificed 4 hrs later.

Group	NO (nM/g tissue)	LPs (µM/g tissue)	TNF-α (pg/g tissue)
Saline	1.38± 0.2	0.68±0.063	5.64±2.4
Indomethacin	0.92±0.14	6.8±1.14a	135.8±29#
Ranitidine ***(50 mg/kg, p.o.)***	2.84±0.62	1.51±0.197*	236±57
SILD ***(10 mg/kg, p.o.)***	3±0.27	1.38 ± 0.23*	66±9.9
VPM ***(10 mg/kg, p.o.)***	1.75±0.18	0.92 ±0.06*	165.1±24
Propranolol ***(10 mg/kg, p.o.)***	2.07±1.02	0.97 ±0.15*	40.8±11.8

SILD: Sildenafil, VPM: verapamil, NO: nitric oxide, LPs: lipid peroxides, TNF-α: tumor necrosis factor-α

Rats were pretreated with ranitidine, SILD, VPM or propranolol 30 min before indomethacin administration. The stomachs were dissected and 0.5 g of the tissue was frozen and subsequently homogenized for the determination of the measured parameters

Data are expressed as mean±SEM and analyzed using ANOVA followed by Bonfferroni's multiple comparisons test ($n=8$).

#: significantly different from saline group at $p\leq0.05$.

*: significantly different from indomethacin group at $p\leq0.05$.

unexpectedly decreased the LPs production as compared to indomethacin group (p≤0.05, **Figure 3**).

Furthermore, Verapamil could enhance Mucin and GSH and suppress LPs production as compared to Indomethacin-group (p≤ 0.05, **Figure 3**).

Finally, pretreatment with Propranolol could elevate Mucin and LPs suppress production in the stomach tissue as compared to indomethacin group (p≤0.05, **Table 5**).

From the histological point of view, Administration of indomethacin (30 mg/kg, p.o.) induced marked erosion to the mucosal epithelia coupled with congestion and bleeding. Also, inflammation was

Table 5: Degree of microscopic changes in the gastric mucosa of the different experimental groups. The control group was treated with indomethacin (30 mg/kg, p.o.) and sacrificed 4 hrs later.

Experimental group (n=8)	Hemorrhagic damage (score 0-4)	Edema (score 0-4)	Epithelial cell loss (score 0-3)	Inflammatory cells (score 0-3)	Total (scores 14)
Saline	0	0	0	0	0
Indomethacin	4	4	3	3	12
Ranitidine ***(50 mg/kg, p.o.)***	1	1	1	1	4
SILD ***(10 mg/kg, p.o.)***	1.5	2	1	.5	5
VPM ***(10 mg/kg, p.o.)***	1.5	2	1.5	1	6
Propranolol ***(10 mg/kg, p.o.)***	3	2.5	1.5	2	8

SILD: Sildenafil, VPM: verapamil,

Rats were pretreated with ranitidine, SILD, VPM or propranolol 30 min before indomethacin administration. The stomachs were dissected and longitudinal sections were taken and fixed in 10% paraformaldehyde solution and sections were cut along the stomach wall and stained with hematoxylin and eosin and examined for microscopic changes.

Data are expressed as mean±SEM and analyzed using ANOVA followed by Bonfferroni's multiple comparisons test ($n=8$).

#: significantly different from saline group at $p\leq0.05$.

*: significantly different from indomethacin group at $p\leq0.05$.

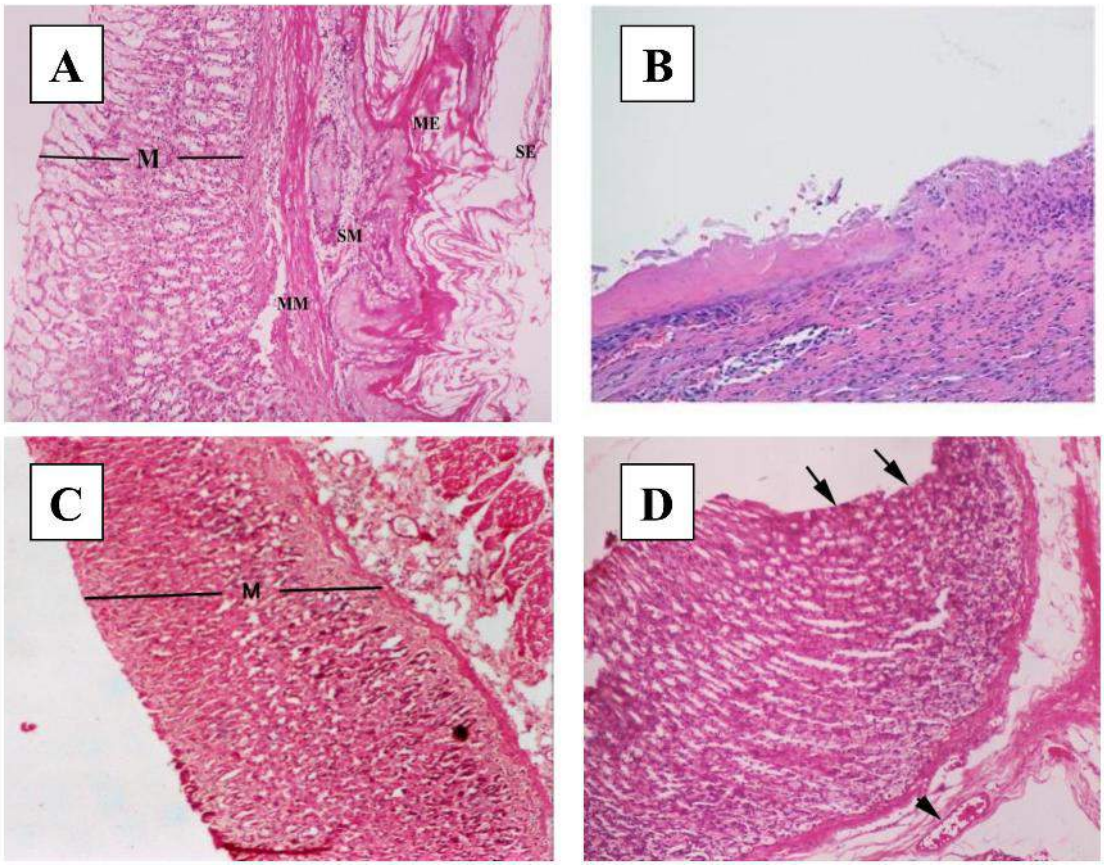

Figure 4: A- stomach showing normal histological appearance mucosa (M), MM (muscularis mucosa), submucosa (SM), muscularis enterna (ME) and serosa (SA). B: stomach group control +v showing erosion of mucosa, degeneration and necrosis of gastric gland and hemorrhage. (C): rantidine group showing failurly normal gastric mucosa. (D): stomach of verapamil group showing erosion of mucosa (arrows) with degeneration and necrosis (covering of epithelium with congestion of blood vessels of submucosa (short arrows).

manifested by the higher number of leukocytes in the field (mainly, neutrophils and macrophages) (**Figure 4**).

Pretreatment with ranitidine and Sildenafil improved the histopathological picture; the erosion was mild and limited to the superficial epithelia (**Figure 4**).

Pretreatment with Verapamil or Propranolol protected against the erosive action of indomethacin, the degree of hemorrhage was, and leukocytic attraction was less than indomethacin group (**Figure 4**).

4. Conclusion

P. aeruginosa are quiet critical micro-organism in the treatment therapy; as the resistance rate is increasing in response to illegal antimicrobial use especially in the developing counties.

API 20NE provide a rapid and accurate species identification. Antibiotic have been monitored and rotated on hospital use to avoid bacterial resistance.

5. Conflict of interest

The authors report no declaration of conflict of interest.

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