EVALUATION OF THE GASTROPROTECTIVE EFFECT OF MISOPROSTOL, CHITOSAN AND THEIR COMBINATION ON INDOMETHACIN INDUCED GASTRIC ULCER IN RATS.

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ABSTRACT

Background: The study was designed to evaluate the anti-ulcer effect of chitosan, misoprostol, and their combination on gastric ulceration induced by indometacin in rats.

Methods and experimental design: Chitosan was prepared from shrimp shells waste products. Thirty rats were divided into 5 groups, 6 rats each. Rats in group 1 (control) were given solid sugar and distilled water for 3 days; group 2 were treated by indometacin (25mg/rat) ; group 3,4, and 5 were treated by misoprostol, chitosan, and by the combination chitosan and misoprostol respectively before treatment with indometacin. Blood were collected before sacrificing the animals and used for estimation of MDA, a marker of oxidative stress. The stomachs were prepared for estimating the total gastric area, ulcerated area, tissue MDA, mucin production as well as for histopathological examination.

Results: Indometacin produced gastric ulcers, and increased the total gastric area in all animals. These effects were associated with a significant elevation of MDA levels in the blood and in stomach tissues, and a significant reduction in mucin production. Misoprostol, chitosan and their combination protected gastric mucosa since they significantly reduced ulcer index. Moreover, the observed anti-ulcer effect was more with the combination in comparison to monotherapy of misoprostol or chitosan.

Treatments by misoprostol, chitosan and their combination before indometacin significantly reduced blood and tissue MDA levels and increased mucin production.

Conclusion: Chitosan, misoprostol and their combination have gastroprotective effects against indometacin-induced gastric ulceration in rats.

INTRODUCTION

Long term use of NSAIDs causes gastric or duodenal ulceration in some patients with serious complications such as hemorrhage or even gastrointestinal perforation.[1] With the development of advanced endoscopy techniques such as capsule endoscopy and balloon enteroscopy, NSAIDs were found to cause damage to the small intestine in about 50% of patients receiving these drugs.[2] The pathophysiology of NSAIDs-induced gastric ulceration can be considered as instability among the injurious factors, direct gastric tissue injury by the drug, gastric acid, pepsin, infection by Helicobacter pylori and the protective factors, bicarbonate secretion, mucus and prostaglandins. The current approach of treatment is directed to the reduction of injurious factors and to strengthen mucosa protection of the stomach and duodenum through cytoprotective agents.[3] Indometacin, one of the NSAIDs groups labeled as powerful prostaglandin inhibitors,[1] is frequently associated with gastrointestinal ulceration and bleeding. The aim of this study is to investigate the gastro-protective effect of chitosan, a polymer with gastro-protective effect,[5] misoprostol, a prostaglandin analogue[4] or their combination in indometacin induced gastric ulceration in rats.

MATERIALS AND METHODS

Animals handling

Thirty males and females albino rats were purchased from the College of Science,

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University of Basrah. They were three month old, 200g average weight, and were considered healthy by general examination. The animals were divided into 5 groups, six animals each

Preparation of drugs

Drugs were administered by oral gavage using modified insulin syringes. Since the body weights of animals ranged from (197-205 g), fixed doses of the indometacin were given[6] as follow: indometacin 25mg/rat, by suspending one capsule of indometacin (Indylon 25mg, Medochemie, Cyprus), in 1 ml distilled water, and misoprostol tablet (Cytotec 200 µg, Searle, UK), suspended in 10ml distilled water, and administered in a dose of 20 µg /rat. Chitosan was prepared locally from shrimp shell wastes according to the method of Weska et al.[7] This yielded high molecular weight (HMW) chitosan, then low molecular weight (LMW) chitosan was obtained according to the method of Yong et al.[8] Chitosan was tested by infrared spectrophotometry. Calculation of molecular weight was performed to satisfy standard specifications.

LMW chitosan was then suspended in 1ml distilled water and given in a dose of 400 mg/rat.

Experimental design

The study protocol was described as follow:

Group	Day 0 –Day 7	Day8	Day 9- Day 12
Group 1 Control	Normal diet	Fasting	Distilled water
Group2 Indometacin (I) treatment	Normal diet	Fasting	Indometacin daily (25mg / rat)
Group 3 Misoprostol (M) treatment	Normal diet + (M) (20µg/day)	Fasting	Indometacin daily (25mg / rat)
Group 4 Chitosan (C) treatment	Normal diet +(C)(400mg/day)	Fasting	Indometacin daily (25mg / rat)
Group 5 Combination (M+C)	Normal diet + (M+C)	Fasting	Indometacin daily (25mg / rat)

All treatments were administered orally. At the end of each study protocol and before sacrificing the animals blood samples were collected directly from the heart, the animals were then sacrificed under light chloroform anesthesia.

Preparation of the stomach

A longitudinal incision from abdomen toward chest cavity was made. The stomach was dissected from the other viscera and separated by means of two incisions, the first one at the cardiac sphincter and the other at the pyloric sphincter.[9] The stomach was then opened along the greater curvature, total gastric area (T.G.A); gastric lesions as ulcer index (U.I.) were measured by digital caliper. Parts of gastric mucosa were removed for measurement of MDA and free mucin levels. Finally the stomach was immersed in 7y10% formalin solution for histopathological examination. The percentages of inhibition of ulcer index in relation to the indometacin treatment were calculated according to Dengizet *al.*,[10] formula, Inhibition percentage = U.I. treatment/U.I. (indometacin) × 100

The collected blood or stomach tissue samples were analyzed as follow:

Measurements of serum MDA level by thiobarbituric acid assay described by Beuge and Aust.[11] Measurements of stomach tissue

MDA according to the method of Cassini et al.[12] Measurements of free mucin in stomach tissue by measuring the amount of Alcian blue dye.[13]

Statistical analysis

One way analysis of variance **(ANOVA)** was carried out by SPSS computer package version 11. The differences were considered significant at $P < 0.05$.

RESULTS

Effect of treatments on total gastric area

The mean total gastric area of the control was (875.1±33.5 mm^2) which was slightly increased to (1003.3±11.9 mm^2) in the group which received indometacin. This difference was statistically significant (P<0.05). The mean total gastric area in the rats which received misoprostol, LMW chitosan and the combination of LMW chitosan and misoprostol were (888.5 ± 113.5 mm^2, 893.8 ± 44.6 mm^2 and 881.1 ± 53mm^2) respectively. These values are not significantly different from the control group value (871.1 ± 33.5 mm^2) (table-1).

Effects of treatments on ulcer index

There were no ulcers found in the rats treated with distilled water (control group). In the rats treated by indometacin (25mg/rat) there were gastric ulcers seen, and the mean calculated ulcer index was (151.8 ± 27.1mm^2). Treatment by misoprostol (20 µg/rat) daily for 7 days administered before indometacin (25mg/rat) daily for 3 days significantly reduced the mean ulcer index to (28 ± 6.7mm^2) in comparison to the mean ulcer index value of indometacin, (P<0.05). In the LMW chitosan treated rats, four animals had no ulcer and in two small areas of ulceration were seen. The mean ulcer index in LMW chitosan treated rats was (3.6 ± 5.7mm^2) which was significantly lower than that of misoprostol (P<0.05) and indometacin (P<0.05). There was no ulcer, necrosis or hyperemia seen in all animals (n=6) which were treated by the combination of LMW chitosan plus misoprostol (LMW chitosan 400 mg/rat + Misoprostol 20 µg / rat daily for 7 days) (table-1).

Effects of treatments on blood MDA

The mean MDA level of the control was (0.92 ± 0.15 µmol/L). In indometacin treated group the mean MDA level significantly increased to (2.28 ± 0.22 µmol/L) (P<0.05). Misoprostol prior to indometacin treatment has resulted in a mean MDA level of (2.01 ± 0.07 µmol/L) which was slightly but significantly lower than that of indometacin (P<0.05), but, still was higher than the MDA level of the control treated group. When LMW chitosan was administered prior to indometacin, the mean blood MDA became (1.48 ± 0.93 µmol/L) which was significantly lower than that of indometacin (P<0.05). The mean MDA level measured for LMW chitosan plus misoprostol administration prior to indometacin was (0.98 ± 0.18 µmol/L) which was reduced towards the value of the control group (0.92 ± 0.15 µmol/L) (table-1).

Effects of treatments on tissue MDA

Stomach tissue MDA was measured for the control group and was (2.1±0.91 ηmol /mg). In indometacin treated group the mean value of tissue MDA significantly increased to (15.7 ± 2.58 ηmol/mg) (P<0.05). Misoprostol prior to indometacin treatment has resulted in a tissue MDA level of (2.7 ± 1.42 ηmol/mg) which was significantly lower than that of indometacin, but, was slightly higher than that of the control group. The value of tissue MDA of the two groups, which were treated by LMW chitosan alone and chitosan plus misoprostol prior to indometacin was found to be (0.8 ± 0.98 ηmol/mg) and (0.7 ± 0.05 ηmol/mg) for to the two treatments respectively. These values were significantly lower than the level of the tissue MDA of the control group (P<0.05) (table-1).

Effects of treatments on mucin secretion

The mean value of mucin in the control group was (0.134 ± 0.002 ml/cm^2). There was a great and statistically significant reduction of mucin

(0.063 ± 0.028 ml/cm^2) in the group of rats treated with indomethacin. Treatment with misoprostol, LMW chitosan and the combination of chitosan plus misoprostol respectively for 7 days administered before indometacin (25 mg/rat daily for 3 days) had significantly increased mucus secretion compared to indometacin treated group (P<0.05). The respective mean values of mucin for misoprostol, LMW chitosan and the combination of LMW chitosan plus misoprostol were (0.205±0.028 ml/cm^2), (0.351 ± 0.034 ml/cm^2) and (0.374± 0.028 ml/cm^2) respectively (Table-1).

Table 1. The effect of LMW chitosan, misoprostol and their combination on various measurements in indometacin induced gastric ulceration in rats.

MEASUREMENTS	CONTROL	INDOMETACIN (25 MG / RAT)	MISOPROSTOL (20 µG / RAT)	CHITOSAN (400 MG / RAT)	CHITOSAN (400 MG / RAT) + MISOPROSTOL (20 µG / RAT)
Total gastric area (mm^2)	875.1 ± 33.5	1003.3 ± 111.9^{a}	888.5 ± 113.9^{b}	893.8 ± 44.6^{b}	881.1 ± 53^{b}
Ulcer index (mm^2)	0	151.8 ± 27.1^{a}	28 ± 6.7^{a,b}	3.6 ± 5.7^{a,b,c}	0
Blood MDA (µmol/L)	0.92 ± 0.15	2.28 ± 0.22^{a}	2.01 ± 0.07^{a,b}	1.48 ± 0.39^{a,b,c}	0.98 ± 0.18^{a,b,c,d}
Gastric tissue MDA (ηmol/mg)	2.1 ± 0.91	15.7 ± 2.58^{a}	2.7 ± 1.42^{b}	0.8 ± 0.98^{b,c}	0.7 ± 0.05^{a,b,c}
mucin secretion (µg Ab. /cm^2 gastric tissue)	0.134 ± 0.002	0.063 ± 0.028^{a}	0.205 ± 0.028^{a,b}	0.351 ± 0.034^{a,b,c}	0.374 ± 0.028^{a,b,c}

(a): Significantly different from the control (P<0.05).
(b): Significantly different from indometacin treated group (P<0.05).
(c): Significantly different from misoprostol treated group (P<0.05).
(d): Significantly different from chitosan treated group (P<0.05).

Effect of treatments on histopathological features

There were no histopathological changes detected in the mucosa in all rats in the control group (Figure-1). In the indometacin treatment there was an area of ulceration in glandular region, with some disappearance of mucous membrane associated with heavy infiltration of inflammatory cells mostly polymorph nucleated leukocytes. The area of inflammation is divided into three parts. The first part consisted of complete loss of gastric mucous membrane with heavy infiltration of inflammatory cells. The second part is characterized by disappearance of gastric mucous membrane with fibrosis and hyalinization in lamina properia, and the third part consisted of severe infiltration of inflammatory cells adjacent to normal glandular mucosa. None of the gastric ulcers perforate throughout the stomach layers (Figure-2). The histopathological examination of the stomach in the group of rats treated by misoprostol before indometacin, showed an area of gastritis associated with infiltration of inflammatory cells mostly polymorph nucleated leukocyte and an area of mucosal membrane loss which could be due to necrosis. The field also shows normal mucosal membrane adjacent to areas of gastritis (histopathology is not presented). In rats which were pretreated by chitosan before indometacin the histopathological examination of the stomach reveal slight infiltration of single inflammatory cells in the lamina properia (Figure-3). While in those treated by the combination chitosan plus misoprostol before inducing gastric ulceration by indometacin the stomach appeared normal and histopathological examination revealed normal stomach tissue (Figure-4).

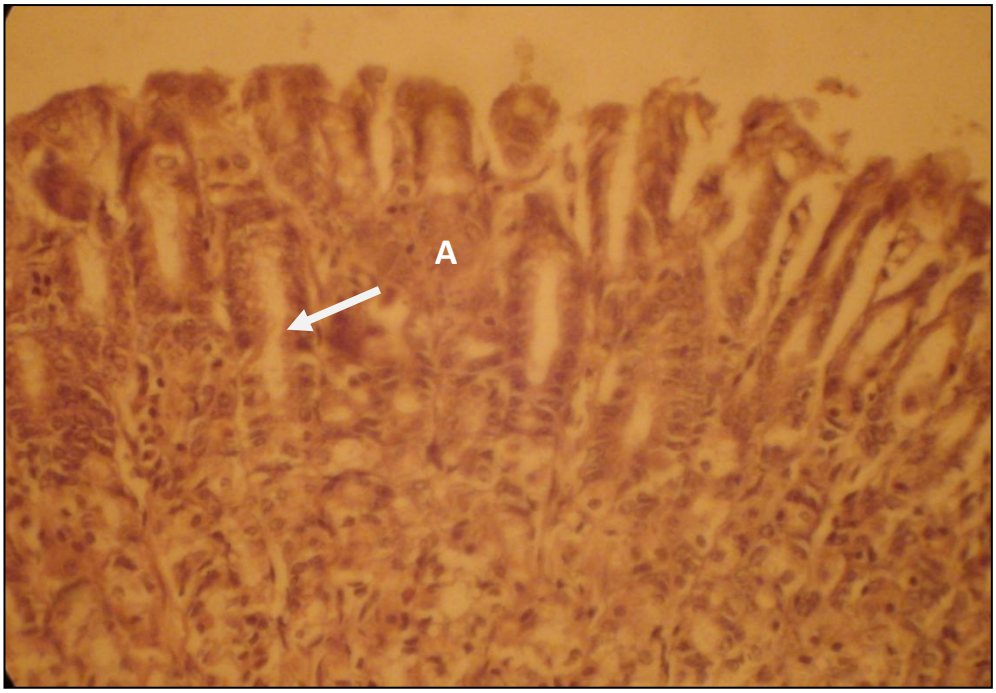

Fig 1. A cross section of normal gastric mucosa of rats in the control group. A: normal gastric gland (Hematoxyline and eosin X 400).

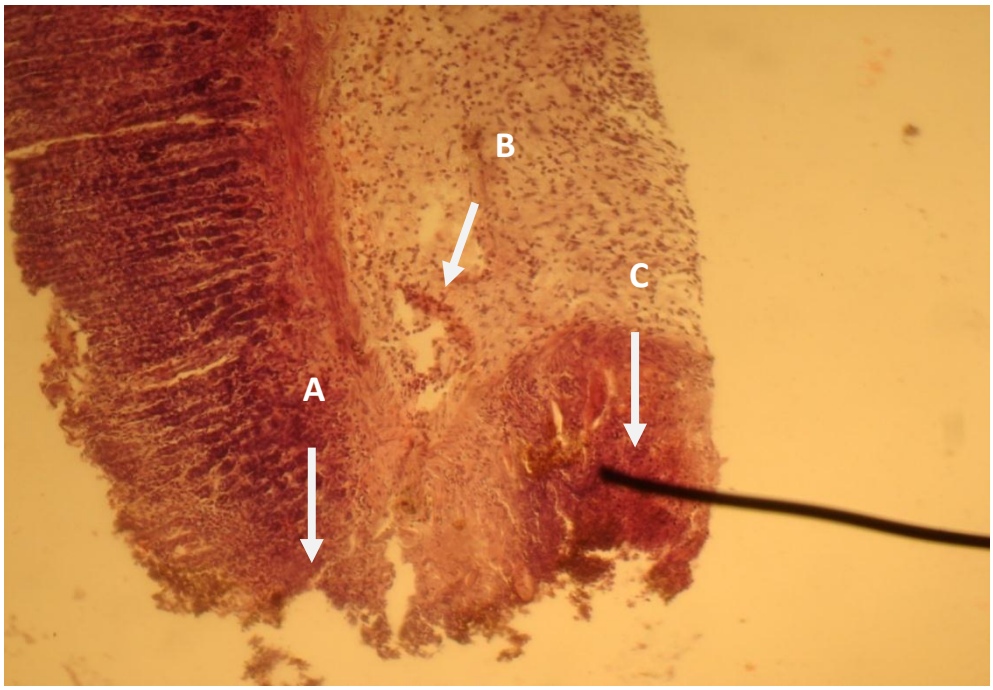

Fig 2. A cross section of stomach which shows ulcerated area in a rat treated by indometacin (Hematoxyline and eosin X 100), A: Loss of top mucous membrane, B: infiltration of Inflammatory cells C: Fibrosis and hyalinization of lamina properia.

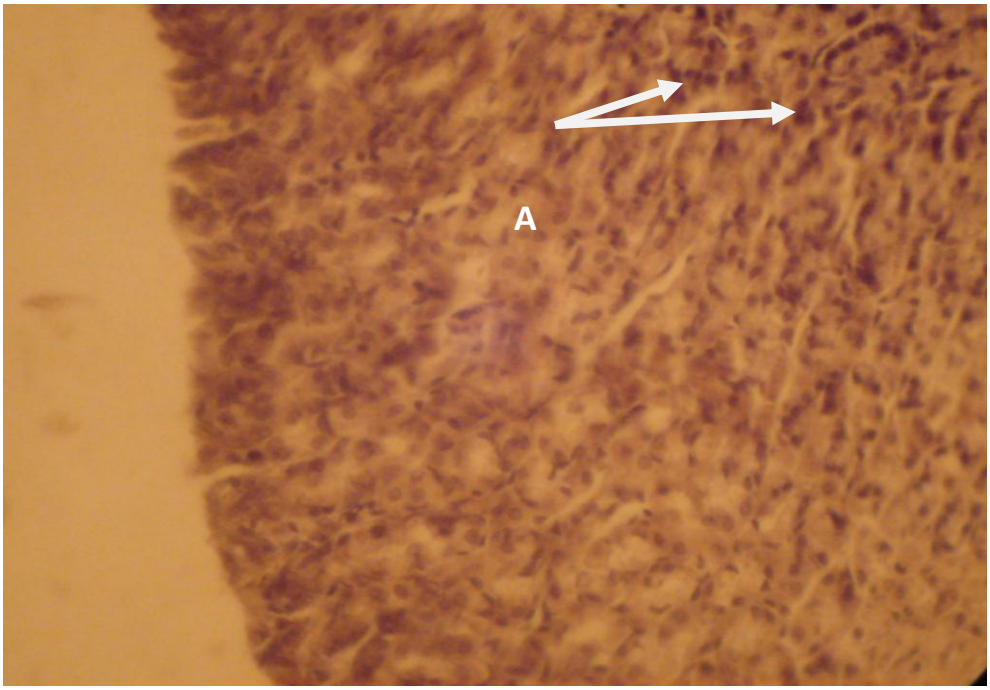

Fig 3. A cross section of stomach of rat treated by chitosan before indometacin treatment, no sign of gastric ulceration but the prominent feature is slight infiltration of inflammatory cells in the mucosa (A). (Hematoxyline and eosin X 400).

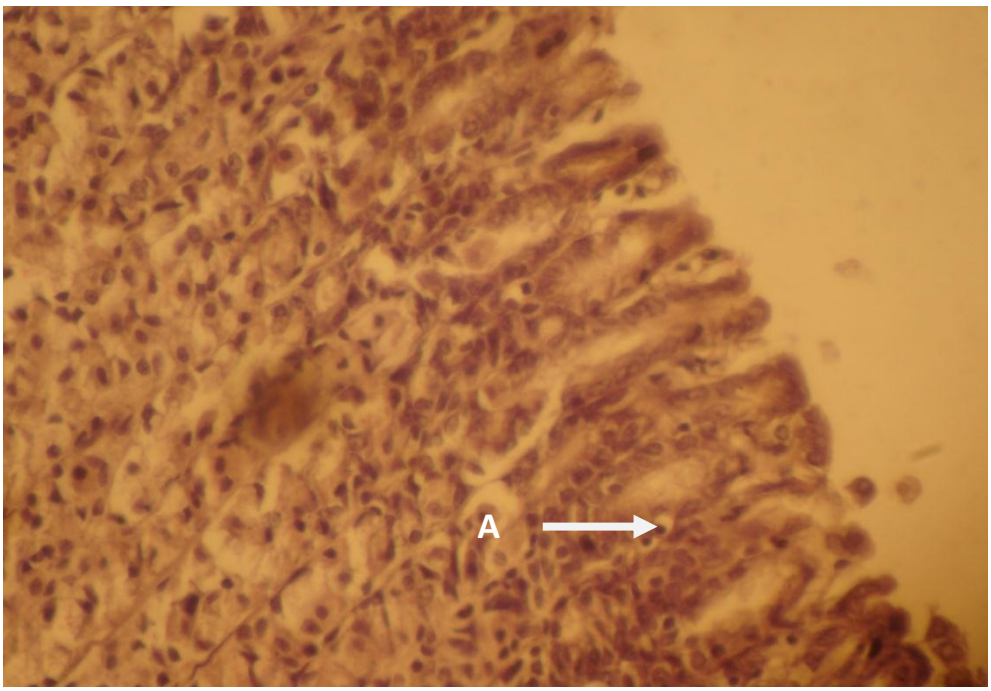

Fig 4. A cross section of stomach of rat treated by combination of chitosan plus misoprostol before indometacin treatment. The epithelium appears normal with no signs of ulcer, necrosis or congestion. A: normal gastric gland. (Hematoxyline and eosin X 400).

DISCUSSION

From the many models available for inducing gastric damage in animals, such as those using HCl, ethanol or acetic acid, indometacin has been selected in the present study for inducing gastric damage in rats.[10] Indometacin which is a non-steroidal anti-inflammatory drug (NSAIDs) is frequently prescribed for the treatment of various musclo-skeletal diseases as analgesic and anti-inflammatory such as in rheumatoid arthritis. However, as a potent gastric irritant its use is frequently associated with gastric ulceration. Hence indometacin induced gastric ulceration model is superior to other models because it mimics gastric ulceration that occurs in individuals receiving indometacin.[14] Chitosan was selected because it is an inert natural polymer, non absorbable, easily isolated and prepared, relatively cheap in addition to its ability to covalently cross-link other compounds to its free amino groups. This characteristic feature was invested by researchers in cross-linking chitosan to indometacin to produce a slow release compound which has the advantage of once daily dosing with minimal gastric irritation.[15] The present study revealed that all rats treated by indometacin at a dose of (25 mg/rat) daily for 3 days have shown gastric ulceration. This effect is associated with increased MDA levels, a marker of oxidative stress and lipid peroxidation. An association between gastric ulceration and increased oxidative stress is reported by Everett *et al*.[16] Hence the observed anti ulcer effect of chitosan was reported previously,[5] which demonstrated that the anti-ulcerogenic effect of chitosan can be attributed to the improvement of the antioxidant status of rats due to scavenging activity of free radicals. The present study provided a further support to the antiulcer and antioxidant effect of chitosan. Moreover, parameters of oxidative stress, in the present study, were measured in the blood as well as in tissues taken from ulcerated area of the stomach. Reduction in both gastric tissue and serum MDA were found in all animals given chitosan. Another possible mechanism of the anti ulcer effect of chitosan is probably due to inhibition of reduction in mucus formation by indometacin. This effect is in agreement with other reported results.[17] Chitosan has other minor effects which may contribute to its anti ulcer effects such as antacid activity,[17], antibacterial activity against *H. pylori*[18] Formation of chitosan into gel in the stomach and protection of the stomach from the digestive effect of acid and pepsin.[17] Treatment by misoprostol caused a significant reduction in indometacin induced gastric ulceration such cytoprotective effect is expected from a prostaglandin analogue (misoprostol). Misoprostol produced an increase in mucin production which was seen in the present study and it is also reported to increase bicarbonate and decreased acid secretion.[6] As the cytoprotective effect of misoprostol is well documented,[19] it is not yet known if this effect involves antioxidant potential. However, misoprostol in this study decreased MDA in the serum and in gastric tissue. Whether, misoprostol has a direct antioxidant activity or the reduction in MDA results indirectly from minimizing the stressful condition imposed by indometacin resulting from inhibition of indometacin induced gastric ulcer needs to be clarified. We also noticed that accidental treatment of pregnant rats by misoprostol causes vaginal bleeding and abortion. This is one of the side effects of misoprostol which causes contractions of uterine smooth muscles and intestinal smooth muscles resulting in diarrhea which most animals had.[1] The combination of chitosan and misoprostol completely inhibited gastric ulceration induced by indometacin. This effect was associated with significant reduction in blood and gastric tissue MDA and an increase in mucin secretion. This result may indicate a synergistic action between chitosan and misoprostol that may occur at the site of action

of both. This combination could have a practical significance because a cytoprotective effect can be obtained using modified doses of misoprostol with minimal side effects.

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