# THE CYTOPROTECTIVE EFFECT OF A NITRIC OXIDE DONOR DRUG ON GASTRIC MUCOUS MEMBRANE OF RATS TREATED WITH KETOPROFEN, A NON-STEROIDAL ANTI-INFLAMMATORY DRUG

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**ABSTRACT** – ***Background -*** Non-steroidal anti-inflammatory drugs are considered today a very important group of medication, with a wide variety of therapeutic use, in different areas of modern medicine. Despite their beneficial effects on the patient, these drugs show a high incidence of side effects, mainly in the gastrointestinal tract. The physiopathological mechanisms of non-steroidal anti-inflammatory drugs induced lesions and the gastric mucosa defense mechanism became an important source for medical research, especially those which try to evaluate the role of nitric oxide as a cytoprotective agent. ***Aim*** - To define a possible cytoprotective effect of a nitric oxide donor, isosorbide dinitrate, on the gastric mucous of rats submitted to non-steroidal anti-inflammatory drugs ketoprofen treatment. ***Methods*** - Adult male Wistar rats, previously submitted to starvation for 24 hours and divided in three groups: group I (standard): animals that received isotonic saline solution intragastric by gavage and intravenous. Group II (control-ketoprofen): animals that received isotonic saline solution intragastric by gavage and ketoprofen intravenous. Group III (nitrate/ketoprofen): animals that received 2mM solution of isosorbide dinitrate intragastric by gavage and ketoprofen intravenous. Later on, these animals were sacrificed and had their stomach removed and submitted to macroscopical, microscopical and biochemical studies. The evaluated parameters were: a) gastric lesion index; b) gastric mucous layer thickness; c) gastric tissue nitrate/nitrite (NOx) concentration and d) gastric tissue malondialdehyde concentration. ***Results -*** a) Gastric lesion index evaluation showed a smaller statistically significant incidence on the animals of group III; b) group III showed a thicker mucous layer, which also was statistically significant, when compared to group II; c) the variation on tissue nitrate/nitrite concentration was similar in all three groups, without statistical significance when compared to each other. ***Conclusion -*** Isosorbide dinitrate has a cytoprotective activity on the gastric mucosa of rats submitted to ketoprofen action.

**HEADINGS** – Anti-inflammatory agents, non-steroidal. Ketoprofen. Nitric oxide. Isosorbide dinitrate. Gastric mucosa. Rats.

## INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAID) are presently considered an important group of medication, with a wide variety of therapeutic use, in different areas of modern medicine. Despite their beneficial effects on the patient, these drugs show a high incidence of side effects, mainly in the gastrointestinal tract. The physiopathological mechanisms of NSAID-induced lesions and the gastric mucosa defense mechanism became an important challenge for medical research, especially those which try to evaluating the role of nitric oxide (NO) as a cytoprotective agent. To elucidate those physiopathological injury mechanisms and to understand better the defense mechanisms involved in gastric cells protection are mandatory to develop new drugs with NSAID therapeutics qualities, but quite reducing the undesirable side effects.

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A NO donor, isosorbide dinitrate was administered in rats submitted to NSAID ketoprofen treatment. This experimental study was carried out to define a possible cytoprotective effect on the gastric mucosa.

## MATERIALS AND METHODS

### Animals

Adult male Wistar rats weighing 200 to 250 g were obtained from the Central Animal House of the São Paulo University, Ribeirão Preto, SP, Brazil, and were used in all experiments. The experimental design, handling and use of the animals were accomplished in agreement with the Committee of Ethics in Research of the Faculty of Medicine of Ribeirão Preto, São Paulo University.

### Experimental design

Twenty five adult male Wistar rats previously submitted to starvation for 24 hours were included in the following experimental three groups:

A) group I (control, n = 5): animals that received isotonic saline solution both by gavage and endovenous via (EV);
B) group II (saline/ketoprofen, n = 10): animals that received isotonic saline solution by gavage and ketoprofen (Aventis Pharma, São Paulo, SP, Brazil) by EV;
C) group III (nitrate/ketoprofen, n = 10): animals that received 2 mM solution of 2,5-dinitrato of 1,4:3,6-dianidro-D-glucol (Isosorbide dinitrate - Sigma Pharma, São Paulo, SP, Brazil) by gavage and ketoprofen by EV. Five milligrams of the nitrate were diluted in 21 mL of isotonic saline solution (2 mM) and used by gavage. Seven hours after the beginning experiment, animals of the three experimental groups were sacrificed under diethyl ether anesthesia, the peritoneal cavity was entered and the stomach was excised.

### Histomorphometric evaluation

Each stomach was opened along the greater curvature, gently washed with saline and pinned upon a cork plate with the mucosal surface turned upward. The total extent of gastric mucosal injury was observed, and photographs of the gastric mucosal area were taken with a digital camera (JVC TK 1270, Victor Company of Japan Ltd., Japan). The stomachs were then fixed in 10% formalin buffered for 48 h at 25ºC, and the glandular portion was dissected into 5-mm-wide parallel strips, perpendicular to the lesser curvature. The strips obtained from each stomach were included in paraffin and submitted to routine histological procedure.

The index of gastric mucosal lesion was estimated as the area of damaged mucosa divided by the total area of glandular portion, and expressed as percentage values.

The gastric mucus thickness was measured using the technique described by CARBAJAL et al.[5], and expressed as micrometers. Gastric sections were stained by alcian blue, according the technique described by LEV and SPIDER[10], and PAS according to the method described by MacMANS[11]. Also, the hematoxylin/eosin (H&E) was used to study the histological aspects of the normal or injured mucous membrane.

The gastric images were analyzed with the aid of an IBM microcomputer a Frame Graber KE capture card, using the KS 400 V 2.0 image processing software from Kronton Eletronic GmbH.

### Biochemical study

Gastric mucosal samples were randomly collected before fixation in formalin, and used to measurements of gastric nitrate/nitrite (NOx) and malondialdehyde (MDA) concentrations. The gastric NOx concentration was measured by the chemiluminescence concentrations (Sievers 280i NOA, Sivers, Boulder, CO, USA), and the levels of gastric lipid peroxide were determined by specific commercial assay kit (Lipid Peroxidation Assay Kit - Calbiochem – San Diego, CA, USA).

### Statistical analysis

The results were expressed as means ± standard deviation (SD). Statistical comparisons of groups were performed by non-parametric Mann-Whitney test for two independent samples, or by non-parametric Kruskal-Wallis test followed by Dunn's test for comparisons of more than two independent samples. *P* values less than 0.05 were considered to prove statistical significance of differences.

## RESULTS

The histomorphometric parameters are informed in Table 1. The perimeter and area of glandular portion of stomach did not show statically significant difference among the three experimental groups.

Figure 1 show the mucosal lesion index, which was estimated as the area of damaged mucosa divided by the total area of glandular portion. The control group (group I) did not present any gastric lesions. Nevertheless, groups II and III exhibited broad ulcerous areas, with significant predominance ($P<0.05$) on the saline/ketoprofen-treated rats (group II).

The thickness of the mucus in the glandular region of stomach is given in Figure 2. A continuous mucus layer was observed over the surface of all the glandular epithelium of the stomach. The thickness values varied between 120 and 200 micrometers. In the non-glandular portion of stomach there was a continuation

**TABLE 1 – Histomorphometric parameters**

| Groups | Perimeter (mm) | Glandular area ($mm^2$) | Mucosal lesion index (%) | Mucus thickness (µm) |
|---|---|---|---|---|
| gI (n=5) | 166.4 ± 6.2 | 961.9 ± 70.2 | 0 | 167.2 ± 10.8 |
| gII (n=10) | 175.7 ± 6.9 | 969.5 ± 48.4 | 28.6 ± 19.2 * | 133.6 ± 12.2 * |
| gIII (n=10) | 173.9 ± 8.4 | 954.0 ± 60.6 | 13.5 ± 14.6 | 172.1 ± 18.9 |

mean ± SD *P<0.05

of the non-glandular stratified epithelium characteristic of the esophagus. However, at the interface between glandular and non-glandular regions, it was possible to see the transition from the non-glandular stratified epithelium that had no mucus, to a glandular mucus secreting epithelium that had a mucus layer. The mean (SD) mucus thickness in glandular region of saline/ketoprofen-treated rats (gII) was significantly lower ($P<0.05$) than that of control group (gI), whereas that of nitrate/ketoprofen-treated rats (gIII) was significantly greater ($P<0.001$) than that of saline/ketoprofen-treated rats (gII).

The gastric NOx concentration was similar in all three groups, without any statistical significance among them. (Figure 3) The determination of lipid peroxidation in glandular portion of stomach, by dosing gastric MDA concentration, did not also show statically significant difference among the three experimental groups ($P = 0.37$) (Figure 4).

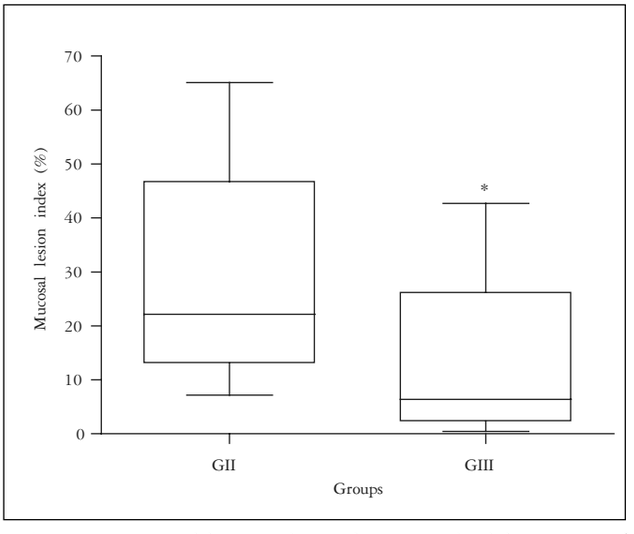


**FIGURE 1** – Mucosal lesion index in the gastric glandular portion of saline/ketoprofen- (group II) and nitrate/ketoprofen-treated (group III) rats. The values represent the mean ± SD for 10 rats per group. * $P$ <0.05 compared to the group II

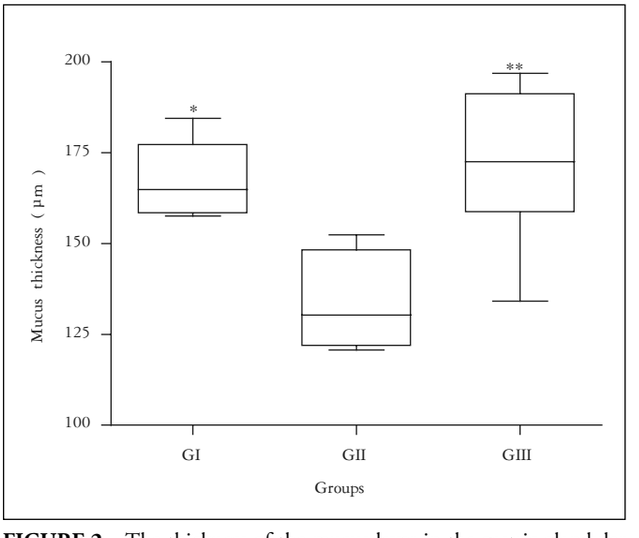


**FIGURE 2 –** The thickness of the mucus layer in the gastric glandular portion of control (group I), saline/ketoprofen- (group II) and nitrate/ketoprofen-treated (group III) rats. The values represent the mean ± SD for 5-10 rats per group. * $P$ <0.05 and ** $P$ <0.001 compared to the group II

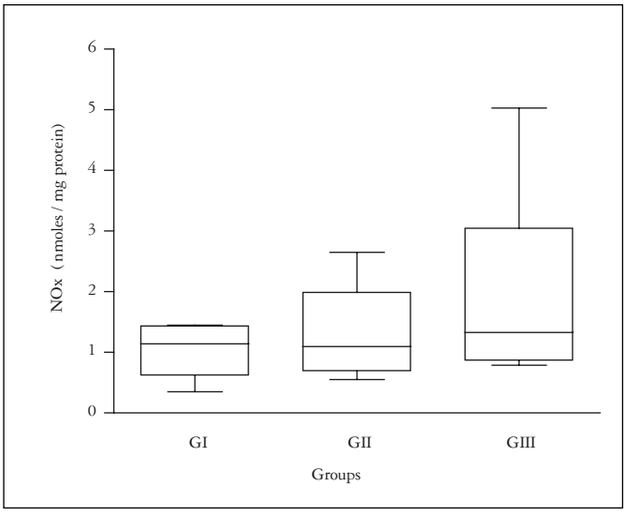


**FIGURE 3** – Nitrate/nitrite (NOx) concentration in the gastric glandular portion of control (group I), saline/ketoprofen- (group II) and nitrate/ketoprofen-treated (group III) rats. The values represent the mean ± SD for 5-10 rats per group

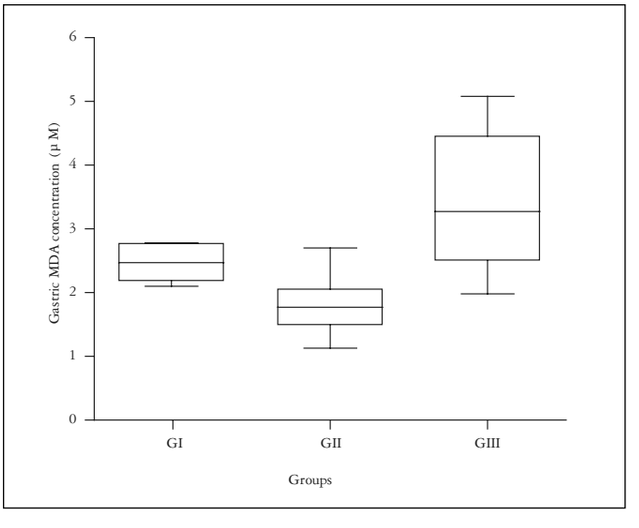


**FIGURE 4 –** Gastric malondialdehyde (MDA) concentration in the gastric glandular portion of control (group I), saline/ketoprofen- (group II) and nitrate/ketoprofen-treated (group III) rats. The values represent the mean ± SD for 5-10 rats per group

## DISCUSSION

The NSAID have been associated with an increase relative risk of gastrointestinal lesions, mainly associated with prostaglandins synthesis inhibition, through the inhibition of the enzymes COX 1 and COX 2 involved in the protection of the mucous membrane. A current proposal for gastropathy NSAID-dependent etiology emphasizes the vascular one, based on the demonstrated capacity of these drugs in reducing the mucous blood flow, to promote an increase in the vascular permeability, with polymorphonuclear activation and its adherence to the vascular endothelium[13].

On the other hand, it was also demonstrated the NO role in the integrity maintenance of the gastric mucous membrane. The continuous NO release, through cNOS expression, contributes to the physiologic gastrointestinal mobility, tonus, permeability and blood flow to the vessels of gastric wall. Some studies suggest that NO should revert gastric mucous membrane alterations caused by NSAID, reducing their toxicity through the activation of cGMP pathway[8, 14].

Other studies demonstrated that NO overproduction by iNOS expression has been implicated in the gastrointestinal inflammatory disorders, suggesting a dualist behavior of its activity. The NO physiologic concentration, released through the cNOS enzymatic expression, has activities on gastric mucous membrane function maintenance, while an NO overproduction through the iNOS expression, would exhibit a cytotoxic activity, interacting with oxygen free radicals. Some other recent studies[4] question this cytotoxic paper of the iNOS, demonstrating that its activity can also be associated to a reduction in the platelet aggregation and leukocyte/endothelium interaction, as well as microcirculatory protection of the gastric mucous membrane. iNOS activity can also be important in the inflammatory process resolution, promoting an increase in the inflammatory cells apoptosis and NO protects from apoptosis induced by TNF-alpha[7].

As NO is one of the gastric mucous membrane protecting factors, the present experimental model was carried out to investigate if dinitrate isosorbide, an oral NO drug donor, would prevent gastric lesions caused by the NSAID ketoprofen, comparing two groups submitted to the treatment with the anti-inflammatory drug. Based on the initial studies published by BROWN et al.[2, 3], isosorbide dinitrate in the concentration of 2 mM was defined as NO drug donor, producing a dose-related stimulation of mucous secretion, without affecting the viability of the isolated cells, and cellular of cGMP activation. The evaluated parameters were: a) gastric lesion index, b) gastric mucous layer thickness, c) gastric tissue Nitrate/nitrite (NOx) concentration and d) gastric malondialdehyde (MDA) concentration.

From the macroscopic point of view, isosorbide dinitrate effectiveness was proved, based on the gastric lesion index differences between groups II (ketoprofen) and III (nitrate/ketoprofen). The microscopic mucous layer thickness evaluation was also statistically significant, reinforcing the isosorbide dinitrate cytoprotective actions through the stimulation of mucous secretor production.

The NO activity, measured by NOx gastric tissue concentration was statistically similar in the three studied groups. The analysis of gastric MDA concentration in lipid peroxidation study also did not reveal any difference among the investigated groups. This data is important because excessive isosorbide dinitrate NO release may be cytotoxic[1, 9]. Even without statistic significance the MDA concentrations revealed an increased tendency to high levels in group III (nitrate/ketoprofen-treated). By the other hand, the MDA analysis and the thiobarbituric test response in lipid peroxidation studies, especially in biological systems, requires caution, discretion, and correlative data from other indexes of fatty peroxide formation and decomposition would be necessary[6, 12, 15].

## CONCLUSION

The experimental model highly suggests that NO drug donors should be effective in the prophylaxis of NSAID-induced lesions, demonstrating a cytoprotective action in rats' gastric mucous membrane, evaluating by the lesion index and of membrane mucous layer thickness.

Villa AL, Ceneviva R, Viaro F, Ramalho F, Campos AD, Evora PRB. Efeito citoprotetor de uma droga doadora de óxido nítrico na membrana da mucosa gástrica de ratos tratados com o antiinflamatório não-esteróide - o cetoprofeno. Arq Gastroenterol. 2006;43(3):233-7.

**RESUMO** – ***Racional*** – Drogas antiinflamatórias não-esteróides são consideradas, atualmente, importante grupo de medicamentos, com ampla variedade de uso terapêutico, em diferentes áreas da medicina moderna. Apesar de seus efeitos benéficos para o paciente, apresentam grande incidência de efeitos colaterais, principalmente no trato gastrointestinal. Os mecanismos fisiopatológicos de lesões induzidas por essas drogas e os mecanismos de defesa da mucosa gástrica tornaram-se uma importante linha de pesquisa médica, especialmente procurando avaliar o papel de óxido nítrico como agente citoprotetor. ***Objetivo*** - Estudar uma droga doadora de ácido nítrico - o dinitrato de isossorbida - e sua ação citoprotetora da mucosa gástrica de ratos submetidos ao tratamento com uma droga antiinflamatória - o cetoprofeno. ***Métodos*** – Ratos machos adultos previamente submetidos a jejum de 24 horas, foram divididos em três grupos: a) grupo I (controle): animais, que receberam apenas solução salina isotônica via intragástrica, por gavagem e via endovenosa; b) grupo II (cetoprofeno-controle): animais que receberam solução salina via intragástrica por gavagem e cetoprofeno via endovenosa, e c) grupo III (nitrato/cetoprofeno): animais que receberam solução de 2 mM de dinitrato de isossorbida a via intragástrica por gavagem e cetoprofeno via endovenosa. Esses grupos foram, posteriormente, submetidos a exames macroscópico, microscópico e bioquímico, avaliando-se os seguintes parâmetros: a) determinação do índice de lesão gástrica; b) determinação da espessura da camada do muco secretor; c) determinação da concentração de ácido nítrico x tecidual, e d) determinação da concentração do malondialdeído tecidual. ***Resultados*** - Encontrou-se menor índice de lesão gástrica nos animais do grupo III (nitrato), assim como maior espessura da camada do muco secretor nos animais deste grupo, do que nos animais do grupo II (cetoprofeno). A variação da concentração do ácido nítrico x tecidual foi semelhante nos três grupos. A taxa de peroxidação lipídica, avaliada pela dosagem do malondialdeído foi maior nos animais do grupo III, do que nos dos demais grupos, não sendo possível, contudo, se estabelecer correlação com os demais parâmetros. Se a utilização do dinitrato de isossorbida, por si próprio, induziu peroxidação lipídica é motivo para especulações. ***Conclusão*** - Analisando o conjunto dos dados obtidos neste trabalho, conclui-se que o dinitrato de isossorbida exerceu atividade citoprotetora.

**DESCRITORES** – Antiinflamatórios não-esteróides. Cetoprofeno. Óxido nítrico. Malondialdeído. Dinitrato de isossorbida. Mucosa gástrica. Ratos.

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