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**ORIGINAL ARTICLE** / ÖZGÜN ARAŞTIRMA

# The effect of simvastatin pretreatment on stress-induced gastric ulcer in rats

## Sıçanda stres ile uyarılan mide ülserinde simvastatin'in etkisi

Elif BAHADIR, Muhammet BULUT, Beyza KABAYUKA, Ece Söylem ŞENKAL, Abdullah AVLAÇ, Meral YÜKSEL, Feriha ERCAN, İnci ALİCAN

**ABSTRACT**

**Objectives:** This study aimed to elucidate the possible protective effect of simvastatin (SIM) pretreatment on stress-induced gastric ulcer in rats.

**Materials and Methods:** Gastric ulcer was produced in Sprague-Dawley rats (250-300 g) by cold-restraint stress. SIM (10 mg/kg/day; per oral) or saline was administered for 21 days prior to stress. On day-21, a group of animals was treated with the non-selective nitric oxide synthase (NOS) inhibitor L-NAME (50 mg/kg; intraperitoneally) or the non-selective cyclooxygenase (COX) inhibitor indomethacin (Indo; 5 mg/kg; subcutaneously) prior to SIM. The stomachs were examined macroscopically and microscopically and stored for biochemical analyses.

**Results:** The severity of the lesions of the stress group was decreased by SIM, but this was not altered significantly by L-NAME or Indo. Stress increased gastric myeloperoxidase activity compared to control level ($p<0.01$); however, SIM did not cause a significant change on this parameter. Stress increased gastric chemiluminescence levels ($p<0.001$) which were reversed by SIM ($p<0.001$) and this effect continued in L-NAME- or Indo-treated animals.

**Conclusion:** SIM pretreatment of rats with cold-restraint stress provided partial protection against gastric lesion formation via suppression of oxidants derived from sources other than neutrophils without the involvement of NOS and COX systems.

**Keywords:** Ulcer, Nitric oxide synthase, Cyclooxygenase, Rat, Stress

Elif Bahadır, İnci Alican (✉)
*Department of Physiology, School of Medicine, Marmara University, Istanbul, Turkey*
*e-mail. incialican@yahoo.com*

Muhammet Bulut, Beyza Kabayuka, Ece Söylem Şenkal, Abdullah Yalvaç,
*Student, School of Medicine, Marmara University, Istanbul, Turkey*

Meral Yüksel
*Vocational School of Health Related Professions, Marmara University, Istanbul, Turkey*

Feriha Ercan
*Department of Histology and Embryology, School of Medicine, Marmara University, Istanbul, Turkey*

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**ÖZ**

**Amaç:** Bu çalışmada sıçanlarda stres ile uyarılan mide ülserinde simvastatin (SİM)'in olası koruyucu etkisini değerlendirmek amaçlanmıştır.

**Gereç ve Yöntem:** Sprague-Dawley sıçanlarda (250-300 gr) soğuk-kısıtlama stresi uygulaması ile mide ülseri oluşturuldu. Stres öncesi 21 gün süreyle SİM (10 mg/kg/gün; oral yolla) veya fizyolojik tuzlu su (1 ml; oral yolla) uygulandı. Bir grup sıçan 21. günde SİM öncesi selektif olmayan nitrik oksit sentaz (NOS) inhibitörü L-NAME (50 mg/kg; intraperitoneal) veya selektif olmayan siklooksijenaz (COX) inhibitörü indometazin (İndo; 5 mg/kg; subkutan) verildi. Mideler makroskopik ve mikroskopik olarak incelendi ve biyokimyasal analizler için saklandı.

**Bulgular:** Stres grubunda lezyonların şiddeti SİM ile azaldı ancak L-NAME veya İndo ile anlamlı bir değişiklik göstermedi. Stres mide miyeloperoksidaz aktivitesini kontrol düzeyine kıyasla artırdı ($p<0,01$); ancak, SİM bu parametre üzerinde anlamlı bir değişikliğe neden olmadı. Stres mide kemiluminisans düzeylerini artırdı ($p<0,01$); bu etki SİM ile geri döndürüldü ($p<0,001$); L-NAME veya İndo ile tedavi edilen hayvanlarda ise devam etti.

**Sonuç:** Soğuk-kısıtlama stresine maruz bırakılan sıçanlara SIM öntedavisi mide lezyon oluşumunda NOS ve COX sistemlerinden ayrı mekanizmalarla nötrofil dışı hücrelerden kaynaklanan oksidanları baskılayarak kısmen koruma sağlamaktadır.

**Anahtar kelimeler:** Ülser, Nitrik oksit sentaz, Siklooksijenaz, Sıçan, Stres

## Introduction

Statins are widely used clinically for lowering hypercholesterolemia because of their inhibitory effect on 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the enzyme that catalyzes the rate-limiting step of the cholesterol synthesis in the liver and other tissues [1]. Clinical studies have shown that statins reduce cardiovascular related morbidity and mortality in patients with or without

coronary artery disease with or without high cholesterol levels [2-4]. Besides the therapeutic use in hyperlipidemia, the antioxidant, anti-inflammatory and immunomodulatory benefits of statins have been reported in many studies [5,6]. They promote endogenous nitric oxide (NO) production [7-9], decrease platelet aggregation and inhibit thromboxane formation [10]. Simvastatin (SIM) is a commonly prescribed statin with anti-inflammatory [11,12] and antioxidant effects [13]. Studies on experimental animals revealed that SIM protects against ischemia-reperfusion injury of the lung [14], kidney [15], heart [16] and brain [17]. A study by Tariq et al. [18] demonstrated the gastric antisecretory and antiulcer effects of SIM in pylorus-ligated rats. SIM was also found to protect gastric mucosa from indomethacin-induced ulceration via promoting gastric mucosal NO and prostaglandin E2 levels, stimulating gastric mucin release in addition to its direct antioxidant activity [19].

Stress ulcer characterized by diffuse lesions of the gastric mucosa and duodenum frequently occurs as a result of various stressful events, including extensive burns, shock, sepsis, major surgery, and severe trauma. The pathological basis for the development of stress-induced gastric lesions has been postulated to be multifactorial. The contribution of gastric neutrophil accumulation, inflammatory cytokine production, free radical production, decreased antioxidants and decreased mucosal blood flow have been reported to be involved in the pathogenesis of stress-induced gastric lesions [20].

The present study was conducted to elucidate the possible gastroprotective effect of SIM pretreatment on stress-induced gastric ulcer in rats and the mechanisms underlying this protection.

## Materials and Methods

### Animals

Adult Sprague-Dawley rats of either sex (250-300 g) were housed in a temperature-controlled room (22 ± 1 °C) with a 12 h light/dark cycle and free access to food and water. The study protocol was approved by Marmara University, Animal Care and Use Committee.

### Stress-induced gastric ulceration model

Gastric ulcer was produced in rats by cold-restraint stress, as described previously [21]. Briefly, the animals were immobilized in metallic restraining devices for 3 h at 4 °C following a starvation period of 24 h in mesh-bottomed cages to minimize coprophagia. The control group was kept at room temperature without any stress.

### Experimental design

Rats were randomized into five groups: Control group (n=7), stress group (n=7), SIM+stress group (n=7), L-NAME+SIM+stress group (n=7) and indomethacin (Indo)+SIM+stress group (n=7). In treatment groups, the animals were given SIM (10 mg/kg/day) or saline daily for 21 days by a gastric tube. On day-21, among SIM-treated rats, a group of animals were treated with the non-selective nitric oxide synthase (NOS) inhibitor L-NAME (50 mg/kg; intraperitoneally) or the non-selective cyclooxygenase (COX) inhibitor Indo (5 mg/kg; subcutaneously) 60 min prior to SIM administration. Then, gastric ulceration was induced by cold-restraint administration. The effect of L-NAME and Indo alone was also assessed in animals exposed to stress (n=5 per group). Immediately after the induction of cold-restraint stress, all rats were decapitated. The stomach was removed, opened along the greater curvature and washed with ice-cold saline. Gastric lesions were examined macroscopically to measure the length of ulcers. The length (mm) of each lesion was summed per stomach and used as lesion index. Tissue samples were taken for histologic evaluation of the lesions by light microscopy or stored at -70 °C for subsequent measurement of malondialdehyde (MDA) and glutathione (GSH) levels, and myeloperoxidase (MPO) activity. Formation of reactive oxygen species in gastric samples was monitored by using chemiluminescence (CL) method.

### Histological evaluation

For light microscopic investigation, tissue samples from the fundic region of the stomach were placed in 10% formaldehyde, dehydrated in ascending alcohol series (70%, 90%, 96% and 100%), and embedded in paraffin. For each animal, four randomly taken tissue sections (5 μm) were stained with hematoxylin and eosin (H&E) and examined under an Olympus BH-2 photomicroscope. The gastric injury based on epithelial desquamation, mucosal hemorrhage, glandular damage and inflammatory cell infiltration was scored using a scale ranging from 0 to 3 (0: none, 1: mild, 2: moderate, and 3: severe) for each criterion. The total score was 12 [22]. All tissue sections were examined by an experienced histologist (F.E.) who was unaware of the treatment groups.

### Measurement of MDA and GSH levels

The gastric samples were homogenized in 10 volumes of ice-cold 10% trichloracetic acid and centrifuged at 700 g for 15 min at 4 °C. Supernatant was removed and recentrifuged at 10,000 g at 4 °C for 8 min. GSH was determined by a spectrophotometric method which is a modification of Ellman procedure [23]. Lipid peroxide levels are expressed in terms of MDA equivalents as nmol MDA/g tissue [24].

### Measurement of MPO activity

Gastric MPO activity- an indicator of neutrophil accumulation- was assessed by measuring the $H_2O_2$-dependent oxidation of o-Dianisidine 2HCl. One unit of enzyme activity was defined as the amount of MPO present that causes a change in absorbance of 1.0 unit min at 460 nm and 37 $^0$C and expressed in units per g tissue [25].

### CL assay

Chemiluminescence assay is a direct noninvasive method for measuring reactive oxygen species. Due to limitations, i.e., potential variability and low intensity of native CL, luminol and lucigenin can be used as enhancers. Due to their high quantum efficiency after oxidation, they function as bystander-substrates for oxygenation and form high levels of excited-state products and CL, when added to an in vitro biological system. The excited electrons in these compounds revert to their ground state with the emission of energy as light CL and can be detected by a luminometer.

In this study, CL of the gastric samples was recorded at room temperature using Mini Lumat LB 9506 luminometer (EG&G Berthold, Germany) in the presence of luminol or lucigenin probes 0.2 mM each. All counts were obtained at 15 s intervals for 5 min and the results were expressed as area under the curve (AUC) of relative light unit (rlu) for 5 min per mg tissue [26]. The calculation was based on the integration of the curve by the trapezoidal rule (a linear approximation).

### Statistical analysis

All data are expressed as means ± S.E. The histological data were compared by Mann-Whitney U non-parametric test and other parameters were compared by two-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. Values of $p < 0.05$ were regarded as significant. Calculations were done using Instat statistical analysis package (GraphPad Software, San Diego, CA, USA).

## Results

### Evaluation of the severity of gastric lesions

Cold-restraint stress resulted in production of gastric lesions. Although it did not reach a statistically significant level, the lesion index of the stress group (6.16±2.28 mm) showed a marked decrease by SIM pretreatment (2.32±1.74 mm). The ulcer index did not change significanty in SIM+stress groups treated with L-NAME or Indo in comparison to SIM+stress group. However, L-NAME or Indo treatment *per se* augmented the stress-induced lesion formation (Figure 1).

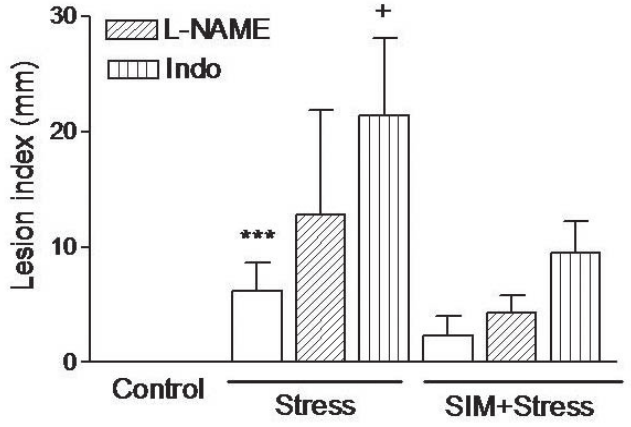


**Figure 1.** The macroscopic evaluation of the gastric lesions in experimental groups. $^{***}p<0.001$, compared to control group; $^{+}p<0.05$, compared to untreated stress ulcer group.

In accordance with macroscopic examination, SIM pretreatment was also found to be beneficial on the severity of the lesions upon microscopic evaluation when compared with the untreated stress group (6.71±0.28 vs. 9.33±0.55; $p<0.01$). However, this effect did not seem to be modified by L-NAME or Indo administration along with SIM (Figure 2). As seen in Figure 3, the gastric samples of the stress group were characterized with severely damaged epithelium with diffuse hemorrhage and inflammatory cell infiltration. In SIM-treated stress group, the epithelial layer had regular morphology with mild glandular epithelial cell damage, mild vascular congestion and less inflammatory cell infiltration in comparison to the untreated stress group. L-NAME- or Indo-treated SIM groups revealed a similar gastric morphology when compared with SIM-treated stress group. L-NAME or Indo treatments alone did not change the severity of the mucosal lesions of the stress group (Figure 3).

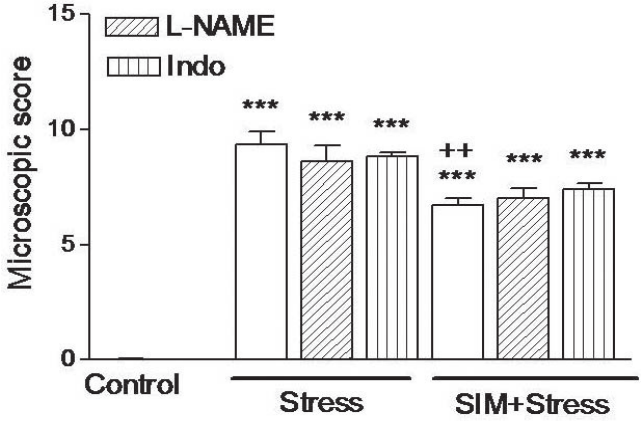


**Figure 2.** The microscopic evaluation of the gastric lesions in experimental groups. $^{***}p<0.001$, compared to control group; $^{++}p<0.01$, compared to untreated stress ulcer group.

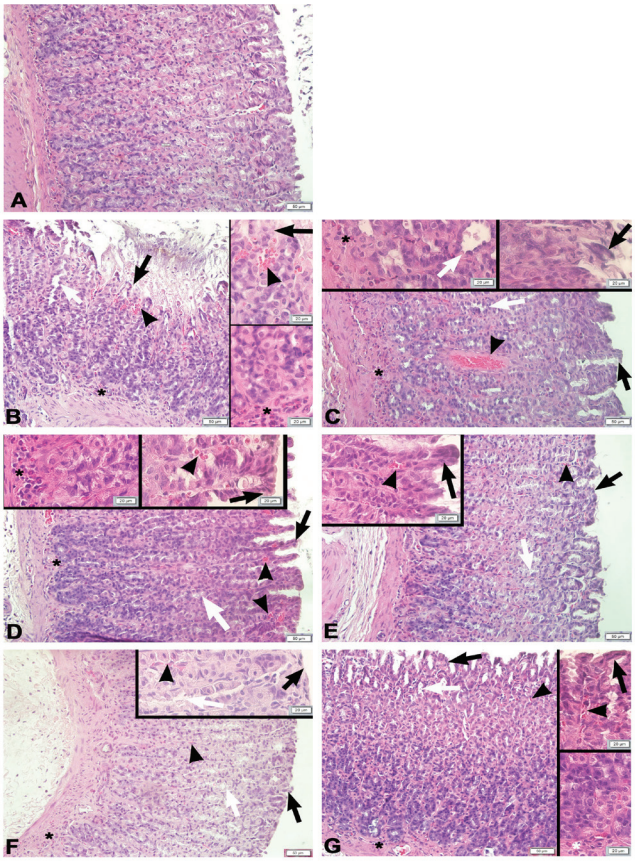


**Figure 3.** Photomicrographs depicting the histological characteristics of gastric samples representing the experimental groups. *Control group (A):* Surface epithelium and glandular epithelium with regular morphology; *Stress group (B*): Severely damaged surface epithelium (black arrow) and glandular epithelium (white arrow), hemorrhage (arrow head) and inflammatory cell infiltration (*); *Indo+Stress group (C):* Desquamation of the surface epithelium (black arrow), damaged glandular epithelium (white arrow), vascular congestion (arrow head) and inflammatory cell infiltration (*); *L-NAME+Stress group (D):* Partially damaged surface epithelium (black arrow) and glandular epithelium (white arrow), vascular congestion (arrow head) and inflammatory cell infiltration *(*); SIM+Stress group (E):* Surface epithelium with regular morphology (black arrow), mild damage of the glandular epithelium (white arrow), mild vascular congestion (arrow head) and mild inflammatory cell infiltration (*); *Indo+SIM+Stress group (F):* Mild damage of the surface epithelium (black arrow) and glandular epithelium (white arrow), mild vascular congestion (arrow head) and mild inflammatory cell infiltration (*); *L-NAME+SIM+Stress group (G):* Surface epithelium with regular morphology (black arrow), mild damage of the glandular epithelium (white arrow), mild vascular congestion (arrow head) and mild inflammatory cell infiltration (*). Hematoxylin and eosin staining; original magnifications: X200, inserts X400.

### Evaluation of gastric MDA and GSH levels

Gastric MDA levels did not show any statistically significant difference among the experimental groups (Table I).

**Table I.** The gastric malondialdehyde (MDA) and glutathione (GSH) data of the experimental groups.

| | MDA (nmol/g) | GSH (μmol/g) |
|---|---|---|
| Control group | 10.16±1.34 | 13.24±1.01 |
| Stress ulcer group | 11.01±1.81 | 9.67±0.58** |
| Stress ulcer + treatment groups | | |
| SIM+Stress | 10.78±0.42 | 9.58±0.64** |
| L-NAME+SIM+Stress | 11.16±1.37 | 9.09±1.01** |
| Indo+SIM+Stress | 13.93±2.43 | 7.91±0.24** |
| L-NAME+Stress | 11.01±1.81 | 10.85±0.97 |
| Indo+Stress | 8.22±1.40 | 10.23±1.17 |

**p<0.01, compared to control group.

Gastric GSH content of the control group (13.24±1.01 μmol/g) decreased markedly in the stress group (9.67±0.58 μmol/g; p<0.01). However, SIM pretreatment alone or along with L-NAME or Indo did not cause a significant change in the gastric GSH content of the stress group (Table I).

### Evaluation of gastric MPO activity

Cold-restraint stress caused a significant increase in gastric MPO activity in comparison to control level (145.80±11.48 U/g vs. 102.40±8.64 U/g; p<0.01). In SIM-treated stress group, the gastric MPO activity was not significantly different from that of the untreated stress group (188.80± 20.82 U/g) (Figure 4).

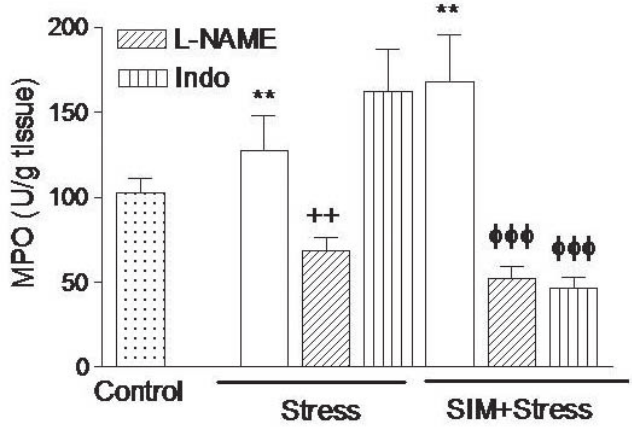


**Figure 4.** The gastric myeloperoxidase (MPO) activity of the experimental groups. **p<0.01, compared to control group; ++p<0.01, compared to untreated stress ulcer group; ϕϕϕp<0.001, compared to SIM+Stress ulcer group.

### Evaluation of gastric luminol and lucigenin CL levels

As demonstrated in Table II, cold-restraint stress caused marked elevations in both luminol and lucigenin CL levels which were reversed by SIM pretreatment. The beneficial effect of SIM on luminol CL levels continued in animals pretreated with L-NAME or Indo prior to SIM.

**Table II.** The gastric chemiluminescence (CL) data of the experimental groups.

| | Luminol CL (rlu/mg) | Lucigenin CL (rlu/mg) |
|---|---|---|
| Control group | 6.77±0.77 | 12.38±0.79 |
| Stress ulcer group | 26.50±2.37$^{***}$ | 31.70±2.15$^{***}$ |
| Stress ulcer + treatment groups | | |
| SIM+Stress | 11.60±1.06$^{**+++}$ | 12.84±1.25$^{+++}$ |
| L-NAME+SIM+Stress | 8.57±0.95 | 20.05±1.66$^{\phi\phi}$ |
| Indo+SIM+Stress | 8.60±1.84 | 11.92±1.03 |
| L-NAME+Stress | 10.26±1.32$^{+++}$ | 12.80±0.70$^{+++}$ |
| Indo+Stress | 6.42±1.06$^{+++}$ | 14.58±1.75$^{+++}$ |

$^{***}p<0.001$, compared to control group; $^{+++}p<0.001$, compared to stress ulcer group; $^{\phi\phi}p<0.01$, SIM+Stress group.

## Discussion

The cold-restraint stress model in rats mimics clinical acute gastric lesions, that may appear in the gastric mucosa as a consequence of major trauma, surgery or sepsis and it is widely accepted for studying the mechanism of stress-induced gastric lesions [27,28]. An acute inflammation of the gastric mucosa plays an important role in the pathogenesis of stress-induced ulcerogenesis, being responsible for the enhanced permeability of blood vessels to activate neutrophils resulting in an excessive infiltration of gastric mucosal tissue [29-31]. In the present study, the cold-restraint stress model caused severe mucosal injury in the stomach of rats along with increased neutrophil infiltration and consumption of tissue glutathione content. Increased gastric luminol- and lucigenin-enhanced CL levels in cold-restraint animals revealed the involvement of oxidants in the pathogenesis of gastric injury.

The anti-inflammatory actions of the statins were substantiated by in vivo studies. In a murine model of acute inflammation, oral administration of SIM (3 mg/kg) resulted in a significant decrease in foot-pad edema, with a potency and effectiveness comparable to that of indomethacin [32]. In a previous study performed in our laboratory, both SIM and fluvastatin were found to be beneficial in trinitrobenzene sulfonic acid-induced colonic inflammation in rats [33].A study by Heeba et al. [11] comfirmed that SIM, at a dose of 10 mg/kg for 2 weeks, is gastroprotective against indomethacin-induced ulcer formation via scavanging free radicals, increasing NO and prostaglandin E2 levels, and increasing gastric juice mucin production. In line with these observations, in our study, SIM pretreatment (10 mg/kg for 21 days) reduced the severity of the gastric mucosal lesions induced by cold-restraint, as confirmed by macroscopic and microscopic examination. Although SIM pretreatment did not seem to affect the endogenous antioxidant glutathione consumption and neutrophil infiltration due to cold-restraint, there was marked suppression of oxidant production in the gastric samples. These findings may imply that SIM shows a beneficial effect on stress-induced gastric ulcer formation via scavanging oxidants which may be derived from sources other than neutrophils (e.g. macrophages, endothelial cells, epithelial cells, or mast cells).

Stress ulcer increases the formation of reactive oxygen metabolites and promotes inhibition of prostaglandin synthesis, leading to alterations in gastric NO levels [34]. NO is one of the most efficient mediators of gastric defense mechanism. Previous studies have shown that gastric lesions induced by various chemical agents are attenuated by administration of NO donors or augmented by suppression of the NO pathway [35]. However, in our study, the non-selective NOS inhibitor L-NAME given alone or prior to SIM to animals with cold-restraint ulcer did not show a statistically significant effect on the severity of the gastric lesions, gastric GSH content and gastric CL levels.

On the other hand, prostaglandins also play an essential role in gastric mucosal defense. This effect is dependent on the prostaglandin-induced stimulation of bicarbonate and mucous secretion, inhibition of gastric acid secretion, and regulation of maintaining epithelial cell restitution and mucosal blood flow [36]. Early studies have shown that inhibition of COX activity by non-selective nonsteroidal anti-inflammatory drugs (NSAIDs) leads to gastric ulcers and delays healing of the gastric mucosa [36,37]. In the present study, we used Indo- the non-selective inhibitor of COX-1 and COX-2- to examine if the actions of SIM are mediated via the COX-derived prostaglandins. According to our data, the suppression of the COX pathway by Indo did not cause a statistically significant change on the extent of

gastric lesions and gastric CL levels of the SIM-treated cold-restraint group.

In conclusion, pretreatment of rats with cold-restraint-induced gastric ulcers with SIM showed partial protection against gastric lesion formation which might be attributed to its effect to suppress the generation of oxidants derived from sources other than neutrophils and that NOS and COX systems do not take part in this process.

**Conflict of interest:** The authors have declared that there is no conflict of interest.

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