

The Protective Effects of Probiotic Bacteria on Indomethacin-Induced Gastric Ulcer in Rats

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Abstract

Indomethacin is an anti-inflammatory drug that causes ulcers on the gastric mucosa as a result of its use. For this reason, many experimental studies have been performed to search for new agents in order to treat or prevent gastric ulcers. Probiotic bacteria are live microorganisms, and it has been stated by various studies that these bacteria have antioxidant and anti-inflammatory effects. In this study, we investigated the possible protective effect of various types of probiotic bacteria against acute gastric mucosal damage caused by indomethacin. Our research used 40 female Wistar albino rats that were divided into four groups, with 10 in each group: Control group - Physiological saline was administered daily for 10 days. Indo group - Physiological saline was administered daily for 10 days. On day 11, a single 100mg/kg dose of indomethacin was given. Ranitidine + Indo group - A ranitidine dose of 5mg/kg was administered daily for 5 days. On day 11, a single dose of 100mg/kg of indomethacin was given to the same group. Probiotic + Indo group - A dose of 1 ml/kg of oral probiotic bacteria was administered daily for 10 days. On day 11, a single 100mg/kg dose of indomethacin was given to the same group. After application, rats were killed in appropriate conditions, and stomach tissues were obtained. The obtained gastric tissues were used in the biochemical and histopathological analyzes discussed below. As a result, the administration of indomethacin caused gastric damage, stimulating oxidative stress, inflammation, and apoptosis. However, we found that the use of probiotic bacteria reduces oxidative stress (TOC), increases the activity of antioxidant enzymes (TAC), suppresses inflammation (IL-6, IL-1 β , Tnf- α , and COX-2) and inhibits apoptosis (Bax and Bcl-2). This suggests that probiotic bacteria inhibit indomethacin-induced apoptosis. Probiotic treatment can mitigate gastric damage and apoptosis caused by indomethacin-induced gastric damage in rats. Probiotic also enhances the restoration of biochemical oxidative enzymes as it has anti-inflammatory, antioxidant and antiapoptotic properties.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in the treatment of diseases such as rheumatic disorders and osteoarthritis (Pal et al. 2010). They are also given as an antineoplastic drug for the purpose of prevention and treatment of ischemic heart disease (Gladding et al. 2013). However, the use of NSAIDs leads to a number of gastrointestinal complications in the organism, such as gastric mucosal bleeding, decreased gastric mucosal blood flow, and induced mucosal cell apoptosis (Pal et al. 2010; Bindu et al 2013; Lanar et al. 2005; Musumba et al. 2009; Wallace, 2000; Yadav et al. 2012). It is thought that these drugs cause gastric injury via the inhibition of cyclooxygenases (COXs), an increase in prostaglandin (PG) synthesis, and stimulation of gastric mucosal apoptosis associated with increased NSAID-induced reactive oxygen species (ROS) (Pal et al. 2010). Indomethacin (IND), a strong NSAID, was reported to induce ROS production and increase gastric damage (Chatterjee et al. 2007). This results in oxidative stress, mitochondrial permeability transition pore formation, mitochondrial dysfunction, and a consequent increase in proinflammatory cytokine production. Thus, inflammation occurs. This is related to the formation of mitochondrial oxidative stress (Pal et al. 2010; Bindu et al. 2013). Studies have reported that inflammation plays an important role in the pathogenesis of indomethacin-induced gastric

mucosal injury (Musumba et al. 2009; Uc et al. 2012). Increased leukocytes at the site of gastric injury is a determinant in the initiation of pathogenesis of gastric mucosa (La et al. 2000; Luster 1998; Marise et al. 1999; Nishida et al. 1997). Neutrophils are also activated in patients with indomethacin-induced gastric injury. Activated neutrophils can physically occlude small vessels by producing many proinflammatory and pro-oxidative enzymes (Musumba et al. 2009; Uc et al. 2012; Wintebourn 2002). This increases the oxidative load of the gastric mucosa and damages the endothelium (Uc et al. 2012; Demir et al. 2003; Kristal et al. 1998; Salvemini et al. 1389). Therefore, antioxidant, anti-inflammatory and anti-apoptotic treatment will be an effective approach in the prevention or treatment of NSAID-induced gastric injury. Several recent studies have reported that antioxidant and anti-inflammatory agents have prevented indomethacin-induced gastric injury. Probiotic bacteria are live microorganisms with anti-inflammatory and antioxidant biological uses (Matsuzaki and Chin. 2000; Karamese et al. 2016; Wang et al. 2017; Martarelli et al. 2011; Abu-Elsaad et al. 2015; Salva et al. 2014; Sengül et al. 2019). The aim of this study is to investigate the gastroprotective effects of the application of probiotic bacteria on gastric mucosal damage induced by indomethacin in rats.

Material And Method

Animals

Six-week-old female Wistar albino rats were housed in a cage maintained at 23°C, with a 12/12-hour light/dark cycle under specific pathogen-free conditions. After one week of adaptation, rats weighing 250 to 300g were used for the experiments. All experimental procedures were approved by the of Kafkas University (KAÜ-HADYEK/2017/076).

Analaysis and Preparation of Probiotic Mixture

For the isolation of lactobacilli from the kefir, Man, Rogosa, and Sharpe (MRS, Merck, Germany) were seeded with agar media and incubated anaerobically at 30°C for forty-eight hours. At the end of the incubation, white and opaque colonies were tested for catalase. Catalase-negative colonies were identified by APICH50. *Lactobacillus rhamnosus*, *Lactobacillus fermentum*, and *Lactobacillus brevis* were isolated as lactic acid bacteria. The bacteria were separated from the supernatant culture by centrifugation, washed with an cold phosphate saline buffer, and resuspended in PBS (10^5 lactic acid bacteria in 1ml).

Experimental design

At an experimental study, forty female Wistar albino rats were used and equally divided into four groups as following; Gp. I (Control group) was kept on physiological saline (1 ml) for 10 days as a negative control group. Gp. II (Indo group) was administered physiological saline for 10 days and on day 11, was given a single dose of indomethacin (100mg/kg BW). Gp. (III) (Ranitidine+Indo group) was received ranitidine (5mg/kg BW) for 5 days and on day 11 given indomethacin as. Gp. (IV) (Probiotic+Indo group) orally taken probiotic bacteria (1 ml/kg BW) for 10 days and on day 11 given indomethacin as. At the end

of dosing, rats of all groups were anesthetized, sacrificed and stomach was picked up, opened, and washed with physiological saline and ulcer scoring was done (Table 2 and Figure 3). Then a fragment of the stomach was homogenized for biochemical analysis and another fragment was taken on neutral formalin 10% for histopathological and immunohistochemical examination.

Tissue homogenization

The gastric tissues (0.5 g) were homogenized by a tissue homogenizer (WINGER HAUSER/Ser no. 177002) in 5 mL of cold phosphate buffer saline (pH 7.4, 0.1 M). The homogenates were centrifuged (Hettich zentrifugen, D-78532-Tuttlichgen, GERMANY) at 10,000g for 20 min at 4°C. The supernatants were collected and stored at -20°C until further use in bioassays.

Measurement of gastric TOC and TAC

Gastric total oxidant capacity (TOC) and total antioxidant capacity (TAC) were determined using kits of (TOC (MY130380) and TAC (MT13033) kit, Gaziantep, Turkey) according to the manufacturer's protocol.

Inflammatory cytokines analysis

Rat-specific ELISA kits of (Sunlong biotech Co., Ltd) were used to measure interleukin-6 (IL-6), interleukin-8 (IL8), interleukin-1 β (IL-1 β) and Tumor necrosis factor (TNF- α), (rat IL-6 (201704), IL-8 (201704), IL-1 β (201704) and TNF- α (201704) ELISA, kit Sunlong Biotechnology, Shanghai, China) and Cyclooxygenase-2 (COX-2) (COX-2 (E-EL-H1414) ELISA, kit Elabscience, USA) with. respectively following the manufacturer's protocols.

Histopathological and immunohistochemical examinations

For histopathological analysis, stomach tissues were fixed in 10% formalin. After 72h of fixation, the tissue samples were dehydrated, cleared, and embedded in paraffin. The paraffin was cut into 5 μ m thick blocks using a LeicaRM2125RT microtome (*Leica Microsystems, Wetzlar, Germany*) and stained by Mallory's triple stain, modified by Crossman for assessment of architectural damage and inflammatory process. A rabbit polyclonal antibody against Bax (dilution:1/50,Abcam,Cambridge,UK) and a rabbit polyclonal antibody against Bcl-2 (dilution:1/100,Abcam,Cambridge,UK) were used to estimate apoptosis and cellular proliferative activity in the stomach tissue. The stained specimens were examined under a light microscope (*Nikon eclipsei50, Tokyo, Japan*) and photo images were taken for histopathological and immunohistochemical evaluation. At least ten high-power field in the for each slice was observed, and the number of positive cells was counted and averaged to reflect the intensity of positive expression. The sections were evaluated as none (-), mild (+), moderate (++) , and severe (+++) according to their immunity positivity.

Statistical analysis

All data were statistically evaluated by one-way ANOVA using SPSS 20.00, followed by Duncan *Post hoc* test. The data were expressed as mean \pm SD. $P < 0.05$ was considered statistically significant.

Results

Effect of Probiotic Bacteria on oxidative stress

The TAC values were significantly lower in the Indo group compared to the control group. The Probiotic+Indo group's values were lower than the control group but higher than the Indo group ($P < 0.05$), Figure 1A. This suggests that the application of probiotic bacteria activates the antioxidant defense system. When the TOC values were compared among the groups, there was a significant increase in the Indo group compared to the other groups ($P < 0.05$), Figure 1B. This shows that probiotic bacteria treatment also reduces oxidative stress.

Effect of Probiotic Bacteria on Inflammation

When IL-6 levels were compared among groups, it was observed that indomethacin applied significantly increased compared to other groups ($P < 0.05$).

When IL-8 levels were compared among groups, it was observed that the indomethacin-treated group had significantly increased values compared to control and Probiotic+Indo groups ($P < 0.05$). This shows that the administration of probiotic causes a decrease in IL-8 levels. When the levels of IL-1 β were compared among the groups, we found that the indomethacin-administered group experienced an increase compared to the other groups, but this was not statistically significant ($P > 0.05$).

There was, however, a significant increase in the indomethacin-treated group compared to the others when the levels of TNF- α were compared ($P < 0.05$). We found a significant decrease in ranitidine and probiotic groups. This suggests that probiotic administration had prevented an indomethacin-induced increase.

COX-2 levels were significantly lower in the Indo group compared to the control group ($P < 0.05$). In the probiotic group, there was a increased compared to the indomethacin-administered group, but it did not show any statistical significance ($P > 0.05$), Table 1.

Table 1. Inflammation markers in the gastric tissues for all groups. IL-6, IL-8, IL-1 β ; TNF- α , and COX-2 the letters indicate the statistical differences among groups ($P < 0.05$, $n = 10$), the results were expressed as mean \pm SD.

Mean±SD	IL-6 (pg/ml)	IL-8 (pg/ml)	IL-1 β (pg/ml)	TNF- α (pg/ml)	COX-2 (ng/ml)
Control	97.68±4.26 ^a	53.15±2.84 ^a	54.11±5.07 ^a	42.14±4.12 ^a	156.23±12.16 ^a
Indo	151.42±8.84 ^b	79.05±3.12 ^b	63.17±4.16 ^a	71.08±3.71 ^b	114.21±15.71 ^b
Ranitidine+Indo	86.14±3.21 ^a	61.12±4.03 ^{bc}	60.04±3.14 ^a	59.13±2.89 ^c	112.32±10.82 ^b
Probiotic+Indo	89.45±5.16 ^a	51.16±5.01 ^{ac}	61.21±2.12 ^a	49.16±3.12 ^c	139.64±11.84 ^{ab}

Macroscopic findings of indomethacin-induced gastric mucosal injury

Representative macroscopic photographs of the rat stomachs are shown in Fig.3. Macroscopic findings revealed the normal structure of the gastric mucosa in the control group (Fig. 3A). The Indo group presented severe mucosal injuries and the largest ulcer area (Fig. 3B). The Ranitidine+Indo (Fig. 3C) and Probiotic+Indo (Fig. 3D) groups showed fewer gastric erosions or ulcers compared with the indomethacin group.

Table 2. Ulcer index and preventive index in the studied groups

Groups	Ulcer Index
Control	-
Indo	19.19±1.42 ^a
Ranitidine+Indo	4.43±0.74 ^b
Probiotic+Indo	3.95±0.23 ^b

Histopathological findings

In the histopathologic analysis, the stomach sections of the control group revealed normal histologic structure (Fig. 4A). However, in the same section of Indo group, there were active chronic gastritis findings in the rat gastric mucosa, consistent with the macroscopic appearance of chronic gastric erosion. Common mononuclear inflammatory cells were observed as foci between the lamina propria and submucosa. Also, widespread necrosis, with loss of surface epithelium and submucosal edema, was seen (Fig. 4B). However, fewer lesions were visible in the gastric mucosa in the treatment groups. Histological examination indicated that treatment with Ranitidine or probiotic promoted the healing of gastric lesions, with the base of the

ulcer covered by regenerating mucosa and fewer inflammatory cells. The results obtained showed that the group receiving probiotic exhibited lower gastric erosion and better efficiency than the Ranitidine+Indo group (Fig. 4C, D).

Immunohistochemical findings

The representative images of Bax and Bcl-2 immunoreactivity are depicted in Fig. 5.

Immunohistochemistry revealed that Bax and Bcl-2 immunoreactive products presented as brown-reddish fine granules, located in the cytoplasm. Minimal expression of Bax and massive expression of Bcl-2 protein were observed in the control group, but Bax expression increased and Bcl-2 protein expression decreased in the section of Indo group. Furthermore, immunopositivity of Bax was significantly decreased in the Ranitidine+Indo and Probiotic+Indo groups compared with Indo group. Moreover, immunopositivity of Bax in the Probiotic+Indo group was lower than the Ranitidine+Indo group. Immunopositivity of Bcl-2 was significantly increased in the Ranitidine+Indo and Probiotic+Indo groups compared with Indo group, and immunopositivity of Bcl-2 in the Probiotic+Indo group was higher than Ranitidine+Indo group (Fig. 5). The positive cell intensity of Bax and Bcl-2 in the groups is shown in Table 3.

Table 3: Immunohistochemical findings and their scores in stomach tissue.

Groups	Bax	Bcl-2
Control Group	-	++
Indomethacin Group	+++	+
Ranitidine+Indo Group	++	++
Probiotic+Indo Group	+	+++

According to immunohistochemical findings: none (-), mild (+), moderate (++) and severe (+++)

Discussion

NSAIDs are among the most widely used drugs worldwide due to their anti-inflammatory and analgesic effects (Cashin et al. 1977; Bjamasan et al. 1993). Indomethacin is used in a group of NSAIDs. However, indomethacin use causes extensive and severe erosions and ulcers in the gastric mucosa (Beck et al. 1990; Tenenbaum 1999). Free oxygen radicals and lipid peroxide production and inflammation play an important role in the formation of gastric mucosal lesions originating from indomethacin (Langenbach et al. 1999; Lanza 1984; Vananen et al. 1991). Biochemical and immunohistochemical data obtained from this study indicate that probiotic bacteria have anti-inflammatory, antioxidant, and antiapoptotic effects on indomethacin-induced gastric mucosa damage. Oxidative stress has significant impact on the

pathophysiology of indomethacin-induced gastric injury. Previous studies have shown that the amount of lipid peroxidation and superoxide dismutase (SOD) has changed. In addition, indomethacin-induced gastric mucosal damage has been reported to be associated with enzyme activity, such as catalase and glutathione peroxidase (Delsoldata et al. 1986; Iwasaki et al. 2004). For these reasons, the use of substances that can increase the activity of antioxidant enzymes and reduce oxidative stress is an important approach in order to protect the gastric mucosa from the effects of indomethacin.

In our study, according to histopathological evaluations, the rats in the group exposed to indomethacin had more ulcers in their stomachs, while the probiotic bacteria group had smaller and more superficial ulcers. This important result provides good evidence for the protective effect of probiotic bacteria on the gastric mucosa. Our research, combined with that previously published, confirms that indomethacin-induced gastric injury is prevented as a result of the application of various substances with antioxidant and anti-inflammatory (Delsoldata et al. 1986; Iwasaki et al. 2004; Ellinge et al. 2002). For example, the antioxidant effect of selenium is well known, and its curative effect on gastric mucosal oxidative stress has been previously reported (Ellinge et al. 2002).. In another study, the antioxidant properties and healing effect of L-carnitine on gastric mucosal damage were determined (Derin et al. 2006).

Other research (Kim et al. 2013) has reported that grape seeds have protective and healing properties on indomethacin-induced gastric damage, and this effect is achieved by an increase in GSH levels. In addition, Kim et al. found that the application of selenium caused GSH levels to rise and the MDA level to drop (Kim et al. 2011). In our study, we determined that TAS increases the level of TOS compared to the indomethacin group in this group. These findings suggest that probiotic bacteria increase antioxidant activity and suppress oxidative stress, thereby inhibiting indomethacin-induced gastric mucosal damage.

Changes in the concentration of local inflammatory mediators (proinflammatory cytokines) such as IL-16, TNF- α , IL-1 β , IL-8, and COX-2 are associated with this NSAID. The role of proinflammatory cytokines in the pathogenesis of gastric damage in relation to the cellular signaling pathways is still being researched. Cytokine secretion is the mediator of inflammation and contributes to the pathogenesis of tissue injury (Lavery et al. 2010; Lacour et al. 2005). It has been reported that proinflammatory cytokines are associated with a significant increase in serum IL-16, TNF- α , IL-1 β , IL-4, IL-10, and COX-2 levels following indomethacin treatment in rats (Sirhendu et al. 2012). Previous studies reported the inhibitory effect of probiotic bacteria on IL-1 α , TNF- α , and IL-6 (Sengül et al. 2019). In the current study, indomethacin treatment markedly increased TNF- α , IL-1 β , and IL-6 levels. Conversely, probiotic bacteria treatment caused a significant reduction in TNF- α , IL-8, and IL-6 levels in indomethacin-administered experimental rats. NSAIDs induce gastric injury partly by inhibiting the cyclooxygenases (COX). COX-1 regulates basal prostanoid levels, while COX-2 is associated with the inflammatory response (Takeuchi 2012). NSAIDs induce gastric injury partly by inhibiting the cyclooxygenases (COX). COX-1 regulates basal prostanoid levels, while COX-2 is associated with the inflammatory response (Farnai et al. 2011). When COX activity is blocked by indomethacin, the PG level decreases and impairs gastroprotection mechanisms, reducing mucus and bicarbonate secretion, decreasing mucosal blood flow and leucocyte infiltration, deteriorating microvascular structures, and increasing gastric acid secretions (Jainu et al. 2006). In our study, COX-2

levels were lower in indomethacin-treated groups than control groups, and probiotic bacteria significantly prevented decreases in COX-2 levels; these results were in agreement with previous research (Wu et al. 2018; Chatterjee et al. 2012). This is possibly owing to its anti-inflammatory properties. In the light of these findings, it can be deduced that probiotic bacteria gastric mucosal inflammation is caused by indomethacin.

Gastric ulcers are a common multiplex diseases. Pathogenesis is closely related to apoptosis in gastric mucosal epithelial cells. Bcl-2 regulation is one of the key factors affecting cell apoptosis. Bcl-2 and Bax proteins are important representatives of the Bcl-2 family and play a major role in determining cell life (Szabo and Tamawski. 2000). When the expression of the Bax protein is increased, apoptosis can be induced. In contrast, however, when the Bcl-2 protein is increased, apoptosis is suppressed. In previous research on Bcl-2 protein, when acute gastric mucosal damage was repaired, Bax expression was reported to be reduced (Kontuerek et al. 1999). In this study, we found that Bax expression decreased in the gastric tissues of the group treated with probiotic bacteria compared to indomethacin-administered group, whereas Bcl-2 expression was increased. This suggests that probiotic bacteria inhibit indomethacin-induced apoptosis. Probiotic treatment can mitigate gastric damage and apoptosis caused by indomethacin-induced gastric damage in rats. Probiotic also enhances the restoration of biochemical oxidative enzymes as it has anti-inflammatory, antioxidant and antiapoptotic properties. Further studies are warranted to investigate its future clinical applications.

Declarations

Compliance with ethical standards

The study was designed and conducted according to ethical norms approved by the Kafkas University Animal Experiments Local Ethics Committee (Kars, Turkey), (KAÜ-HADYЕК/2017/076).

Conflict of Interest The authors declare that there are no conflicts of interest.

Author contributions V.G. E.Ş. S.G. M.M. S.U.G. G.A.U. H.U. K.A. : experiment design, experiment application, samples collection. V.G. G.A.U. H.U. M.M. : serum markers and tissue antioxidant estimation, data curation and analysis, final reviewing. S.G.: histopathological and immunohistochemical investigation. All authors contributed to the writing and editing, and they read and approved the final manuscript.

Data availability The authors confirm that the data and materials supporting the findings of this study are available within the article.

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Consent to participate All authors voluntarily participated in this research study.

Consent to publish All authors have consent for the publication of the manuscript.

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Figures

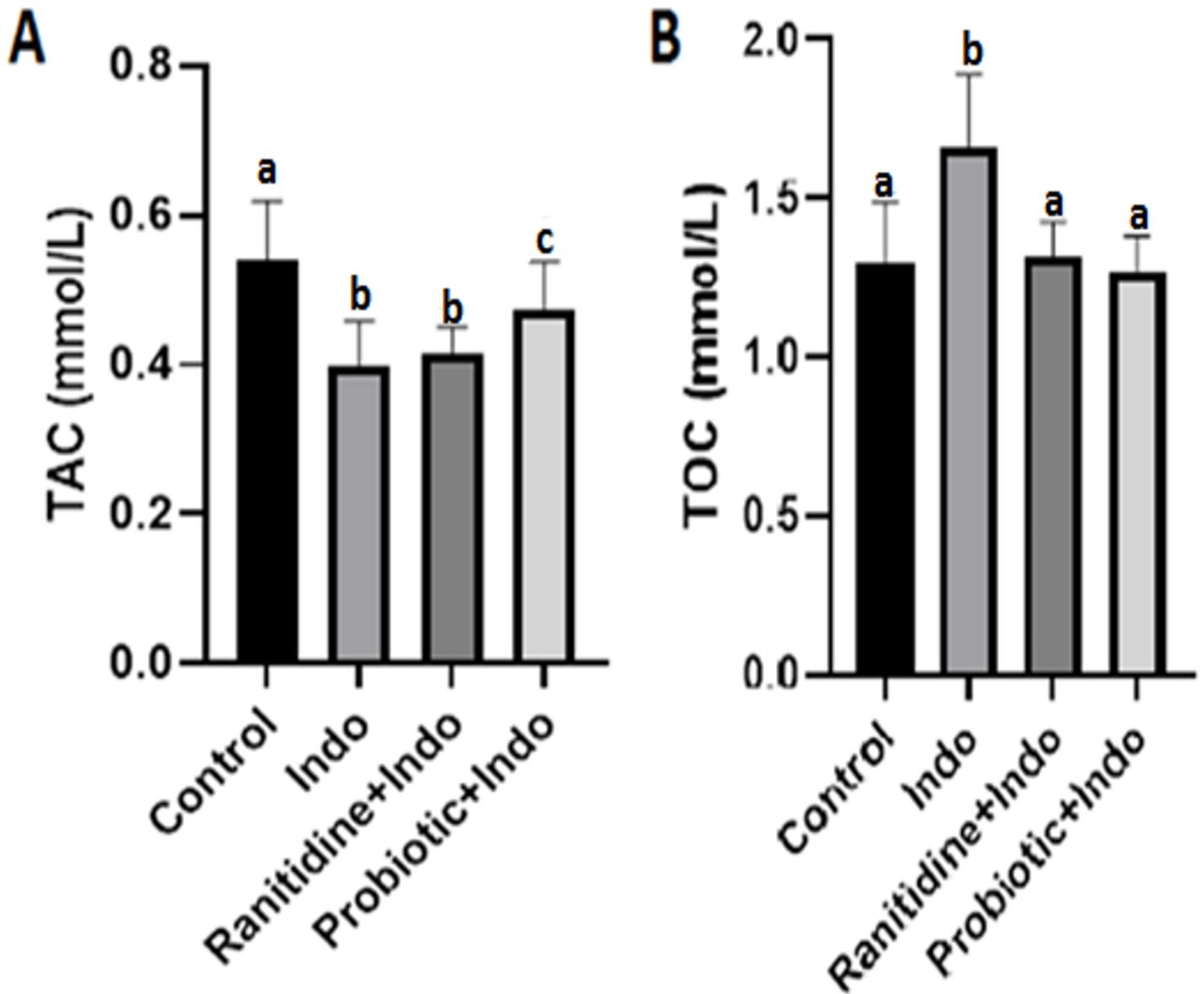


Figure 1

Illustration of levels of oxidative parameters (TAC and TOC) for all groups in the gastric tissues. A; TAC level, B; TOC level, the letters indicate the statistical differences among groups ($P < 0.05$, $n = 10$), the results were expressed as mean \pm SD.

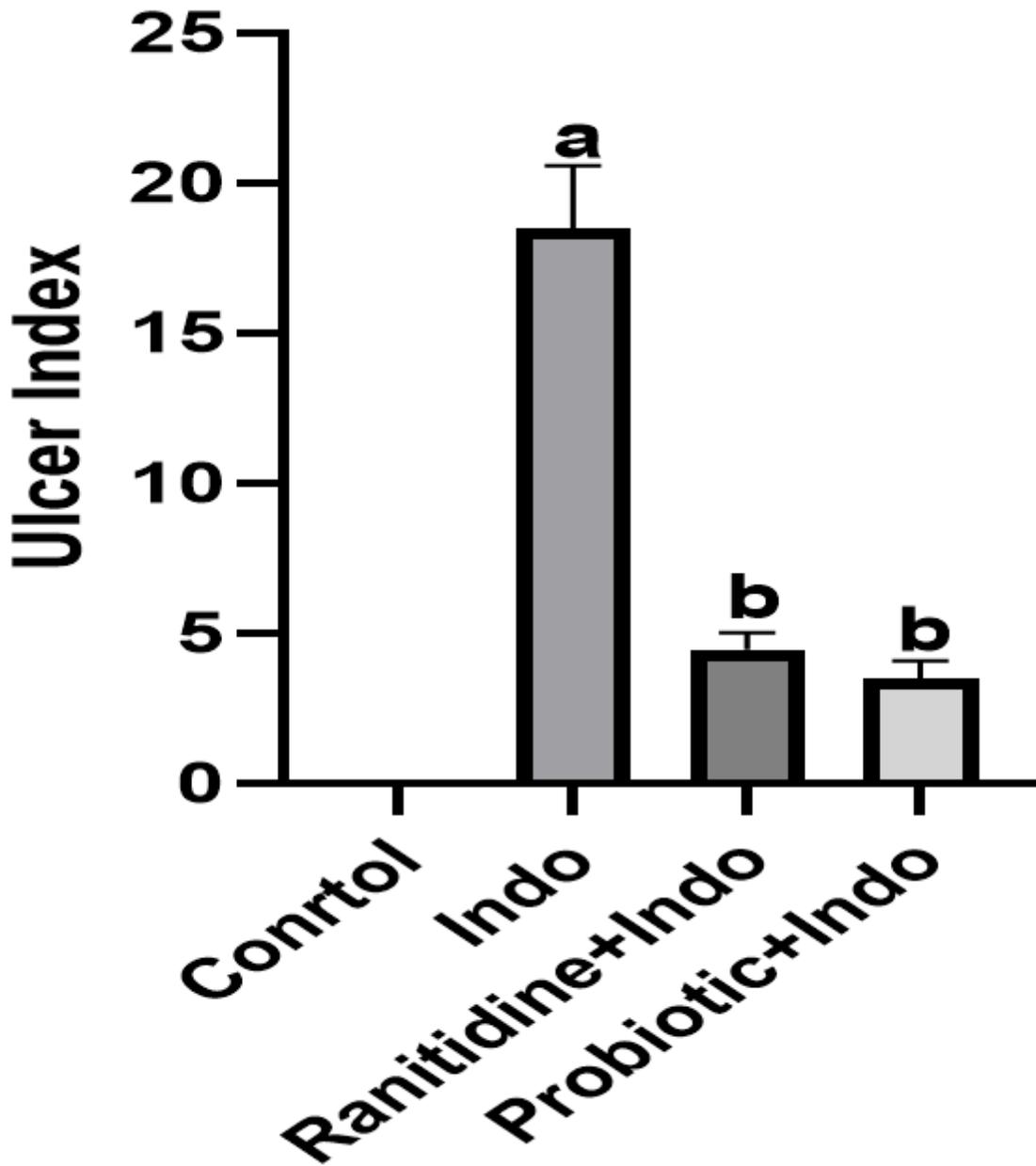


Figure 2

Effect of Probiotic on the severity of gastric lesion (UI) in Indo-induced gastric ulcer.



Figure 3

Representative macroscopic findings of the stomachs in Control group (A), Indomethacin group (B), Ranitidine+Indo group (C), and Probiotic+Indo (D) group rats.

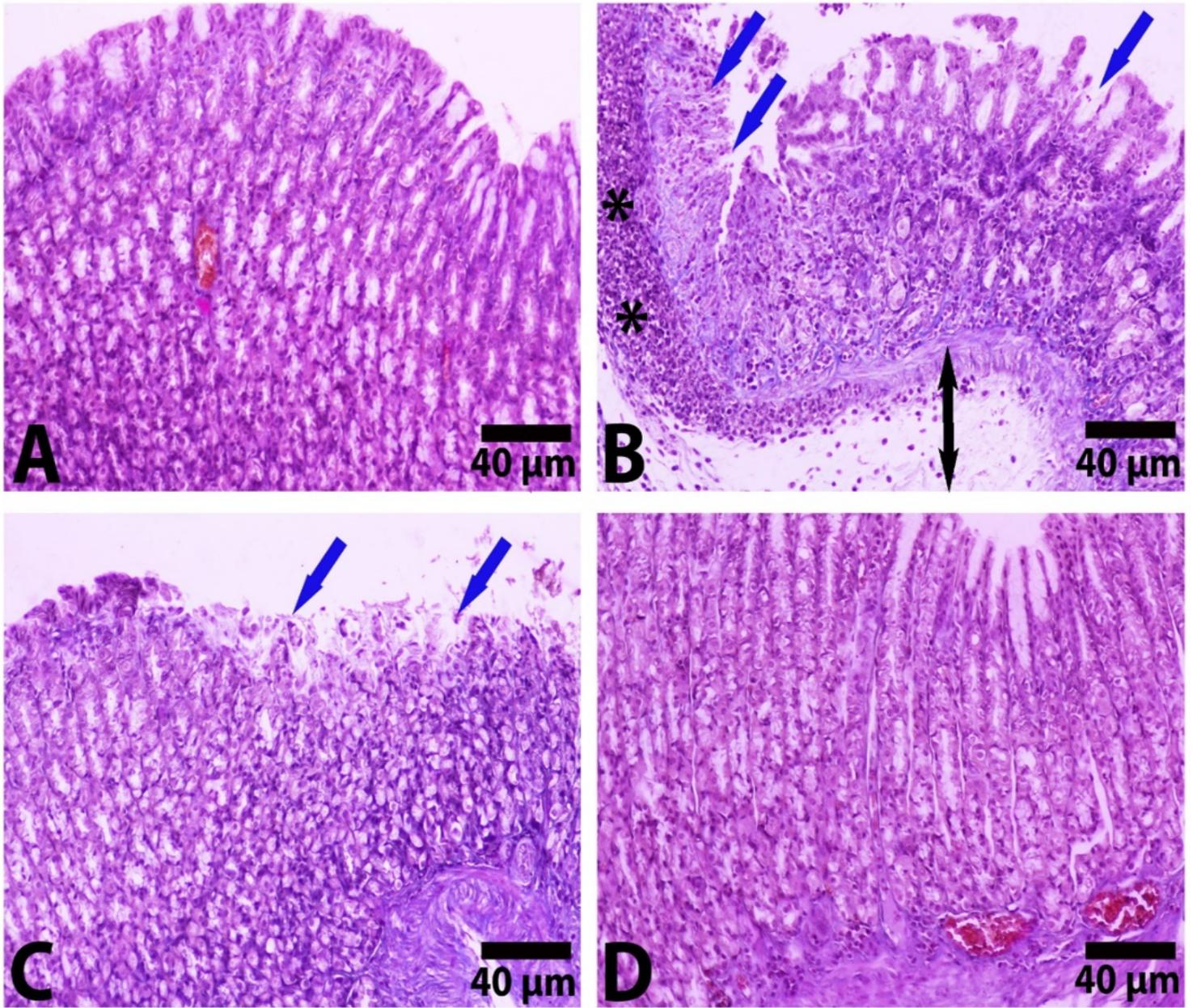


Figure 4

Micrograph of stomach section of the Control (A), Indomethacin (B), Ranitidine+Indo (C), and Probiotic+Indo (D) groups. asterisks: Mononuclear inflammatory cells; blue arrows: Widespread necrosis with loss of surface epithelium. Stain: Crossman's modified Mallory triple staining.

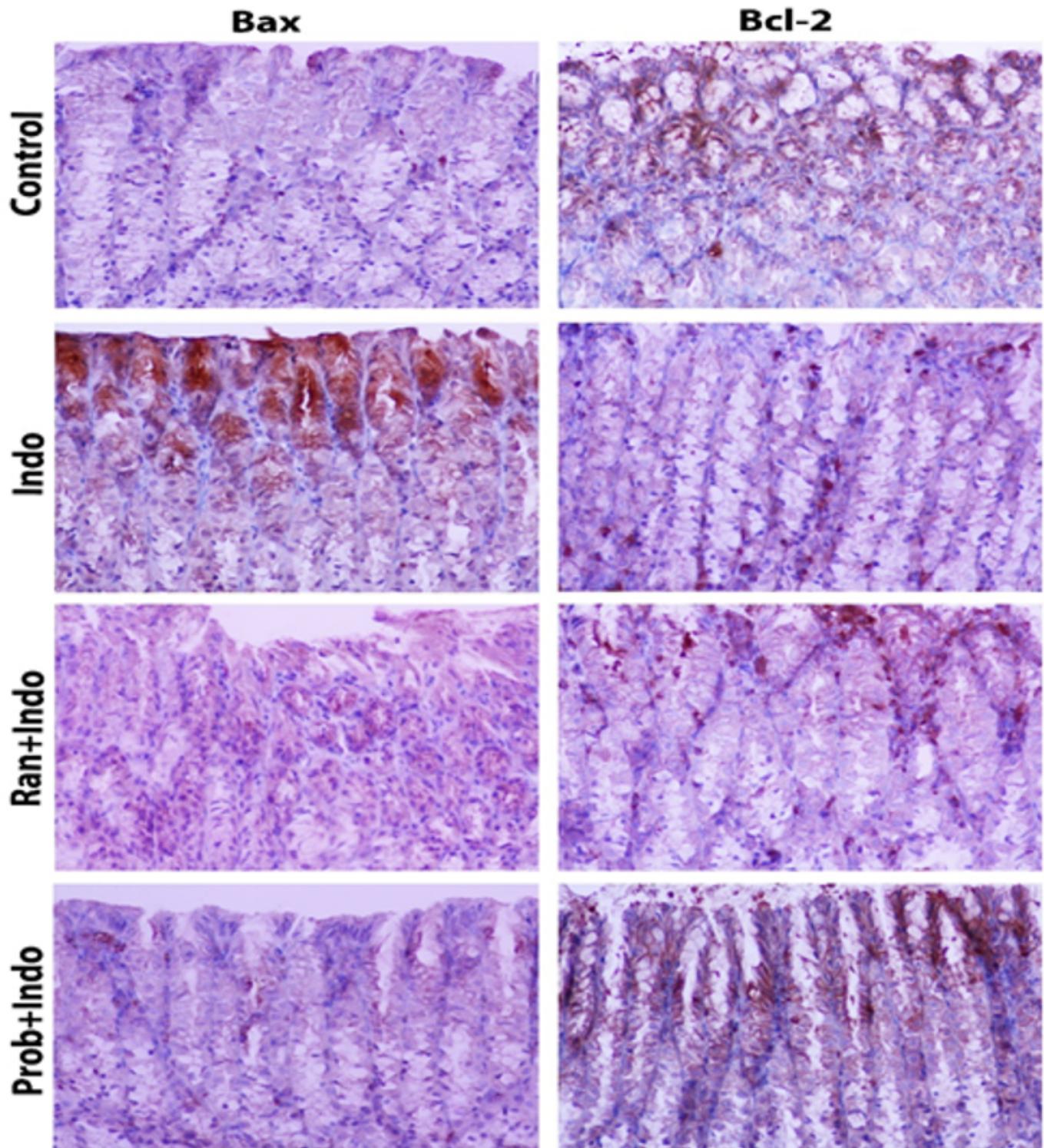


Figure 5

Immunohistochemical staining for the Bax and Bcl-2 for the Control, Indomethacin, Ranitidine+Indo and Probiotic+Indo groups. Streptavidin–biotin peroxidase staining.