Effect of probiotics on aspirin-induced gastric mucosal lesions

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Background/aims: We aimed to investigate the role of a probiotic mixture, including 13 different bacteria, in the prevention of aspirin-induced gastric mucosal injury. Methods: Forty rats were allocated into 4 groups: normal control, aspirin, probiotic control, and probiotic plus aspirin. Normal control and aspirin groups received 0.2 ml of skim milk by daily gavage for 14 days. Probiotic control and probiotic plus aspirin groups were administered 0.2 ml/day of probiotic mixture (1.3 x 10^8 cfu/ml) suspended in skim milk by daily gavage for 14 days. On day 15, gastric lesions were induced by administration of aspirin (200 mg/kg) in the aspirin and probiotic plus aspirin groups. Normal control and probiotic control groups were given saline. Results: Pretreatment with probiotic mixture reduced aspirin-induced gastric damage scores (4.50 ± 0.43 and 2.60 ± 0.40, p<0.01) and exerted tendency of downregulation of proinflammatory cytokines elicited by aspirin (p>0.05). We also found that the probiotic mixture increased sIgA production approximately 7.5-fold in the stomach, and significantly reduced the malondialdehyde (MDA) increase in the gastric mucosa elicited by aspirin (p<0.001). Additionally, pretreatment with the probiotic mixture alleviated aspirin-induced reduction of mast cell count in the gastric mucosa. Conclusions: Probiotic mixture pretreatment attenuates the aspirin-induced gastric lesions by reducing the lipid peroxidation, enhancing mucosal sIgA production, and stabilizing mucosal mast cell degranulation into the gastric mucosa.

Key words: Probiotic, aspirin, gastric lesion, cytokines, mast cells

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs), particularly aspirin, are widely used as anti-inflammatory, analgesic agents. Despite their therapeutic benefits, NSAIDs can cause inflammation...
and ulceration in the gastrointestinal tract (1). Aspirin damages the gastrointestinal mucosa by several mechanisms. Suppression of endogenous prostaglandin (PG) production by cyclooxygenase (COX) inhibition is considered to be important (2). However, there is much evidence that aspirin exerts direct topical damage by a non-PG-mediated mechanism (3). In the pathogenesis of aspirin-induced mucosal injury, neutrophil-dependent microvascular injury is one of the important events (4,5). Inhibition of neutrophil accumulation significantly attenuated the gastric mucosal injury induced by aspirin (5,6). On the other hand, oxygen-derived free radicals are directly implicated in the mechanism of aspirin-induced acute gastric mucosal injury, and scavenging these free radicals protects against injury (7,8). Tumor necrosis factor (TNF)-α is a proinflammatory cytokine and has been shown to be a crucial mediator of NSAID-induced gastric mucosal injury (9,10). TNF-α augments neutrophil-derived superoxide generation (11), leading to oxygen radical-mediated tissue damage. Pretreatment with TNF-α inhibitors suppresses the gastric mucosal injury caused by aspirin and other NSAIDs (5,10).

Probiotics are defined as live microorganisms which, when consumed in adequate numbers, confer a health benefit on the host beyond basic nutrition (12). Probiotics have been applied as an alternative approach in the prevention and therapy of several intestinal inflammatory disorders, including inflammatory bowel disease (IBD). Probiotics decrease new or recurrent bacterial infections in high-risk patients (13), and prevent antibiotic-induced diarrhea (14). In acute gastric damage induced by intragastric administration of ethanol in rats, probiotic strain Lactobacillus rhamnosus GG (LGG) pre-treatment was shown to increase the basal gastric mucosal PGE₂ level (15). Probiotics may induce mucin gene expression in colonic epithelial cell lines (16,17) and may enhance the mucus-secreting layer in the gastric mucosa of rats (15). Some species of lactobacilli or bifidobacteria produce anti-oxidant substances that could have a protective effect against intestinal mucosal barrier damage (18,19). Treatment of interleukin (IL)-10-deficient mice with oral probiotic VSL#3 DNA resulted in a reduction of colonic mucosal secretion of TNF-α and interferon-γ and improvement in histologic disease (20). On the basis of this evidence, probiotics may represent a potential alternative treatment for aspirin-induced gastropathy. The potential role of probiotics in prevention of aspirin-induced gastric injury has not been investigated previously. In this study, we examined the effect of a probiotic mixture, including 13 different bacterial strains, on aspirin-induced gastric mucosal injury. The effects of probiotics on the levels of proinflammatory cytokines TNF-α and IL-2, anti-inflammatory cytokine IL-4, and malondialdehyde (MDA), as an index of lipid peroxidation, in the gastric mucosa were studied. In addition, gastric mucosal secretory (s)IgA levels and degranulated mast cell count were determined.

MATERIALS AND METHODS

Animals

Male Wistar albino rats (200-250 g) were fed on standard laboratory diet and water ad libitum and kept in cages at a temperature of 22 ± 2°C with a 12-hour (h) dark–light cycle before and during experiments. Experiments were approved by Suleyman Demirel University School of Medicine Ethical Committee. During this experimental study, we acted according to the principles of the Guide for the Care and Use of Laboratory Animals (www.nap.edu/catalog/5140.html).

Probiotic microorganisms

Thirteen bacteria with resistance to low pH and bile salts between the 10⁷ probiotic bacterial strains were selected from the healthy human stool (21) (four strains of Lactobacillus fermentum (BB16-75, AK2-8, AK5-22, AK6-26), 3 strains of Lactobacillus plantarum (AA17-73, AK7-28, AK8-31B), and 6 strains of Enterococcus faecium (AB6-21, AB16-68, AK4-120, AK7-31, BK9-40, BK13-54)). Molecular identification of these strains was made with 16S rRNA analysis (22). Each strain was inoculated in MRS broth medium and incubated at 37°C for 24 h until the cell number reached 10⁶ cfu/ml. The cells were pelleted by centrifugation at 5000 x g for 10 minutes (min) at 20°C, and the pellets were washed in phosphate-buffered saline solution (PBS, pH 7.4) twice. Finally, the probiotic mixture was adjusted to 1.3 x 10⁹ cfu/ml in 10% reconstituted sterile skim milk.

Study design

Forty rats were allocated into four groups, each consisting of 10 animals, with different pre-treatments for 14 days, as follows: (i) normal control group rats received 0.2 ml/day of skim milk by daily gavage; (ii) probiotic control group rats received 0.2 ml/day of probiotic mixture (1.3 x 1010 cfu/ml)
suspended in skim milk by daily gavage; (iii) aspirin group rats received 0.2 ml/day of skim milk by daily gavage; (iv) probiotic plus aspirin group rats received 0.2 ml/day of probiotic mixture (1.3 x 10^10 cfu/mL) suspended in skim milk by daily gavage. On day 15, gastric lesions were induced by administration of aspirin (200 mg/kg) in the aspirin and probiotic plus aspirin groups. Normal control and probiotic control groups were given saline (1 ml). Coprophagy was avoided. All agents given orally were administered by gavage through an intragastric tube. Twelve hours before aspirin (or saline) administration, rats were deprived of food but had free access to water 2 h before aspirin (or saline) administration.

**Macroscopic analysis**

The animals were sacrificed by intramuscular (i.m.) 100 ml/kg ketamine hydrochloride (Ketalar®, Parke-Davis, Eczacibaşı, Istanbul, Turkey) and i.m. 25 mg/kg xylazine hydrochloride (Rompun®, Bayer, Germany) 3 h after aspirin (or saline) administration, and the stomach was removed. Stomachs were opened along the greater curvature, and mucosae were rinsed with cold PBS to remove blood concomitant, if any. A damage score was assigned using a scale of 0 to 6 described by Coleman et al. (23).

Gastric mucosal tissues were removed by scraping with a glass slide and immediately frozen in liquid nitrogen and stored at -80°C until determination of gastric TNF-α, IL-2, IL-4, sIgA, and MDA concentrations. For mast cell counts in the stomach, stomach tissue specimens were fixed in 10% formalin solution.

**Histological study**

Samples from the gastric mucosa of each stomach were fixed in 10% formalin solution and embedded in paraffin after completion of the routine follow-up. Serial sections of 5-μm thickness were obtained and stained with hematoxylin/eosin (HE) to evaluate gastric morphology.

**Quantitation of gastric mast cells**

Routine histological procedures were followed, and 5 μm thick sections were made and stained in 1% aqueous solution of toluidine blue. The mast cells in the mucosal layer could be easily identified under the microscope by their metachromatic purple stain. Using a calibrated ocular micrometer, the cells were counted under oil immersion objective and expressed for 1 mm² of gastric mucosa (24).

**Determination of cytokines and sIgA**

Gastric tissues were weighed and homogenized (1:10, wt/vol; Ultra Turrax T25; IKA-Labotechnik, Staufen, Germany) in 100 mmol/L of phosphate buffer (pH 7.4) containing 0.05% sodium azide in an ice bath. The homogenate was sonicated (Bandelin; Berlin, Germany) for 30 seconds (s) and centrifuged (5000 x g for 10 min). The gastric mucosal levels of rat TNF-α, IL-2 and IL-4 were measured using a commercial ELISA kit (Biosource International Inc., California, USA), according to the manufacturer’s instructions without modification. A standard curve was constructed from a series of known concentrations of TNF-α, IL-2 and IL-4 solution provided by the kit’s manufacturer, and the TNF-α, IL-2 and IL-4 concentrations of the unknown samples were determined by interpolation. The gastric mucosal levels of sIgA were measured using a commercial ELISA kit (Immunodiagnostik AG, Bensheim, Germany), according to the manufacturer’s instructions without modification.

The levels are expressed as picograms for TNF-α, IL-2 and IL-4 and nanograms for sIgA concentrations per milligrams total protein in the supernatant, where the total protein levels are determined by the Lowry method (25).

**Determination of MDA**

500 mg tissue was homogenized by Vetra-Turrax in volume of 1.15% KCl, 2400 rpm. For alkaline hydrolysis of protein-bound MDA, 200 μL 6 M NaOH were added to 1 ml homogenate in an Eppendorf cup and the sample was incubated in a 60°C water bath for 45 min. An aliquot of 1 ml was diluted with an equal volume of acetonitrile to precipitate proteins. The resulting suspension was then vortex mixed for 30 s and centrifuged at 15000 g for 10 min. The upper clear supernatant (0.25 ml) was transferred to a 2 ml Eppendorf cup, mixed with 25 μL DPNH solution, and incubated for 10 min. After derivatization, the sample was filtered through a 0.2 μm filter. Aliquots of 20 μL were injected into the high performance liquid chromatography (HPLC) system.

The samples were analyzed on a thermo Finnigan series HPLC apparatus (USA). The analytical column was ODS 2 C18 (5 μm particle size, 125x4). The mobile phase was acetonitrile-distilled water.
(38/6; v/v) containing 0.2 % (v/v) acetic acid. HPLC apparatus was isocratic at a flow rate 1 ml/min and UV detector was set at 310 nm. MDA peaks were determined according to retention time and confirmed by spiking with added exogenous standard. Concentrations of MDA were calculated from standard curve prepared from 1,1,3,3 tetrachloroxypropane and expressed as nmol/mg protein for tissue.

Statistical analysis
All data are expressed as mean±SEM. The significance of differences in gastric mucosal concentrations of TNF-α, IL-2, IL-4, sIgA, and MDA between groups was determined by one way analysis of variance (ANOVA), followed by a post hoc Tukey’s test when the analysis of variance suggested a significant difference between groups. Kruskal–Wallis analysis of variance was applied to assess differences in macroscopic damage score and mast cell counts in the stomach among the experimental groups. When the Kruskal-Wallis test indicated a significant difference, multiple comparisons were performed using the Mann-Whitney u test to determine which group differed from the others. Spearman’s correlation was calculated to assess associations between gastric mucosal damage scores, mucosal mast cell counts and other parameters. The association between gastric mucosal concentration of cytokines and sIgA was analyzed using the Pearson’s correlation test. Values of p<0.05 were considered significant.

RESULTS
Intragastric administration of aspirin induced congestion and multiple hemorrhagic erosions in the rat stomachs. Negligible damage was observed in the control and probiotic groups. In the aspirin group, the mean gastric mucosal damage score was 4.50±0.43. Probiotic mixture administration reduced aspirin-induced gastric damage to 2.60±0.40 (p<0.01). Figure 1 shows the gastric mucosal damage scores.

Effects of probiotics on gastric mucosal concentration of cytokines
Tissue TNF-α and IL-2 concentration in the gastric mucosa increased 3 h after aspirin administration. In the aspirin group, TNF-α and IL-2 concentrations (respectively, 4.94±1.38 and 6.66±2.24 pg/mg protein) were higher than those of the normal control group (respectively, 4.00±0.64 and 4.39±0.77 pg/mg protein). Pro-inflammatory cytokine levels in the probiotic plus aspirin group were lower compared to the normal control, probiotic control, and aspirin groups. However, no statistical difference was observed between the groups by ANOVA. Gastric mucosal concentrations of IL-4 were also not significantly different between the groups (Table 1).

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>TNF-alpha pg/mg protein</th>
<th>IL-2 pg/mg protein</th>
<th>IL-4 pg/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.00±0.64</td>
<td>4.39±0.77</td>
<td>2.07±0.41</td>
</tr>
<tr>
<td>Probiotic</td>
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<td>4.11±0.62</td>
<td>2.17±0.38</td>
</tr>
<tr>
<td>Aspirin</td>
<td>4.94±1.38</td>
<td>6.66±2.24</td>
<td>2.02±0.40</td>
</tr>
<tr>
<td>Probiotic+Aspirin</td>
<td>3.33±0.25</td>
<td>3.27±0.62</td>
<td>1.67±0.20</td>
</tr>
</tbody>
</table>

Table 1. Effect of pretreatment with probiotic mixture on aspirin-induced TNF-alpha, IL-2 and IL-4 concentrations in the gastric mucosa

Effects of probiotics on gastric mucosal concentration of sIgA
In the probiotic control group, gastric mucosal sIgA concentration was higher by approximately 7.5-fold compared to the normal control group (p<0.001). Mucosal concentration of sIgA was not statistically different in the normal control and aspirin groups. Although aspirin administration caused a decrease in augmented sIgA secretion by probiotic mixture, mucosal sIgA was still two-fold higher in the probiotic plus aspirin group compared to the aspirin group. Figure 2 shows the gastric mucosal sIgA concentrations.
Effects of probiotics on gastric mucosal concentration of MDA

In the aspirin group, MDA concentration of the gastric mucosa was more elevated compared to the normal control and probiotic control groups (p<0.001). The increase in MDA concentration in the gastric mucosa elicited by aspirin was significantly suppressed by probiotic pretreatment (p<0.001). Gastric mucosal MDA concentrations are presented in Figure 3.

Effects of probiotics on gastric mucosal mast cell degranulation

The mean number of gastric mucosal mast cells of the normal control and probiotic control groups were 55.70±6.56 and 64.90±12.31, respectively (p=0.05) (Figure 4). Gastric mast cells in the aspirin group were significantly reduced compared with the normal control and probiotic control groups (p< 0.01, p<0.05, respectively). Pretreatment with probiotic mixture partially restored aspirin-induced gastric mast cell reduction (p>0.05). Additionally, mast cell count between the normal control and probiotic plus aspirin groups was not significantly different (Figure 5).

Histological results

The morphology of the gastric mucosa of control animals given probiotic was not different from that of normal control rats (Figure 6). Administration of aspirin produced severe hemorrhagic necrotic lesions in the gastric mucosa. In the group given aspirin, disruption and exfoliation of the superficial gastric epithelium were observed (Figure 7). In the group given probiotic plus aspirin, it was observed histologically that the probiotic was not protective against the lesions caused by aspirin.
Correlation analyses

Pearson’s correlation analysis: Gastric mucosal TNF-α concentration showed significant positive linear correlation with IL-2 concentration in the normal control and aspirin groups (respectively, r=0.800, p=0.01, r=0.934, p<0.001). There was a negative linear correlation between mucosal IL-2 levels and sIgA levels in the probiotic control and the probiotic plus aspirin groups (respectively, r= -0.672, p<0.05, r= -0.637, p<0.05).

DISCUSSION

The gastric mucosa is frequently exposed to various noxious agents. There are few studies investigating the preventive or therapeutic effects of probiotics on gastric mucosal damages. In one study, it was shown that pre-treatment of rats with LGG at 10^9 cfu/ml twice daily for three consecutive days markedly reduced ethanol-induced gastric mucosal lesions (26). In another study, probiotic strain LGG was determined to enhance the healing of acetic acid-induced gastric ulcer in rats, via the attenuation of cell apoptosis to cell proliferation ratio and increase in angiogenesis (27). In a human study, Gotteland et al. (18) showed that regular ingestion of a lactobacillus-containing product protects the integrity of the gastric mucosal barrier against indomethacin.

Many studies have provided evidence regarding multistrain probiotics being more effective than monostrain probiotics (28,29). Moreover, the use of multispecies preparations, containing multiple strains of more than one genus, could even be more effective than that of multistrain probiotics (28). Therefore, we preferred the use of multispecies – multistrain probiotic combination.

The present study demonstrated that probiotics may have a preventive action against aspirin-induced gastric mucosal injury. To our knowledge, this is the first report to assess the effect of probiotics in aspirin gastropathy. In addition, it was determined for the first time that probiotics may stabilize mucosal mast cell degranulation and induce sIgA production in the stomach.

We determined that aspirin administration caused proinflammatory cytokine response in the stomach; however, differences between the rat groups were not statistically significant, while previous studies showed a significant increase in proinflammatory cytokines (30,31). In these studies, acidified aspirin was used to provide more evident inflammatory response, including TNF-α and IL-1 beta (5,30,31). In our model, we also think that peaked levels of TNF-α and IL-2 could precede the sampling time.

Pretreatment with probiotic mixture exerted a tendency of downregulation of proinflammatory cytokines elicited by aspirin. It is well known that TNF-α induces the increase in intestinal permeability and IL-8 secretion (32) and augments neutrophil–derived superoxide generation (11), leading to oxygen radical-mediated tissue damage. Stimulation of intestinal epithelial cells with TNF-α has also induced apoptosis (33). Thus, downregulation of TNF-α and other pro-inflammatory cytokines by probiotic mixture would prevent subsequent events.

It has been determined that some orally administered strains of lactic acid bacteria are able to in-
increase the number of IgA-producing cells in the small intestines of mice in a dose-dependent manner (34). Bifidobacteria has been shown to increase systemic antibody response (35) as well as total IgA production in the intestine (36). In studies of methotrexate-induced enterocolitis in rats, the administration of *Lactobacillus plantarum* strain 299v increased colonic and ileal levels of sIgA, and reduced mucosal inflammation (37,38). It has been shown that oral administration of *Enterococcus faecium* SF68 increases intestinal IgA production in dogs (39).

We also found that probiotic mixture increases sIgA production approximately 7.5-fold in the stomach, which was not reported previously. On the other hand, a negative linear correlation was observed between mucosal sIgA and IL-2 levels both in the probiotic control and probiotic plus aspirin groups. This observation supports the consideration that the probiotic mixture used enhances Th-2 differentiation while suppressing proinflammatory activity.

It is well known that sIgA in the intestine can prevent infection by pathogenic microorganisms and can prevent aberrant absorption of allergic food proteins and carcinogens (40). In our opinion, probiotic-induced sIgA production may play an important role in the prevention of aspirin-induced gastropathy via a different mechanism from that mentioned above. Studies using various intestinal and gastric cell lines have clearly shown that aspirin and other NSAIDs are capable of initiating apoptosis (41,42). Under normal physiologic conditions, intestinal epithelial cell apoptosis causes no disruption of the epithelial barrier (37). However, dysregulated intestinal epithelial cell apoptosis has been implicated in the pathophysiology of several diseases (43,44). Additionally, it is reported that NSAIDs cause gastric mucosal damage through both necrosis and apoptosis of gastric mucosal cells (45). In an *in vitro* study, Diebel et al. (46) reported that sIgA modulates the increased enterocyte apoptotic response. Thus, modulation of gastric mucosal epithelial apoptosis by increased quantity of sIgA may directly serve to maintain epithelial barrier function and decrease the inflammatory response caused by aspirin.

Some species of lactobacilli or bifidobacteria have been reported to produce anti-oxidant substances that could have a protective effect on intestinal mucosal barrier damage (47). Administration of aspirin has been demonstrated to increase oxidative stress and lipid peroxidation status in the gastric mucosa, resulting in mucosal damage at both the cellular and subcellular level (5,7,8). It has also been shown that the provision of superoxide dismutase plus catalase attenuates this injury (48).

We found that aspirin administration increased the concentration of MDA, an index of lipid peroxidation, in the gastric mucosa compared to the normal control and probiotic control groups (p<0.001). Our results support the importance of lipid peroxidation in the pathogenesis of aspirin-induced gastric damage. The probiotic mixture used in our study significantly reduced the MDA increase in gastric mucosa elicited by aspirin (p<0.001). These results suggest that the preventive effect of our probiotic mixture against aspirin damage should be at least due to suppression of lipid peroxidation.

We also determined that probiotics may display mast cell-stabilizing activity in the rat stomach. Pretreatment with probiotic mixture alleviated aspirin-induced reduction of mast cell count in the gastric mucosa. Gastric mucosal damage induced by aspirin, stress, histamine, and concentrated ethanol in guinea pigs has been found to be accompanied by a significant decrease in mast cell counts (49). Mast cells are known to release histamine and other active substances (50). Effects of histamine on cellular permeability are well known and may serve as the initial stimulus for mediating cellular damage. Also, histamine is the final common mediator of gastric secretion (51). A mast cell-stabilizing agent was also found to show gastric mucosal protection and gastric antisecretion activities (52). Previously Kim et al. (53) demonstrated that probiotic bacteria *Bifidobacterium bifidum* BGN4 and *Lactobacillus casei* 911 decreased the ovalbumin-induced mast cell degranulation in ear and tongue tissue samples of mice. Our results support the findings of Kim et al. The mast cell-stabilizing activity of probiotics possibly presents an important contribution to their beneficial effects on aspirin damage.

In conclusion, the probiotic mixture used in this study significantly inhibited the aspirin-induced gastric lesions. This effect seems to be due to a reduction in lipid peroxidation, enhancement of mucosal sIgA production, and stabilization of mucosal mast cell degranulation into the gastric mucosa.

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REFERENCES


