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Effects of rifaximin on indomethacin-induced intestinal damage in guinea-pigs

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Abstract. – AIM: Enterobacterial translocation into the gut mucosa is the first step required for activation of neutrophils and inducible nitric oxide synthase (iNOS), involved in the pathogenesis of indomethacin-induced intestinal lesions. Rifaximin may limit NSAID-associated intestinal damage by decreasing the bacterial load. We aimed to study the effect of rifaximin on indomethacin-induced intestinal damage in guinea-pigs.

MATERIALS AND METHODS: Twenty-four guinea pigs, equally divided in four interventional groups (A-D), received indomethacin, given orally once daily (30 mg/kg) for three consecutive days. In groups B, C, D different doses of rifaximin (50 mg/kg, 100 mg/kg and 200 mg/kg) were given orally two hours before indometachin administration. Semi-quantitative grades were measure for gross findings, degenerative lesions, neutrophils and eosinophils infiltrates and iNOS immunopositivity. Statistical comparisons used Mann Whitney Test, with a Bonferroni correction for alpha ($p \leq 0.016$).

RESULTS: Statistical analysis of graded gross findings, microscopic degenerative lesions, endothelium damage and iNOS immunopositivity found no difference between A and B groups. Significant fewer gross findings ($U = 3$, $p = 0.015$), microscopic degenerative lesions ($U = 2$, $p = 0.008$) and lower grades for iNOS immunopositivity ($U = 0$, $p = 0.002$) were found in group C compared with group A. In group D, significant lower grades for iNOS immunopositivity were obtained ($U = 0$, $p = 0.002$) compared with group A and fewer degenerative lesions without reaching statistical significance ($U = 4$, $p = 0.026$).

CONCLUSIONS: 100 mg/kg of rifaximin proved efficient in preventing gut degenerative lesions induced by indomethacin in a guinea pig model, the iNOS activity being significantly decreased.

Key Words:
Indomethacin, Rifaximin, Gut immunology.

Introduction

The gastrointestinal tract represents a physical convergence of bacteria, dietary antigens and activated cellular components of the immune system[1]. The mechanisms for immune tolerance in the presence of luminal bacteria are not yet fully understood. Epithelial and stromal cells, immunomodulatory T cells and cytokines modulate the immune response[2-4]. Epithelial cells acquired lipopolysaccharides (LPS) tolerance by a decrease in inducible nitric oxide synthase (iNOS) mRNA and protein response to repeated LPS exposure[5]. Cyclooxygenase (COX) dependent arachidonic acid metabolites, especially prostaglandin E2 (PGE2), produced by resident stromal cells in the murine small intestine lamina propria, are in part responsible for immune tolerance[4]. COX2 expression by the small intestine lamina propria is a basal state contributing to the hypo-responsiveness of the intestinal immune response[4].

Nonselective non-steroidal anti-inflammatory drugs (NSAIDs) inhibit both COX-1 and COX-2 expression, decrease prostaglandin synthesis that unbalances the gut tolerant immune profile[4] and upregulate iNOS expression, revealed by necro-inflammatory lesions[6]. Bacterial flora, neutrophils and iNOS are involved in the pathogenesis of NSAIDs small bowel injuries. Germ-free animals treated with indomethacin showed significantly fewer intestinal lesions compared with normal animals, suggesting that enterobacterial translocation is essential for the development of intestinal lesions[7]. Bacterial lipopolysaccharides upregulate iNOS expression that is responsible for nitric oxid (NO) overproduction[8]. NO combines with superoxides to form damaging species like peroxynitrite, which is cytotoxic, causing intestinal degen-

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erative lesions in 18-48 hours after NSAIDs administration[9-12]. Experimental studies documented that iNOS inhibitors reduce or abolish NSAID enteropathy by decreasing iNOS activity[13].

Enterobacterial population could be manipulated with antibiotics or probiotics, in order to reduce NSAIDs small bowel injuries. Previous reports indicate that pretreatment with antibacterial agents like ampicilin, metronidazole or polymyxin B have beneficial effects[14]. Probiotics have also been investigated for their potential role in limiting the severity of inflammatory gastrointestinal disorders, but contradictory effects were documented[15].

Rifaximin, a nonabsorbable oral antibiotic that acts locally in the gastrointestinal tract with minimal systemic adverse effects, may have beneficial effects in preventing NSAIDS enteropathy. *In vitro* studies found that rifaximin possessed activity against *Lactobacillus* spp., *Staphylococcus* spp., *Enterococcus* spp., *Bacillus cereus, Moraxella catarrhalis, Haemophilus influenzae*[16].

This study was designed to assess the potential of rifaximin to protect against indomethacin-induced damage in the guinea pig small intestine.

Materials and Methods

Animals and Study Design

Twenty seven adults, male and female guinea pigs, 26-28 weeks of age, with an average weight of 700 grams were used for the experiments, conducted in the licensed animal house facility of the Faculty of Veterinary Medicine from Cluj-Napoca (Romania). The experiments were approved by the Institutional Animal Ethics Committee, being in accordance with Romanian laws. The animals were divided into 4 interventional groups of 6 animals (named from A to D) and one control group with three guinea pigs. Group A received indomethacin, administered orally once daily (30 mg/kg) for three days. The same dose of indomethacin was associated with different doses of rifaximin orally: 50 mg/kg in group B, 100 mg/kg in group C and 200 mg/kg in group D. Control animals received the vehicle.

Animals were fasted over night. Drugs were given by oral gavage, first antibiotics and after two hours, indomethacin, for three consecutive days. After 72 hours, the animals were sacrificed by deep ether anesthesia.

Necropsy and Assessment of Gross Features

The abdomen of each guinea pig was opened immediately after sacrifice; the entire small intestine was removed and fixed for 10 minutes in 2% formalin. The side opposite to mesentery attachment was incised and the tissue spread out carefully. Small intestines were investigated for congestion areas, erosions and ulcers. A semi-quantitative assessment of gross lesions was used, adapted from a capsule endoscopy score: grade 0 no lesions, grade 1 – red spots corresponding to congestive areas, grade 2 – small erosions (less than 5 mm), grade 3 – large erosions (more than 5 mm), grade 4 – ulcer[17]. Erosion was defined as a superficial necrosis with small blood clots on the mucosa surface. Ulcer was defined as a larger mucosal break with apparent depth, and a definite rim.

Tissue sampling was conducted from the pathological lesions identified at macroscopic examination and also from proximal, middle and distal area of the small bowel if no gross lesions were identified.

Histological and Immunohistochemical Analysis

Samples from small intestine were fixed in 10% phosphate-buffered formalin for 24 hours, embedded in paraffin wax, cut in 3-5 µm sections, and stained with hematoxylin and eosin (H&E) and Periodic acid Schiff (PAS).

The histological samples were evaluated for degenerative lesions, neutrophils and eosinophils infiltrates and vascular endothelium damage. A semi-quantitative assessment was used for degenerative lesions: grade 0 – no lesions, grade 1 - focal necrosis of the surface epithelium, grade 2 – upper villous necrosis, grade 3 – necrosis of > 1/3 of villous height, grade 4 – necrosis of > 1/3 of mucosa[18]. The neutrophils and eosinophils infiltrates were quantified apart: grade 0 – no infiltration, grade 1 – infiltration in lamina propria, grade 2 – infiltration of epithelium beside lamina propria, grade 3 – acute inflammatory cells in muscularis mucosa, and grade 4 – infiltration in submucosa. The vascular endothelium damage was scored in grade 0 – no vascular lesions, grade 1 – hyperemia, grade 2 – endothelial necrosis, thrombosis, acute vasculitis and perivascular inflammation.

The PAS staining depicted the number of goblet cells in three different areas of the surface epithelium, in areas without necrotic lesions.

For the immunohistochemical method, the sections were incubated with primary antibody rabbit polyclonal anti-iNOS/NOS II, (guinea pigs cross reactivity, code 06-573 Millipore) diluted in 1% PBS-BSA (bovine serum albumin) at 1: 1000 at 4ºC overnight. The secondary antibody (labeled streptavidine biotine) was applied, followed by incubation with diaminobenzidine, LSAB System-

HRP kit (Code K0679, Dako, Denmark). After washing with distilled water, the slides were then counterstained with Mayer haematoxylin for 5 min. Negative controls for each sample were prepared by replacing the primary antibody with mouse IgG1 Negative Control (Code X0931, Dako, Glostrup, Denmark).

Immunopositivity for iNOS was scored separately for mucosal epithelium, glandular epithelium and lamina propria according to the published protocols[19], taking in count the extent and the intensity of staining. Grades for the extent of staining were evaluated as: no positive cells = 0; a few dispersed positive cells = 1; clusters of positive cells = 4; (almost) all cells positive = 7. The intensity of staining was graded as faint = 0, moderate (present) = 1 and strong = 2. These values were added up to give a semi-quantitative staining score ranging from 0 to 9[19]. Measurements were performed on the five high power fields in the representative sections by two pathologists.

The slides were evaluated using an Olympus BX51 microscope with Olympus SP 350 (Tokyo, Japan) digital camera.

Statistical Analysis

Histological and immunohistochemistry scoring results were expressed as median ± IQT (interquartile range) and results for villous height and number of PAS positive cells as mean ± (standard error of the mean). Independent groups were analyzed by the Kruskal Wallis test, Mann-Whitney *U*-test or Student's *t*-test. If Kruskal-Wallis test leaded to significant results (regarding the applied semi-quantitative grades on studied groups) a Bonferroni correction for alpha was applied on Mann-Whitney test, exact p values ≤ 0.016 being considered statistical significant. For statistical analysis we used SPSS 9.0 statistical software package (SPSS Inc., Chicago, IL, USA).

Results

An overview of the results, concerning descriptive statistics, is summarized in Tables I and II.

Gross Lesions

In the indomethacin group (A), a perforated ulcer with diffuse suppurative peritonitis (grade 4) was find in one case (Figure 1), large erosions in the middle area of jejunum corresponding to grade 3 were described in two animals, small erosions (grade 2) in both duodenum and jejunum were seen in one case, congestive areas (grade 1) in one guinea pig. The content of the intestinal tract was fluid, yellow-reddish with scattered small blood clots. Only in one case the small bowel was without macroscopic lesions. In group B that received rifaximin 50 mg/kg associated with indomethacin, lesions corresponding to each grade from 1 to 4 were describe in four different animals. Two animals have no macroscopic lesions in the small bowel. In group C (indomethacin + rifaximin 100 mg/kg) no gross findings were identified on small bowel. In group D, (indomethacin + rifaximin 200 mg/kg) one case presented grade 3 erosions.

Statistical analysis of small bowel graded gross findings using Mann-Whitney nonparametric test found no difference between A and B groups ($U = 14.5$, $p = 0.708$), significant differences between A and C groups ($U = 3$, $p = 0.015$) and difference between A and D groups, without reaching the statistical significance ($U = 6.5$, $p = 0.067$).

Histological Assessment

No epithelial changes were observed in negative control group (Figure 2a, b and c). In group A microscopic examination revealed important degenerative lesions: four cases with severe mucosal necrosis that affected more than one third of intestinal mucosa (grade 4) (Figure 2d), one case with upper villous necrosis (grade 2) and one case with focal necrosis of the surface epithelium (grade 1).

In group B histological examination revealed one case with grade 4 degenerative lesions, one case with necrosis affecting more than 1/3 of villous height (grade 3) and three cases with upper villous necrosis (grade 2).

In group C two animals presented discrete degenerative lesions: one case - grade 1 and one case - grade 2. In group D, only one case presented intestinal damage, corresponding to necrotic area of more than 1/3 of mucosa (grade 4).

Statistical analysis of graded microscopic degenerative lesions using Mann Whitney test found no significant difference between group A and B ($U = 10.5$, $p = 0.204$), significant difference between A and C groups ($U = 2$, $p = 0.008$) and fewer necrotic lesions in group D compared with group A, but without reaching statistical significance ($U = 4$, $p = 0.026$).

In each interventional group, the same pattern of neutrophils infiltration of lamina propria and epithelium was noted in at least four animals, so no statistical significant difference was found between graded neutrophils infiltrates.

Table I. Descriptive statistics concerning gross lesions, microscopic degenerative lesions, neutrophils and eosinophils infiltrates, iNOS staining between interventional groups (A-D).

Group	Graded parameters	Minim	Maxim	Percentiles 25th	50th (median)	75th
A	Gross lesions	0	4	0.75	2.50	3.25
	Surface epithelium iNOS	9	9	9.00	9.00	9.00
	Glandular epithelium iNOS	6	9	6.00	9.00	9.00
	Lamina propria iNOS	9	9	9.00	9.00	9.00
	Microscopic degenerative lesions	1	4	1.75	4.00	4.00
	Neutrophils infiltrates	2	4	2.00	2.00	2.50
	Eosinophils infiltrates	1	4	1.75	2.00	2.50
	Vascular endothelium damage	1	2	1.00	2.00	2.00
B	Gross lesions	0	4	.00	1.50	3.25
	Surface epithelium iNOS	9	9	9.00	9.00	9.00
	Glandular epithelium iNOS	6	6	6.00	6.00	6.00
	Lamina propria iNOS	6	9	8.25	9.00	9.00
	Microscopic degenerative lesions	0	4	1.50	2.00	3.25
	Neutrophils infiltrates	2	2	2.00	2.00	2.00
	Eosinophils infiltrates	1	2	1.00	2.00	2.00
	Vascular endothelium damage	0	2	1.50	2.00	2.00
C	Gross lesions	0	0	.00	.00	.00
	Surface epithelium iNOS	4	7	4.00	4.00	5.50
	Glandular epithelium iNOS	1	4	1.00	3.00	4.00
	Lamina propria iNOS	1	4	1.00	1.50	4.00
	Microscopic degenerative lesions	0	2	.00	.00	1.25
	Neutrophils infiltrates	1	2	1.75	2.00	2.00
	Eosinophils infiltrates	1	1	1.00	1.00	1.00
	Vascular endothelium damage	0	2	.00	.00	0.50
D	Gross lesions	0	3	.00	.00	0.75
	Surface epithelium iNOS	2	8	3.50	5.00	7.25
	Glandular epithelium iNOS	0	8	0.75	4.00	6.50
	Lamina propria iNOS	1	5	1.00	2.00	4.25
	Microscopic degenerative lesions	0	4	.00	.00	1.00
	Neutrophils infiltrates	0	4	1.50	2.00	2.50
	Eosinophils infiltrates	1	2	1.00	1.00	1.25
	Vascular endothelium damage	0	1	.00	.00	0.25

Table II. Descriptive statistics concerning PAS cells number between groups.

Group	N	Mean	Standard Deviation	Standard Error	95% Confidence Interval for Mean Lower Bound	Upper Bound	Minim	Maxim
Control	3	219.67	34.819	20.103	133.17	306.16	190	258
A	6	156.83	73.806	30.131	79.38	234.29	35	247
B	6	156.83	34.747	14.185	120.37	193.30	112	205
C	6	158.83	33.879	13.831	123.28	194.39	114	198
D	6	131.83	51.503	21.026	77.78	185.88	47	184

In group A eosinophils were found only in lamina propria (grade 1) in one case, also distributed in epithelium (grade 2) in four cases and were present in the submucosa (grade 4) in one case. In group B, two cases presented scattered eosinophils only in lamina propria (grade 1) and in three cases epithelium was also infiltrated (grade 2). All animals from group C presented eosinophils infiltrate only in lamina propria (grade 1). In group D five animals were classified with grade 1 and one ani-

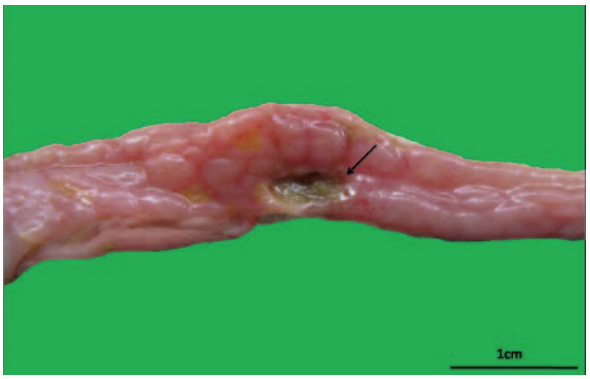

Figure 1. Opened proximal segment of proximal jejunum showing an acute, large (about 0.8 cm) and perforated ulcer (*arrow*) in group A.

mal with grade 4 eosinophilic infiltrates. Statistical analysis of graded eosinophil infiltrates using Mann Whitney test found no difference between A and B groups (U = 13, $p = 0.636$), significant difference between groups A and C (U = 3, $p = 0.015$), and less severe infiltrates with eosinophils in group D compared with group A, without reaching the statistical significance (U = 5.5, $p = 0.067$).

Severe endothelial damage with thrombosis, hemorrhages and perivascular inflammation corresponding to grade 2, was found in five guinea pigs in group A, in four cases in group B, one case by group C and D. Statistical analysis of graded vascular endothelium damage revealed no differences between groups A and B (U = 16, $p > 0.05$), fewer vascular lesions in groups C and D, without statistical significance (U = 4, $p = 0.026$).

Significant fewer PAS stained cells were noted within the surface epithelium in all groups that received indomethacin (Figure 2e and h) compared to control animals (Figure 2b). No statistical significance difference was found between the number of goblet cells between interventional groups.

Imunohistochemical Analysis: iNOS Assessment

In animals receiving only indomethacin (group A), diffuse and intense iNOS staining was noted in the cytoplasma of mucosal surface and glandular epithelium and also in lamina propria, corresponding to neutrophils infiltrates (Figure 2f). The same immunohistochemical pattern was noted in group B. In groups C and D the iNOS expression was observed in clusters cells of the surface epithelium and a few dispersed cells were positive in the glandular epithelium and lamina propria (Figure 2i). The intensity of iNOS staining in these two groups was faint or moderate.

Graded iNOS immunopositivity in the surface epithelium was not significant different between groups A and B (U = 18, $p > 0.05$, Mann-Whitney test). Significant difference was noted comparing group A with group C (U = 0, $p = 0.002$) and D (U = 0, $p = 0.002$). Statistical analysis for graded iNOS expression in glandular epithelium found no difference between groups A and B (U = 6, $p = 0.061$), but significant difference between groups A and C (U = 0, $p = 0.002$) and A and D (U = 3, $p = 0.013$). Regarding the iNOS immunopositivity corresponding to neutrophils infiltrates in lamina propria the statistical analysis found significant difference between groups A and C (U = 0, $p = 0.002$) and A and D (U = 0, $p = 0.002$).

Figure 2 shows comparative histological and immunohistochemical features for iNOS of the guinea pigs' small intestine between controls, group A and C.

Discussion

NSAID-induced small intestinal damage has become a topic of great interest to gastroenterologists, since capsule endoscopy and balloon enteroscopy are available for the detection of small intestinal lesions. The treatment and prevention of NSAIDs enteropathy are intensively studied in clinical and experimental research.

The oral dose for indomethacin enteropathy is well established in rats, being 10 mg/kg, but the dose for guinea pigs is controversial, since an older report, from 1978 found guinea pigs to be resistant to gastric and intestinal indomethacin ulcerogenesis, even in high doses (50-100 mg/kg). Other researchers were able to induce indomethacin gastric ulceration[20] and to prove apoptotic and necrotic effect of indomethacin on primary cultures of guinea-pig gastric mucosal cells[21,22]. In mice, another species considered resistant to indomethacin, the same lesions were obtained by giving 85 mg/kg of the drug twice daily[23]. Using 30 mg/kg of indomethacin, the same pattern of lesions was observed in guinea pigs small bowel, as previous described in rats[8].

The study shows that rifaximin, an antibiotic that acts locally on enteral microbiota, has the potential to limit necro-inflammatory lesions determined by NSAIDs on guinea pig small bowel. The results seem to conform to pathogenic mechanisms: bacterial translocation after NSAIDs administration release lipopolysaccharides, that enhance iNOS expression[7,8]. Subsequently, NO combines with superoxides to form damaging

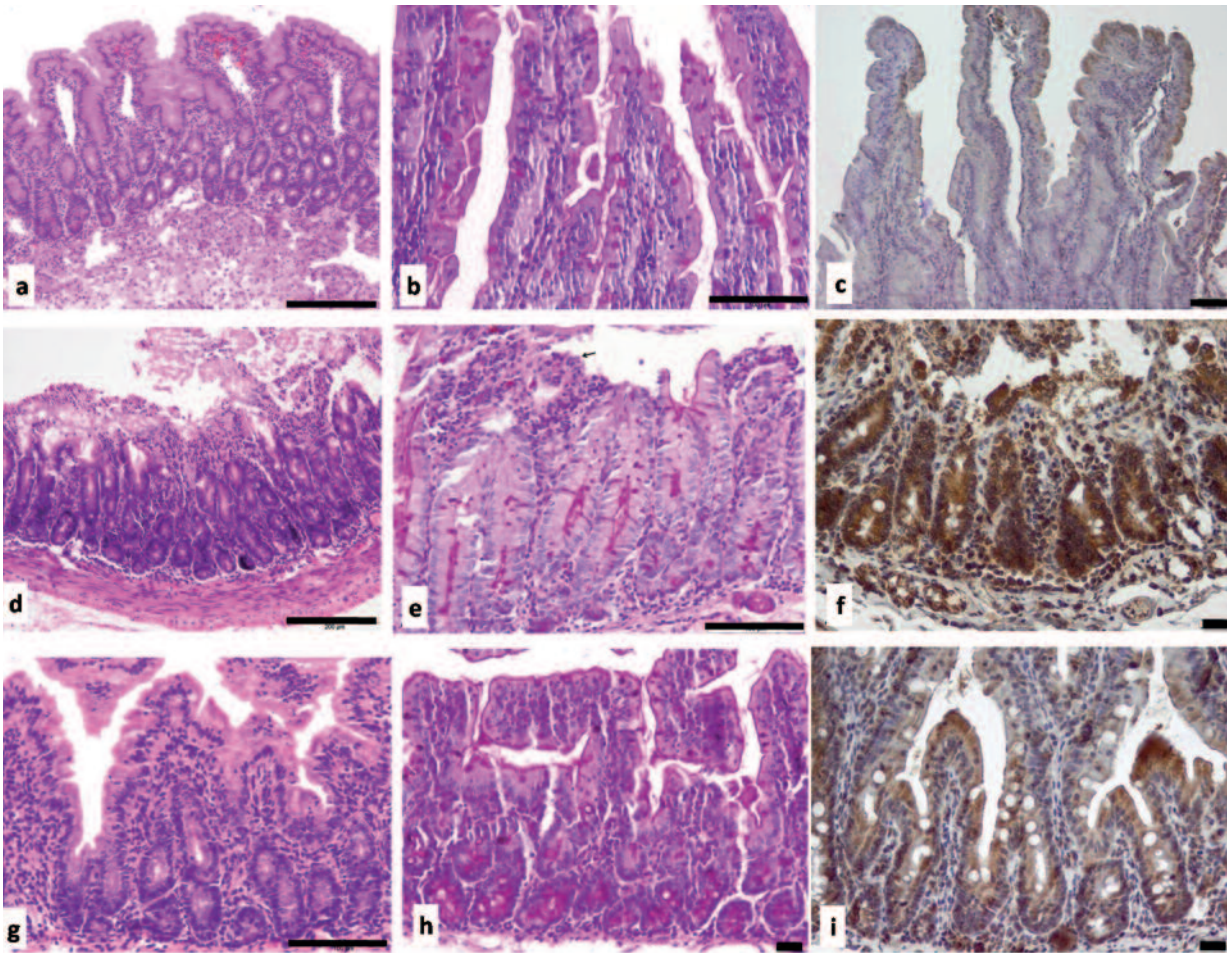

Figure 2. Comparative histological and immunohistochemical features for iNOS of the small intestine in control group (***a-c***), group A ***(d-f)*** and group C ***(g-e).*** Hematoxylin eosin stain showed acute and severe necrosis of necrosis of villous and desquamation of epithelium in group A (***d***, bar = 200 μm) compared with control group (***a***, bar = 200 μm) and the group C (**g**, bar = 100 μm). Histological aspects of the small intestinal mucosa stained by PAS revealed reduced number of goblet cells in group A (***e***, bar = 100 μm) and C (***h***, bar = 20 μm) compared with control group (***b***, bar = 100 μm). ***c*:** Negative immunoexpresion for iNOS was identified in the negative control group. Streptavidin-biotin-peroxidase method. Counterstaining with Mayer's hematoxylin (Bar = 50 μm). **f:** Diffuse and intense iNOS staining in the cytoplasma of surface and glandular epithelium and also in lamina propria, corresponding to neutrophils infiltrates (bar = 20 μm). ***i***: iNOS expression in clusters cells of the surface epithelium and in some dispersed cells in the glandular epithelium and lamina propria in group C. The intensity of iNOS staining is faint (Bar = 20 μm).

species like peroxynitrite, which is cytotoxic, causing intestinal degenerative lesion in 18-48 hours after NSAIDs administration[9-12].

The protective effects of this antibiotic seem to be dose-dependent: 50 mg/kg have no effect on studied parameters; 100 mg/kg significantly reduced degenerative lesions and iNOS expression. Using 200 mg/kg of rifaximin, iNOS expression was significantly decreased, but even fewer degenerative were observed, the difference was not significant compared with indomethacin group.

Rifaximin, a non-absorbable derivative of rifamycin, is an effective antibiotic that acts by inhibiting bacterial ribonucleic acid synthesis. *In vitro* studies found that rifaximin possessed activity against *Lactobacillus* spp., *Staphylococcus* spp., *Enterococcus* spp., *Bacillus cereus, Moraxella catarrhalis, Haemophilus influenzae*[16]. A limit of our study consists in the lack of analysis of the effect of rifaximin on bacterial population, but a previous study documented the ability of this antibiotic to prevent bacterial translocation on experimental colitis model[24]. Previous studies documented a beneficial effect of other antibiotics (ampicile, aztreonam, metronidazol and polymixin B) on NSAIDs enteropathy by decreased the number of Gram-negative bacteria in the small intestinal contents[12,14,25-27]. In clinical gastrointestinal practice,

these antibiotics are not extensively used for this indication, as they have side effects. Rifaximin, a nonabsorbable oral antibiotic that acts locally in the gastrointestinal tract, proved efficacy in travelers' diarrhea[28], bacterial enteritis in children[29], small bowel overgrowth syndrome[30], hepatic encephalopathy[28], irritable bowel syndrome[31] due to its ability to act on pathogenic intestinal flora. As it has minimal systemic side effects, it could be an attractive alternative for NSAIDs enteropathy.

Probiotics are controversial. In one experimental study, probiotics didn't prove their efficacy in preventing NSAIDs gut injuries: *Lactobacillus rhamnosus* exacerbated intestinal ulcerations and Bifidobacterium lactis Bb12 had no beneficial effect[15]. Another study[32] found that *Lactobacillus casei* strain Shirota exhibits a prophylactic effect on indomethacin-induced enteropathy by suppressing the lipopolysaccharide/Toll-like receptor 4 signaling pathway.

Interesting, in our study, using 200 mg/kg of rifaximin, fewer gross and microscopic degenerative lesions were obtained compared with indomethacin group, but without reaching the statistical significance, as in group treated with 100 mg/kg of rifaximin, even iNOS expression was significantly lower. A possible hypothesis might presume that certain doses of rifaximin select bacterial strains that enhanced inflammatory response of NSAIDs by other mechanisms. A previuos study documented that nonpathogenic, Gram-positive bacteria, such as L. rhamnosus, were able to induce chemokine production in human macrophages and to enhance leukocyte chemotaxis[33]. As in our study no analysis was performed on bacterial population, further research is needed to investigate this hypothesis.

It has been proposed that infiltration of the bowel mucosa by neutrophils is an essential stage in NSAIDs injuries[18]. In our study, semi-quantitative assessment of neutrophils infiltrates was not different between interventional groups, suggesting that other mechanisms are also involved. Watanabe et al[27] reported that lipopolysaccharides (LPS)/toll-like receptor 4 (TLR4)/MyD88-dependent signaling pathway on macrophages plays an important role in the activation of an inflammatory cascade induced by NSAIDs.

Eosinophils have also been reported to infiltrate the small bowel epithelium and undergo degranulation after indomethacin administration. As they induce mast cell degranulation, with collagenase release, they might be in part responsible for degeneration of the basal lamina and separation of the epithelium from underlying structures[17]. Our results found significant different results between group A and C regarding eosinophils infiltration grades, data that converged with degenerative lesions degree. Even fewer eosinophils were present into the gut mucosa in group D compared with group A, the results were not statistical significant, reflecting similar comparative degenerative findings. The number of goblet cells within the surface epithelium between interventional groups was similar, lower than in control group, as indomethacin inhibits basal synthesis of mucus, gradually depleting the cells of granules[33].

Conclusions

Rifaximin in a dose of 100 mg/kg proved efficient in preventing small bowel degenerative lesions induced by indomethacin in a guinea pig model, the iNOS activity being significantly decreased. The protective effect seems dose dependent, but further research on bacterial population, NSAIDs and rifaximine interplay is needed in order to explain this observation.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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