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ORIGINAL ARTICLE

Protective Effects of Leflunomide on Intestinal Ischemia-Reperfusion Injury

Leflunomide against intestinal ischemia-reperfusion

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Abstract *Aim* The aim of this study was to investigate the possible protective effects of leflunomide, which has antioxidant and anti-inflammatory properties, against intestinal IR injury in rats. *Materials and Methods* Forty female Wistar albino rats were divided into six groups: control ($n = 5$), drug control ($n = 7$), sham operated ($n = 7$), IR alone ($n = 7$), IR plus vehicle (IR + vehicle, $n = 7$) and IR plus 20 mg/kg leflunomide (IR + Leflunomide, $n = 7$). While rats were pretreated intragastrically with leflunomide (20 mg/kg) and vehicle in three doses prior to the experiment, respectively, in the IR + Leflunomide and IR + vehicle groups, no additional application was done in the IR alone group. Intestines were exteriorized, and the superior mesenteric artery was occluded for 45 min ischemia, and then the clamp was removed for 120 min reperfusion. After the experiment, the intestines were removed for biochemical and histological examinations. Additionally, blood samples were taken for measurements of antioxidant parameters. *Results* The intestinal IR significantly increased the MDA level and MPO activity; however, treatment with leflunomide reversed those findings ($P < 0.05$). The CAT activity of the IR + Leflunomide group was significantly higher than in the IR groups ($P < 0.05$). The SOD activity was increased in the intestinal IR group, and leflunomide treatment reversed that, too ($P < 0.05$). The light microscopic findings showed that IR caused mucosal necrosis and leflunomide treatment reduced the morphological alterations associated with IR ($P < 0.05$). *Conclusion* Intestinal IR injury may be reversed by the anti-inflammatory and antioxidant actions of leflunomide.

Keywords Intestinal ischemia-reperfusion · Leflunomide · Oxidant · Myeloperoxidase · Antioxidants · Antiinflammatory

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Introduction

Intestinal ischemia-reperfusion (IR) injury is a significant problem in a numerous situations, such as acute mesenteric ischemia, abdominal aortic aneurysm surgery, small bowel transplantation, cardiopulmonary bypass, strangulated hernias, neonatal necrotizing entercolitis, hemorrhagic, traumatic or septic shock, or even severe burns [1, 2]. Decreased contractile activity, increased microvascular permeability and dysfunction of the mucosal barrier are all associated with intestinal IR [3]. Despite being essential for the survival of ischemic tissue, reperfusion itself causes additional cellular injury by reoxygenation, which affects the endothelial cells in the inner surface of blood vessels that are vulnerable to the deleterious effects of both hypoxia (ischemia) and reoxygenation (reperfusion). Intestinal ischemia-reperfusion (IR) causes tissue injury in two ways, starting a proinflammatory cascade and

oxidative stress. Intestinal IR causes a complex series of biochemical events, which affect the structure and function of virtually every organelle and subcellular system of the affected cells. In the pathogenesis of intestinal ischemia-reperfusion, various chemicals and cellular mediators play a role, such as the formation and action of reactive oxygen species (ROS) [3–9], inflammatory cytokines, the complement system [3, 11, 12] and neutrophil infiltration [3, 8–12] at the site of damage.

Leflunomide, an isoxazole derivative and a pyrimidine analog as well as a unique immunomodulatory and anti-inflammatory agent, is capable of treating rheumatoid arthritis, allograft and xenograft rejection, systemic lupus erythematosus, Crohn's disease and prostate cancer [13–15]. The studies indicated that its therapeutic effect occurs primarily through its major metabolite, N-(4-tri-urormethylphenyl)-2-cyano-3-hydroxy-crotonic acid amide [16]. The suppression of pro-inflammatory cytokines is the main target action of leflunomide as a function of anti-inflammation and immune regulation [14, 17, 18]. The antioxidative activity of leflunomide occurs especially through the prevention of free radical production, such as from arachidonic acid metabolism [16]. Karaman et al. [15] demonstrated that leflunomide treatment prevented renal ischemia-reperfusion injury in rats. Intestinal tissues are important targets for ischemia-reperfusion injury that is induced by several oxygen radicals and inflammatory processes.

The aim of this study was to determine the protective effect of leflunomide on intestinal IR injury in rats. The effect of leflunomide against intestinal IR was investigated for the antioxidant enzyme activities of catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) as well as the tissue lipid peroxidation index, malondialdehyde (MDA) level and myeloperoxidase (MPO) activity with glutathione (GSH) levels. In addition, the effect of leflunomide against intestinal IR injury was investigated via light microscopic examination with semi-quantitative analysis.

Materials and Methods

Experimentation on animals was carried out following a protocol approved by the ethical animal research committee at Adnan Menderes University. All animals were kept in individual cages in a controlled room (at 25°C, 75% humidity, 12-h light/dark cycle). The rats were fed ad libitum with standard rat food and tap water. Rats were deprived of food overnight before the experiment, but allowed free access to tap water throughout. The study was performed using 40 female Wistar albino rats (weighing 200–250 g) randomly divided into six groups (control group, $n = 5$; all other groups, $n = 7$). The groups were control, drug control, sham operated, IR alone, IR plus vehicle (IR + vehicle) and IR plus 20 mg/kg leflunomide (IR + Lefluomide).

Experimental Design

The rats were anesthetized by intraperitoneal injection of ketamine hydrochloride (75 mg/kg) and xylazine (8 mg/kg). The abdomen was opened with a midline incision. Intestines were exteriorized, and the superior mesenteric artery was dissected carefully and occluded using an atraumatic microvascular clip (ischemia) for 45 min. Then, the clamp was removed, and reperfusion occurred for 120 min. No additional application was done in the IR alone group. Leflunomide (20 mg/kg; IR + Leflunomide group) or vehicle (1% sodium carboxymethylcellulose; IR + vehicle group) was administered intragastrically for three doses with 8-h interval prior to the experiment. Leflunomide (Arava, Aventis Pharmaceuticals) was insoluble in water; therefore, 1% sodium carboxymethyl cellulose was used as a carrier. Sham-operated animals were submitted to the abdominal incision, but not to IR. In the drug control group, the rats were treated with leflunomide without IR. In the plain control group, nothing was done to the rats, and they were just used as controls by being killed at the same time. After reperfusion, all rats were killed, and their intestines, beginning from duodenum to the middle of the colon, were removed. They were separated into two parts. The first part was used for determination of enzyme activity, and the second part was utilized for histopathological analysis. Blood samples were collected via cardiac puncture for plasma antioxidant measurements.

Biochemical Study

Tissues were homogenized in 50 mM phosphate buffer, pH 7.0, at 4°C (w/v = 1/10). A portion of the homogenate was removed for the thiobarbituric acid reactive substances (TBARS), myeloperoxidase (MPO), protein, catalase (CAT), glutathione reductase (GR) and glutathione (GSH) assays; the remainder was centrifuged at 10,000 rpm for 15 min at 0–4°C and the supernatant used for the enzymatic assay of Cu, Zn-SOD activity. The remaining homogenate was centrifuged at 700 g for 10 min, and the supernatant was used for the assay of TBARS, MPO, protein, CAT and GSH. Protein levels were determined using the method of Lowry [19].

Determination of MDA Level

The breakdown product of lipid peroxidation, TBARS, was measured by the method of Buege and Aust [20]. Briefly,

the stock solution contained equal volumes of 15% (w/v) trichloroacetic acid in 0.25 N hydrochloric acid and 0.37% (w/v) 2-thiobarbituric acid in 0.25 N hydrochloric acid. One volume of the sample and two volumes of stock reagent were mixed in a tube and heated for 15 min in a boiling water bath. After cooling on ice, the precipitate was removed by centrifugation at 1,000 g for 15 min, and absorbance was measured at 532 nm against a blank containing all the reagents except the test sample.

Determination of Tissue Cu, Zn-SOD Activity

Cu, Zn-SOD activity was measured using the method of Sun et al. [21]. The final volume of the reaction systems is 3.0 ml and contains, per liter, 0.1 mmol of xanthine, 0.1 mmol of EDTA, 50 mg of bovine serum albumin, 25 μmol of NBT, 9.9 nmol of xanthine oxidase and 40 mmol of Na_2CO_3 (pH 10.2). The production of formazan is determined at 560 nm and 25°C. One unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. Data were expressed as U/mg protein.

Determination of CAT Activity

The CAT activity was measured by the method of Aebi [22]. The principle of the assay is based on the determination of the rate constant (s^{-1},k) of hydrogen peroxide decomposition by catalase enzyme. The rate constant was calculated from the following formula: $k = (2{,}3/\Delta t)\log(A1/A2)$. In this formula, $A1$ and $A2$ are the absorbance values of hydrogen peroxide at $t1$ (0th s) and $t2$ (15th s). Data were changed to k/s/mg protein after k values were determined.

Determination of GR Activity

GR activity in tissue samples was determined using the method of Carlberg and Mannervik [23], modified by Husain et al. [24]. Briefly, 50 μl of NADPH (2 mM) in 10 mM Tris buffer (pH 7.0) was added to a cuvette containing 50 μl of GSSG (20 mM) and 850 μl of phosphate buffer (0.5 M, pH 7.0, 0.1 mM EDTA) at 37°C. Then 50 μl of tissue extract was added to NADPH-GSSG-buffered solution and measured at 340 nm for 3 min. Data are expressed as μmol/min/mg protein. One unit of activity is equal to mM of NADPH oxidized min^{-1} mg $protein^{-1}$.

Determination of GSH Level

The glutathione (GSH) level was estimated by monitoring the reduction of DTNB (dithiobis-2-nitrobenzoic acid), forming a yellow-colored anion at 412 nm [25].

Determination of Myeloperoxidase (MPO) Activity

We used the method described by Suzuki et al. [26]. Briefly, the homogenate was centrifuged at 15,000 g, and the pellet was resuspended in an equal volume of a detergent-containing buffer (50 mM potassium phosphate, pH 6, 0.5% hexadecyltrimethylammonium bromide, 10 mmol/l EDTA). A standard reaction mixture contained 1.6 mmol/l tetramethyl benzidine. The reaction was started by the addition of H_2O_2 to a final concentration of 0.88 mmol/l (0.003%). The rate of the myeloperoxidase-catalyzed oxidation of tetramethyl benzidine was followed by recording the absorbance increase at 655 nm. Considering the initial linear phase of the reaction, we calculated the absorbency change per minute, and the enzyme activity was expressed as the amount of enzyme producing one absorbency change per minute under assay conditions. Data were expressed as U/g wet tissue.

Histological Procedure and Assessment

The intestinal specimens were fixed in 10% neutral formaldehyde solution at 4°C for 24 h. Afterwards, tissues underwent routine histological procedure (dehydration in ethanol and clearing in xylene) and were embedded in paraffin blocks. Tissues in paraffin blocks were randomly cut in 5-μm sections by a microtome (Leica RM 2135). These sections were stained with hematoxylin-eosin and mounted with entellan. Screen shots were taken with an Olympus DP20 Digital camera attached to an Olympus BX51 microscope.

A histological grading scale was used to determine the extent of ischemia-reperfusion according to microscopic criteria described by Chiu et al. [27] as follows: grade 0: normal mucosa; grade 1: subepithelial edema, partial separation of apical cells; grade 2: epithelial cell slough from the tips of villi; grade 3: progression of slough to the base of villi; grade 4: partial mucosal necrosis of the lamina propria; grade 5: total mucosal necrosis. Microscopic scoring of tissue samples was performed by an observer unaware of the treatment groups.

Statistical Analysis

A computer program (SPSS 14.0) was used for data analysis. Because all groups showed normal distribution with the Kolmogrov-Simirnov test, a parametric test was performed for analyzing data. One-way ANOVA with post hoc test LSD was performed to analyze differences among groups. The results were expressed as mean ± SEM, and $P < 0.05$ was regarded as significant.

Table 1 Antioxidants in all groups

Group	CAT (k/s/mg protein)	GSH (mg/g protein)	SOD (U/mg protein)	GR (μmoles of NADPH oxidized/min/mg protein)
Control	0.532 ± 0.025	57.38 ± 3.65	4.297 ± 0.458[b]	22.41 ± 0.72[c]
Drug control	0.685 ± 0.092	64.38 ± 1.69	6.436 ± 0.598[b]	19.46 ± 1.44
Sham	0.640 ± 0.123	52.81 ± 2.78	5.511 ± 0.341[b]	20.89 ± 1.32
IR	0.323 ± 0.057[c]	59.56 ± 4.98	13.716 ± 1.754[a,c]	19.44 ± 1.58
IR + vehicle	0.487 ± 0.072[c]	54.87 ± 5.69	10.049 ± 1.219[a]	18.20 ± 1.85
IR + Leflunomide	0.872 ± 0.254[b]	51.64 ± 2.97	9.699 ± 0.681[b]	17.56 ± 0.87

[a] $P < 0.05$ versus all control groups

[b] $P < 0.05$ versus IR

[c] $P < 0.05$ versus IR + Leflunomide. *IR* ischemia-reperfusion

Results

Biochemical Parameters

The CAT activities of the IR and IR + vehicle groups were significantly lower in comparison with the IR + Leflunomide group ($P < 0.05$) (Table 1). However, there was no significant change in the GSH level in the groups. There were high SOD acitivity levels in both the IR and IR + vehicle groups in comparison with control groups and the IR + Leflunomide group ($P < 0.05$). The IR + Leflunomide group had lower GR than the control group ($P < 0.05$).

The MDA level of the IR and IR + vehicle groups (229.44 ± 8.24 and 202.69 ± 19.50 nmol/g wet tissue) were significantly increased compared to other groups (141.54 ± 2.73, 163.00 ± 9.22, 154.90 ± 7.34 and 168.66 ± 11.52 nmol/g wet tissue for the control, drug control, sham and IR + Leflunomide groups, respectively) ($P < 0.05$) (Fig. 1a). Leflunomide treatment significantly lowered the lipid

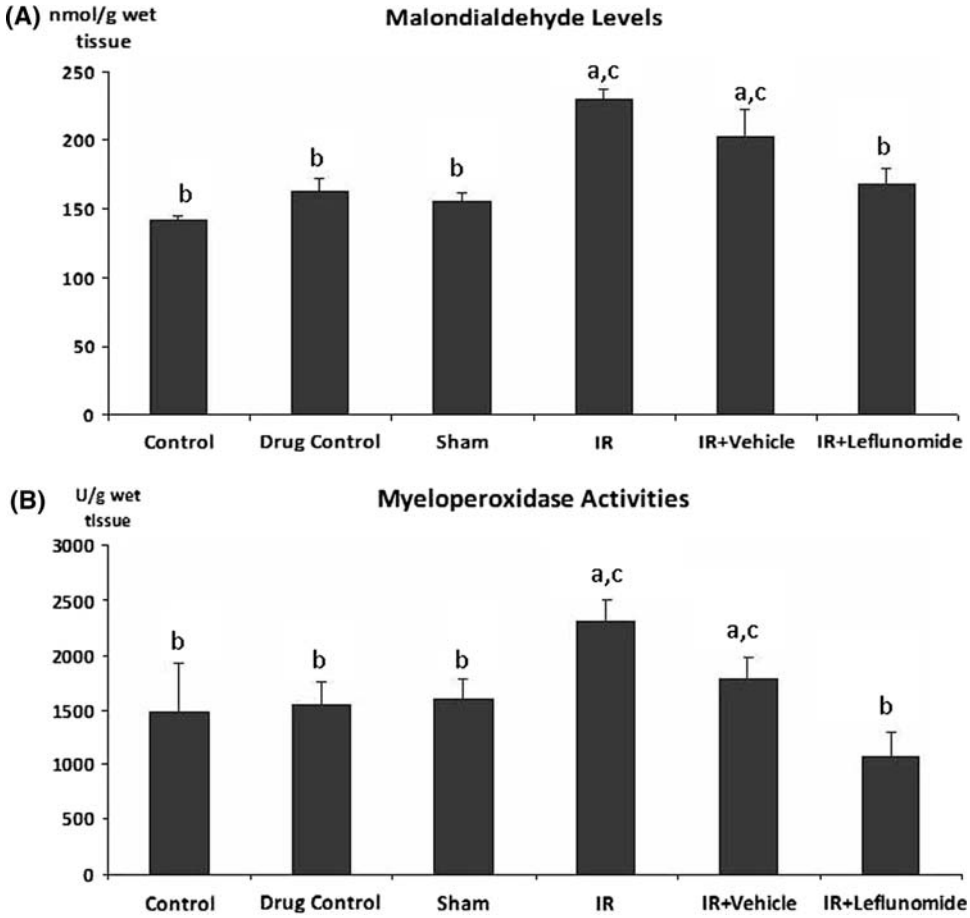

Fig. 1 Tissue malondialdehyde levels and myeloperoxidase enzyme activities in all groups. IR: ischemia-reperfusion. [a]$P < 0.05$ versus all control groups, [b]$P < 0.05$ versus IR and [c]$P < 0.05$ versus IR + Leflunomide groups

Table 2 The mean scores of histological grading in all groups

Group	Mean ± SD
Control	0.20 ± 0.45[b]
Drug control	0.43 ± 0.79[b]
Sham	0.17 ± 0.41[b]
IR	4.67 ± 0.52[a,c]
IR + vehicle	3.57 ± 1.51[a,c]
IR + Leflunomide	1.43 ± 1.13[b]

[a] $P < 0.05$ versus all control groups

[b] $P < 0.05$ versus IR

[c] $P < 0.05$ versus IR + Leflunomide. *IR* ischemia-reperfusion

peroxidation ($P < 0.05$). There were higher MPO activities in the IR and IR + vehicle groups (2,310.44 ± 188.55 and 1,776.72 ± 203.91 U/g wet tissue) in comparison with the control groups (1,475.76 ± 441.83, 1,543.08 ± 204.54 and 1,595.88 ± 176.48 U/g wet tissue for the control, drug control and sham groups, respectively) ($P < 0.05$) (Fig. 1b). The MPO activity of the IR + Leflunomide group (1,074.48 ± 229.86 U/g wet tissue) was significantly decreased compared to the other IR groups (2,310.44 ± 188.55 and 1,776.72 ± 203.91 U/g wet tissue) ($P < 0.05$).

Histological Results

The light microscopic findings showed that there was normal nonischemic intestinal tissue and mucosa in the control, drug control and sham groups. In histological findings, the rats in the control group (0.20 ± 0.447), in the drug control group (0.43 ± 0.79) and in the sham group (0.17 ± 0.41) had essentially normal histological architecture (Table 2, Fig. 2a, b, c). The microscopic results of the IR and IR + vehicle groups were representative of IR injury. There were subepithelial edema, partial separation of apical cells, epithelial cell slough from the tips of villi, progression of slough to the base of villi, and partial and total mucosal necrosis of the lamina propria in the light microscopic findings. There was no difference among the microscopic damage scores of the IR group (4.67 ± 0.52) and the vehicle-treated IR group (3.57 ± 1.51, $P > 0.05$) (Fig. 2d and e). Treatment with leflunomide 20 mg/kg (1.43 ± 1.13) caused a significant reduction (Fig. 2f) in the microscopic damage score compared to the IR group ($P < 0.05$) and vehicle-treated IR group ($P < 0.05$).

Discussion

Intestinal IR induces certain mechanisms that cause tissue injury in intestinal tissue. The laboratory evidence in our study indicated that IR increased lipid peroxidation and MPO activity as well as necrotic changes in mucosa of the intestine. Leflunomide successfully reversed those changes and decreased the MDA level and MPO activity. The treatment with leflunomide protected intestinal mucosal tissues from necrosis. Also, semi-quantitative analysis of light microscopic findings showed that leflunomide treatment significantly decreased grading from 4.67 ± 0.52 to 1.43 ± 1.13 in our study.

Oxygen radicals are produced in different manners in stressful conditions, and one of the ways is arachidonic acid metabolism via cyclo-oxygenase (COX) enzyme activities. Both COX-1 and COX-2 are pharmacological targets of the non-steroidal anti-inflammatory drugs (NSAIDs). However, inhibition of COX-2 activity may well mediate the therapeutic benefits of these drugs, while inhibition of COX-1 may underlie their harmful side effects [28]. Hamilton et al. demonstrated that leflunomide, probably via its active metabolite A771726, *N*-(4-trifluoromethylphenyl-2,2-cyano-3-hydroxycrotoamide), can directly inhibit the activity of cyclo-oxygenase both in vitro and in vivo [16]. Those findings suggested that leflunomide may also decrease oxidative stress via this manner. Our findings indicate lowered oxidative stress with leflunomide treatment in oxidative stress-induced conditions, similar to the results of Karaman et al. [29].

Intestinal IR injury similar to other IR models causes activation and migration of neutrophils to the inflamed area; they induce tissue injury through the production of MPO [30]. Acute inflammatory responses to IR injury are characterized by an inflammatory process with cytokine and ROS production [31]. Activation of inflammatory cells and the subsequent release of cytokines and growth factors probably link the inflammatory processes and ROS; this is known to cause tissue injury. In fact, recent studies showed that together with inflammatory processes, free radicals, oxidative stress and lipid peroxidation are frequently associated with intestinal injury produced by IR. Many studies reported that anti-inflammatory and antioxidant agents may have beneficial effects on persistent inflammation and oxidative damage in intestinal IR [4, 7, 9]. Leflunomide is also one of the possible agents that can affect both the inflammatory process and free radical production together with its anti-inflammatory and antioxidant effects. Previous studies demonstrated that leflunomide had strong anti-inflammatory and immunoregulator effects and had therapeutic actions on experimental acute and chronic disease [32–34].

It was obvious in the present study that leflunomide induced antioxidant mechanisms in the injured area. The increased CAT activity was a good indication for the antioxidant effect in the leflunomide-treated group. IR also induced superoxide anion production in intestinal tissue,

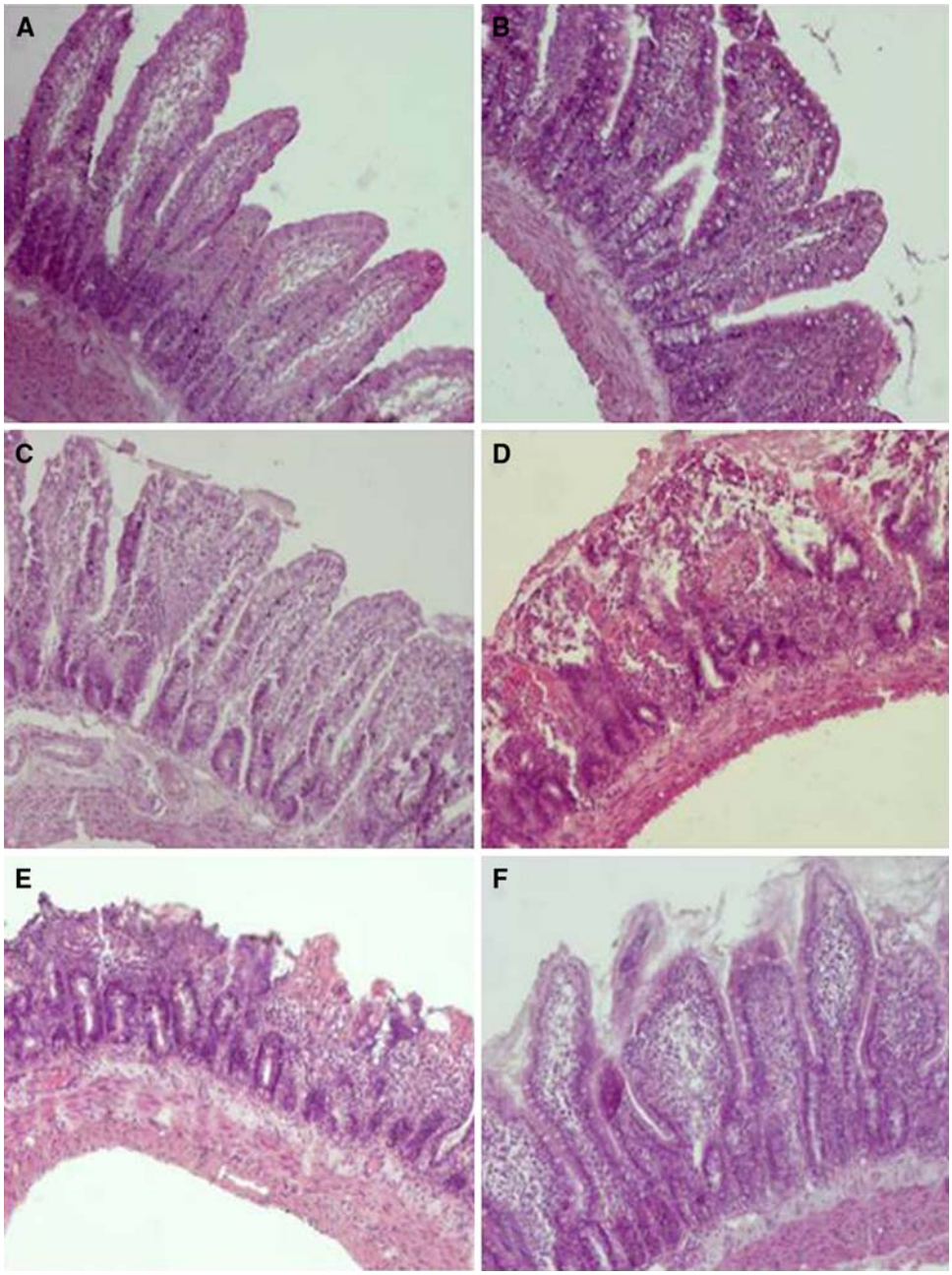

Fig. 2 Representative histological photomicrographs of rat intestinal tissue and the effect of leflunomide on intestinal ischemia-reperfusion injury. **a** Normal nonischemic intestinal tissue in the control group. **b** Histological appearance of nonischemic intestinal mucosa in the drug control group. **c** Nonischemic intestinal tissue in the sham group. **d** Histological appearance of rat intestinal mucosa after ischemia-reperfusion (IR). **e** Ischemia-reperfusion treated with vehicle. **f** IR + Leflunomide (20 mg/kg). Original magnification was 10× (a–f) on hematoxylin and eosin (H&E)-stained preparations. The histological grading scale used to determine the extent of ischemia-reperfusion was as follows: grade 0: normal mucosa; grade 1: subepithelial edema; partial separation of apical cells; grade 2: epithelial cell slough from the tips of villi; grade 3: progression of slough to the base of villi; grade 4: partial mucosal necrosis of the lamina propria; grade 5: total mucosal necrosis

and then high SOD activity tried to convert this molecule to hydrogen peroxide in the present experiment. High SOD activity was an expected result in the IR group as previously reported [35], because the induction of ROS production may also stimulate SOD enzyme activity. The leflunomide treatment also reversed this high SOD activity. Ozturk et al. [34] demonstrated that leflunomide treatment against a sepsis model in rats reversed the MDA level and MPO activities in lung tissue. They found that sepsis caused the decrease in antioxidant enzyme activities, including CAT, GSH-Px and SOD. SOD is a antioxidant, but has different results than the other two antioxidants. CAT and GSH-Px are two major endogen antioxidant enzymes that detoxify hydrogen peroxide to water. However, SOD catalyzes the specific reaction that yields hydrogen peroxide, one of the major ROS [34]. Overexpression of SOD results in increased dismutation of superoxide anion to hydrogen peroxide. NF-kB is a multiprotein complex that is known to activate a great number of genes involved in the early cellular defense reactions of higher organisms [36]. The activation of NF-kB is triggered by a great variety of agents, including the cytokines interleukin-1 and tumor necrosis factor. SOD-overexpressing cells showed a hyperinduced NF-kB activation, possibly resulting in an enhanced conversion of superoxide anion to hydrogen peroxide [37]. The intestinal IR may

possibly induce this pathway. Our results also show that leflunomide may block this pathway, which includes NF-kB and SOD overexpression.

In conclusion, the present study has demonstrated that inflammatory and oxidative stress induced by intestinal IR could be reversed by leflunomide treatment in rats. The treatment with leflunomide acts in two major ways: anti-inflammatory action and prevention of oxygen radical production. However, it is still not clear which signal pathways are potent against intestinal IR injury in leflunomide treatment.

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