ANZ J. Surg. 2008; **78**: 476–481 doi: 10.1111/j.1445-2197.2008.04358.x

ORIGINAL ARTICLE

EFFECT OF BENCYCLANE FUMARATE ON INTESTINAL ISCHAEMIA REPERFUSION INJURY

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Background: Post-ischaemic intestinal tissue damage appears to be due to the formation of oxygen radicals. Free radical-initiated lipid peroxidation following intestinal ischaemia/reperfusion (I/R) may disrupt mucosal integrity. Indirectly, the radicals trigger the accumulation of neutrophils within the affected tissue, initiating inflammatory processes that lead to severe mucosal lesions. We have investigated the protective effect of bencyclane fumarate, a vasodilating Ca^{2+} channel blocker, which has been used for the treatment of peripheral arterial occlusive diseases, on intestinal ischaemia reperfusion (IR) injury in rats.

Methods: Forty-eight Wistar albino rats were divided into three groups: a sham-operated group (no IR injury, $n = 16$), an ischaemic control group (IR, $n = 16$), and BF-treated group (pretreatment 5 mg/kg bencyclane fumarate + IR, $n = 16$). A marker for lipid peroxidation, namely malondialdehyde; free radical scavengers, glutathione peroxidase, catalase and superoxide dismutase levels; an index of polymorphonuclear neutrophils, myeloperoxidase activity and mucosal damage were investigated.

Results: Malondialdehyde levels, myeloperoxidase activity and the severity of mucosal damage were decreased in the BF group. In addition, in the BF group, glutathione peroxidase, catalase and superoxide dismutase levels were higher compared with the IR group.

Conclusion: The pretreatment of rats with bencyclane fumarate before intestinal ischaemia attenuates the mucosal damage in intestinal IR injury, probably by altering lipid peroxidation, neutrophil accumulation and antioxidant activity.

Key words: bencyclane fumarate, free radical scavenger, intestinal ischaemia, reactive oxygen species, reperfusion injury.

Abbreviations: BF, bencyclane fumarate; GSH-Px, glutathione peroxidase; IR, ischaemic reperfusion; MDA, malondialdehyde; MPO, myeloperoxidase; SOD, superoxide dismutase; TBA, thiobarbituric acid.

INTRODUCTION

Intestinal ischaemia is more frequently the result of non-occlusive processes, like those in situations of low mesenteric flow, which occurs in cardiac insufficiency, sepsis and the administration of α-adrenergic agents or digitalics.[1] A decrease in the blood flow in the intestines results in ischaemic damage. When blood flow is restored a more pronounced damage, the so-called reperfusion injury occurs.[2–4] Regardless of the cause, intestinal ischaemia is a serious and growing clinical problem with an unacceptable mortality rate of more than 60%.[5]

Several mechanisms seem to be instrumental in the development of ischaemic reperfusion (IR)-induced lesions of the gut. First, oxygen radicals, generated by the hypoxantine–xantine oxidase system, are the 'molecular triggers'. Second, activation of phospholipase-A_2 constitutes the 'enzymatic trigger'. Both these pathways lead to the accumulation and activation of neutrophils in intestinal tissue.[3,6] Sources of radicals in the gastrointestinal tract include mucosal xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase found in the resident phagocytotic leucocytes (macrophages, neutrophils and eosinophils) of the lamina propria.[7]

Increased myeloperoxidase (MPO) activity is indicative of neutrophil activation because MPO is found almost exclusively within neutrophils.[8]

Oxygen radical-initiated lipid peroxidation (LP) and protein damage may contribute to the impaired cellular function and necrosis associated with reperfusion.[9] Malondialdehyde (MDA) is one of the final non-specific products of LP. MDA can be dosed in both tissue and blood and its concentration is directly proportional to the cell damage caused by free radicals.[10]

During reperfusion, the oxygen molecule (O_2) is reintroduced into the tissue and reacts with hypoxanthine and xanthine oxidase to produce the superoxide anion (O_2-), which is transformed into hydrogen peroxide (H_2O_2) by the action of the enzyme superoxide dismutase (SOD) and through a reaction with catalase or glutathione peroxidase (GSH-Px), H_2O_2 is further transformed into H_2O.[11] Antioxidant enzymes, including GSH-Px, SOD and catalase (CAT) protect tissues from reperfusion injury by destroying reactive oxygen species.[12] These measurements can be carried out on tissue, blood and other fluids.[11]

Platelets, similar to leucocytes, roll and firmly adhere to the endothelial surface of microvessels during post-ischaemic reperfusion. Local accumulation of platelets was observed reaching a maximum within minutes after onset of reperfusion, indicating that platelets are among the first cells recruited to the site of injury.[13] Platelet endothelial adhesion influences leucocyte

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Accepted for publication 17 April 2007.

trafficking because platelet depletion decreases IR-induced leucocyte emigration.[14]

Another factor involved in the lesions of IR is serotonin (5-HT). Serotonin is a bioactive amine, which acts, in several physiological phenomena like neurotransmission, intestinal movement, platelet activation and vasoconstriction. Teramoto *et al.* showed an increase in the plasmatic levels of 5-HT after mesenteric IR in rats, probably due to an increase in the release of 5-HT by the injured intestine. According to the authors, 5-HT together with other bioactive substances could carry out an important function in the pathogenesis of intestinal IR.[15]

There are several endogenous mechanisms to inhibit IR lesions and many drugs have shown protective effects. These agents act through various pathways: elimination of free radicals; inhibition of free radicals production; neutrophilic inhibition; blocking phospholipase-A_2, cycloxygenase and lypoxygenase; platelet aggregation factor inhibition; production of blockers for chemotaxic agents and monoclonal antibodies to counter adhesion molecules.[16]

Bencyclane fumarate (BF), with a formula of *N*-[3-(1-benzyl-cycloheptyloxy)-propyl]-*N*,dimethyl-ammonium hydrogen fumarate (Angiodel®, Organon, Turkey) is a non-selective calcium entry blocker that acts on calcium and fast sodium channels.[17] It has been used as a vasodilator drug for the treatment of peripheral arterial occlusive disease,[18] has been shown to stabilize human erythrocytes and in doses of 0.5–15 mg/kg inhibited platelet aggregation[19,20] BF reduces intracellular accumulation of sodium and calcium and shows striking inhibitory effect both on platelet aggregation induced by ADP, adrenaline or collagen and on platelet adhesiveness to glass or collagen.[21] Egawa *et al.* found that bencyclane produced an anti-serotonergic action in mesenteric strips.[22]

To the best our knowledge, there are no previous studies that have examined the effect of BF on IR injury of the intestine. Therefore, we have investigated the protective effect of BF, a vasodilating Ca^{2+} channel blocker, which has been used for the treatment of peripheral arterial occlusive diseases.[18]

MATERIALS AND METHODS

Animal model

Forty-eight Wistar albino rats weighting between 220 and 280 g were included for this study. Rats were standardized conditions for food, water, light and temperature. All animals were fed standard rat chow and water ad libitum and were given only water for 14 h before surgery. The ethics committee of Suleyman Demirel University Medical School approved the experimental procedures in this study. Animals were obtained from the breeding unit of Suleyman Demirel University, School of Medicine and 'All of the guiding principles in the care and use of laboratory animals' were strictly adhered throughout the entire study.

Experimental design

The animals were divided into three groups of 16 rats as follows:

- Group 1, sham-operated group (S): these animals underwent laparotomy and superior mesenteric artery (SMA) dissection without IR injury.
- Group 2, ischaemic control group (IR): these animals underwent laparotomy plus 60 min of ischaemia and 60 min of reperfusion.
- Group 3, IR and BF treatment group (IR + BF): these animals received 5 mg/kg BF (Angiodel) i.v. 15 min before the induction of ischaemia

Technique of ischaemia and reperfusion

After an overnight fast, the rats were anaesthetized using i.m. injections of 50 mg/kg ketamine hydrochloride (Ketalar; Parke-Davis, Eczacibasi, Istanbul, Turkey) and 10 mg/kg xylazine (Rompun; Bayer, Leverkusen, Germany). The right internal jugular vein was catheterized using 24-G catheters. Throughout the study, 10 mL/kg Ringer's lactate was infused from this catheter to all groups. After shaving the abdomen and preparation with 10% povidone–iodine solution, a midline laparotomy was carried out. The small bowel was exteriorized and the ligament of Treitz cut to expose the SMA. Collateral arcades from the right colic artery and the jejunal arteries proximal to the site of occlusion were ligated to avoid the variable contribution of collateral circulation to the distal ileum as described by Megison *et al.*[23] The SMA was dissected. An atraumatic microvascular clamp (Aesculap BH 21, Tuttlingen, Germany) was then placed across the SMA just after its origin from the aorta, avoiding occlusion of the superior mesenteric vein. Mesenteric ischaemia was confirmed when the mesenteric pulsations were lost and intestines become pale. The bowel was returned to the abdominal cavity and the incision was closed with interrupted atraumatic 4/0 silk sutures. After 60 min of ischaemia, a re-laparotomy was carried out and the microvascular clamp on the SMA was removed for 60 min of reperfusion. Mesenteric reperfusion was confirmed with the restoration of pulsation and colour. The bowel was then returned to the abdominal cavity once more and the incision was closed with 4/0 silk sutures. The bowel was left in the abdomen during IR. After completing the experimental procedure the animals were killed.

Thirty centimetres of small bowel was resected from 5 cm proximal to the caecum. A 5-cm ileal segment from the distal end of the resected small bowel was fixed in 10% formaldehyde for histopathological examination. The rest of the resection material was stored at the −78°C until tissue MDA, MPO, GSH-Px, catalase and SOD assays.

Histopathological evaluation

The tissue specimens were fixed in 10% formaldehyde. Samples of intestine were embedded in paraffin, sectioned and stained with haematoxylin and eosin. They were then submitted for histopathological evaluation carried out in a blinded fashion by the pathologist (N. K.). Mucosal lesions were graded on a scale from 0 to 5 as described by Chiu *et al.*[24]

- Grade 0, normal mucosal villi
- Grade 1, development of subepithelial Gruenhagen's space, usually at the apex of the villus; often with capillary congestion
- Grade 2, extension of the subepithelial space with moderate lifting of epithelial layer from the lamina propria
- Grade 3, massive epithelial lifting down the sides of villi. A few tips may be denuded
- Grade 4, denuded villi with lamina propria and dilated capillaries exposed. Increased cellularity of lamina propria may be noted
- Grade 5, Digestion and disintegration of lamina propria; haemorrhage and ulceration

Biochemical determination

For biochemical analyses the intestines of rats were washed with physiological saline. They were then homogenized for 3 min (Ultra-Turrax T25, Janke & Kunkel, Staufen, Germany) in cold phosphate buffer to provide a 10% homogenate. These homogenates were centrifuged at 6000 *g* for 10 min to obtain supernatants. The levels of protein and MDA were determined in the supernatants. Protein content of homogenates was determined by the Lowry method.[25]

Malondialdehyde, as a marker for LP, was determined by the double-heating method of Draper and Hadley.[26] The principle of the method was spectrophotometric measurement of the colour produced during the reaction to thiobarbituric acid (TBA) with MDA. For this purpose, 2.5 ml of 100 g/l trichloroacetic acid solution was added to 0.5 mL erythrocytes in a centrifuge tube and placed in a boiling water bath for 15 min. After cooling in tap water, the mixture was centrifuged at 1000 *g* for 10 min, and 2 mL of the supernatant was added to 1 mL of 6.7 g/L TBA solution in a test tube and placed in a boiling water bath for 15 min. The solution was then cooled in tap water and its absorbance was measured using a Shimadzu UV-1601 spectrophotometer (Shimadzu Corp., Tokyo, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA–TBA complex 1.56×10^5/cm per mole and expressed in nmol/gHb.

Tissue-associated MPO activity in intestinal mucosa was determined using the method of Grisham *et al.*[6] Approximately 300-mg samples of intestinal mucosa were homogenized in 5 mL of ice-cold 0.02 mol ethylenediaminetetraacetic acid (pH 4.7) for 60 s. Five millilitres of mucosal homogenate was centrifuged at 20 000 rpm for 15 min at 4°C to pellet the insoluble cellular debris. The supernatant, which contained less than 5% of total MPO activity, was discarded. The pellet was then rehomogenized in an equivalent volume of 0.05 mol/L potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. MPO activity was assessed by measuring the H_2O_2-dependent oxidation of *O*-dianisidin. One unit of enzyme activity was defined as amount of MPO present that caused a change in absorbance of 1.0/min at 410 nm and 37°C.[27] The determination of GSH-Px activity was based on the method of Paglia and Valentine.[28] The principle of the method was as follows: GSH-Px catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance of NADPH was measured at 340 nm.

Catalase activity was measured according to the method of Aebi.[29] 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 with following formula:

$$k = (2.3/\Delta t)(a/b)\log(A_1/A_2).$$

In this formula, A_1 and A_2 are the absorbance values of hydrogen peroxide at t_1 (0th second) and t_2 (15th second) times, 'a' is the dilution factor and 'b' is the haemoglobin content of erythrocytes.

The measurement of SOD was based on the principle in which xanthine reacts with xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazon dye. The SOD activity is then measured by the degree of inhibition of this reaction.[30]

An autoanalyser, Abbott Aeroset (Abbott, Illinois, USA), was used to determine the activities of SOD and GSH-Px and the spectrophotometer, Shimadzu UV-1601 (Japan), was used to estimate the levels of MDA.

Data analysis

All statistical analysis was carried out by using SPSS for Windows statistical software (version 13.0, SPSS, Chicago, IL, USA). The results were evaluated using a one-way ANOVA and differences among the groups were analysed with the Tukey honestly significantly different test. For the latter, differences among the groups were evaluated with the Mann–Whitney *U*-test. All values were expressed as mean ± standard deviation. Differences were considered significant when the *P*-value was less than 0.05.

RESULTS

Histopathology

In the sham group, the results of histopathological examinations of the small intestinal epithelium and villi were normal. The specimens from this group were classified as grade 0 according to the Chiu classification. The histopathological injury in the BF group (2.3 ± 1.1) was significantly lower than the IR group (4.6 ± 0.5) ($P < 0.001$). In BF group, four specimens were detected as grade 4. However, PMN infiltration was not seen (Figs 1,2).

Malondialdehyde

The mean MDA levels were 11.6 ± 3.6, 36.7 ± 18.8, and 16.3 ± 5.7 mmol/g tissue in the sham, IR and BF groups, respectively (Fig. 3). The difference between the sham and BF groups was not significant ($P = 0.482$), but the MDA levels in the IR group were significantly increased in comparison to those in the sham and BF groups ($P < 0.001$).

Myeloperoxidase

The mean MPO levels were 29.7 ± 2.3, 45.6 ± 3.7, and 37.4 ± 7.2 U/g tissue in the sham, IR, and BF groups, respectively (Fig. 4). IR produced a significant (1.5-fold) increase in MPO activity as compared with the sham-operated group ($P < 0.001$). The difference between the sham and BF groups was also significant ($P < 0.001$). However, BF treatment significantly decreased MPO activity in comparison to the IR group ($P < 0.001$).

Glutathione peroxidase

The mean GSH-Px levels were 43.4 ± 23.4, 26.1 ± 5.6, and 42.1 ± 8.0 U/g tissue in the sham, IR and BF groups respectively. The GSH-Px levels of the IR group were found to be decreased by approximately 66% as compared with the sham-operated group and this difference was significant ($P = 0.005$). In the BF group, GSH-Px levels returned to the sham values ($P = 0.01$). The difference between the sham and BF groups was not significant ($P = 0.963$).

Catalase

The mean CAT levels were 3.8 ± 4.0, 1.0 ± 0.6, and 2.2 ± 1.8 U/mg tissue in the sham, IR and BF groups, respectively. The difference between the sham and BF groups was not significant

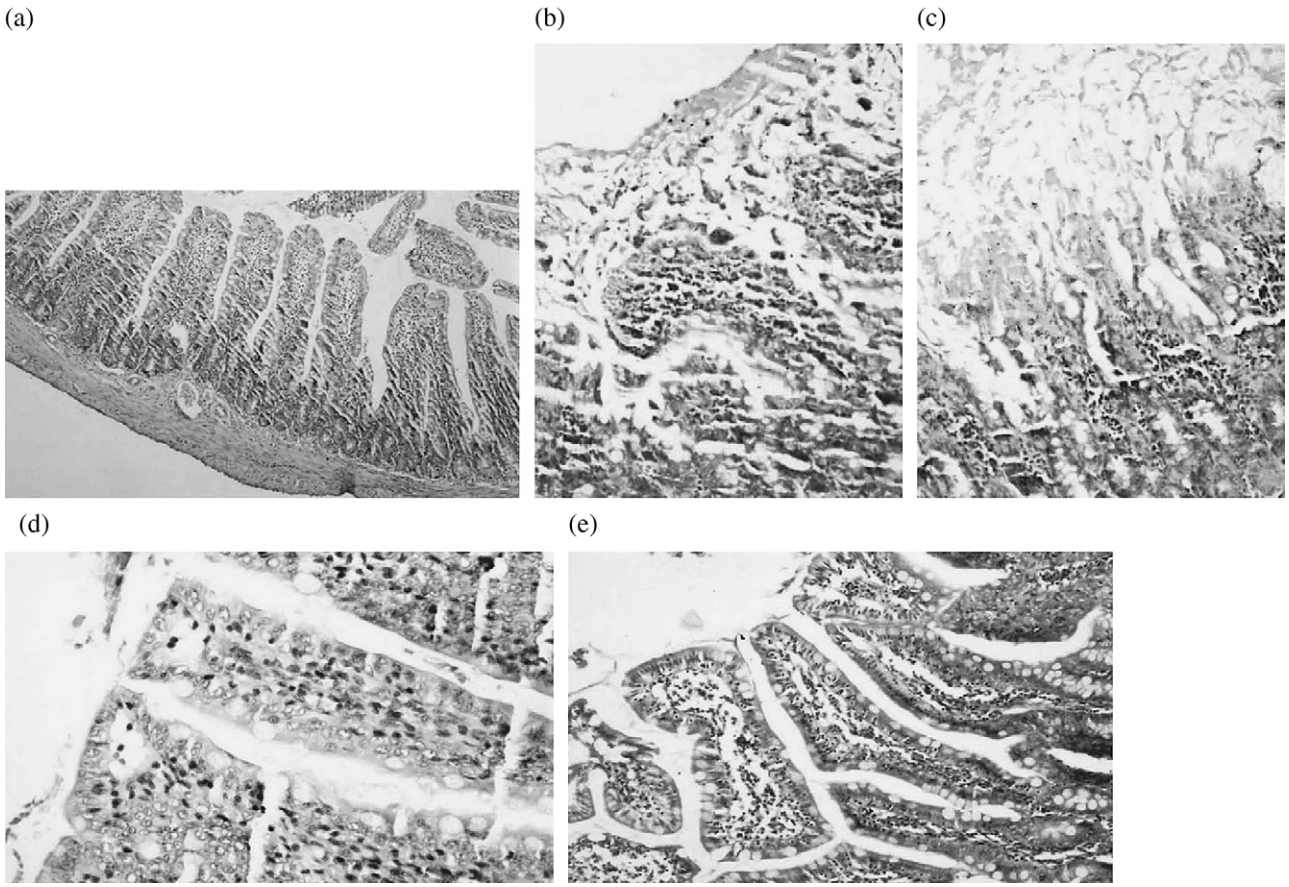

Fig. 1. The microscopic appearance of the mucosal changes. (a) Normal intestinal epithelium and villi (grade 0), (b) severe mucosal damage (grade 4), and (c) severe mucosal damage (grade 5) in the ischaemic reperfusion (IR) group, (d) mild mucosal damage (grade 1), and (e) moderate mucosal damage (grade 2) in the bencyclane fumarate group after IR injury (haematoxylin and eosin ×200).

($P = 0.177$), but the CAT levels in the IR group were significantly decreased in comparison to those in the sham and BF groups ($P = 0.01$).

Superoxide dismutase

The mean SOD levels were 14.9 ± 13.3, 5.9 ± 5.4, and 13.7 ± 8.1 U/mg tissue in the sham, IR and BF groups, respectively. The SOD levels of the IR group were found to be decreased by approximately 250% as compared with the sham-operated group, and this difference was significant ($P = 0.01$). In the BF group, mean SOD levels returned to the control values ($P = 0.025$). The difference between the sham and BF groups was not significant ($P = 0.933$).

DISCUSSION

This study showed that 60 min of reperfusion following 60 min of ischaemia of the rat intestine caused severe mucosal damage. Histopathological damage that occurs after intestinal IR is characterized by shortening of the villus length, loss of villus epithelium, necrosis and invasion of inflammatory cells.[2–4,24,31,32]

The present study clearly showed that BF improved the small intestinal tissue damage against IR injury possibly through positive effects on free radical scavengers and inhibition of neutrophil accumulation.

The MPO levels, an index of tissue-associated neutrophil accumulation in ileal tissue of IR group rats were higher than in the sham-group animals. Our results are in accordance with those in the published work.[2,5,9,12,27,31] BF treatment significantly decreased the MPO levels to the sham levels. The significant decrease in MPO activity indicates decreased leucocyte recruitment.

The significant increase in MDA levels has been observed in various organs, including the liver, kidney and intestine during IR injury.[2,9,27] In our study, the increased levels of MDA in the ischaemic control group supported the notion that LP processes occur during IR injury. The decrease in the level of MDA in the BF group, as compared with the control group, indicated that LP was inhibited by this agent.

Free radical scavengers such as SOD, catalase and GSH-Px have reduced the severity of intestinal IR injury.[9,12,33] In our study, the depletion of free radical scavenger (GSH-Px, CAT, and SOD) levels in IR group was reversed by BF treatment. Several reports indicate that mucosal lesions induced by various stimuli are coupled with depletion of these parameters.[3,4,9,12,33] To the best our knowledge, there are no previous studies suggesting the antioxidant effect of BF.

Although many animal models have been developed to study ischaemic injury to the intestine, rats have been most commonly used. Mesenteric collateral circulation in the rat is analogous to that of the human. But superior mesenteric artery occlusion alone

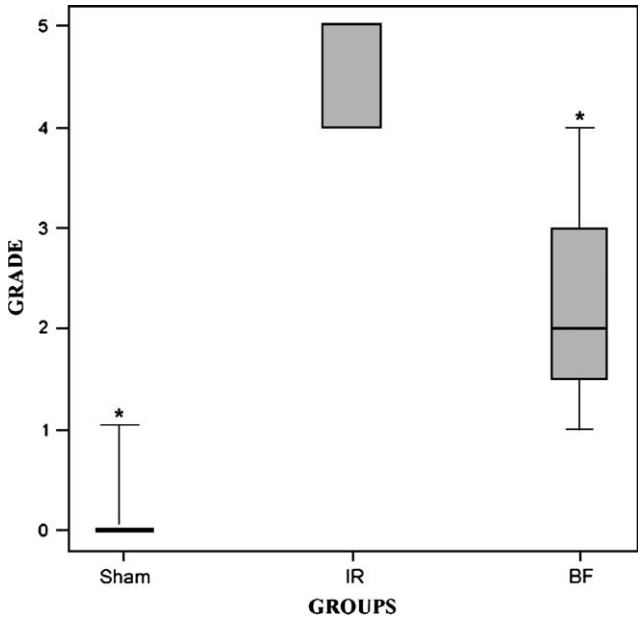

Fig. 2. Histopathological evaluation of the groups. *$P < 0.001$ versus ischaemic reperfusion (IR). BF, bencyclane fumarate.

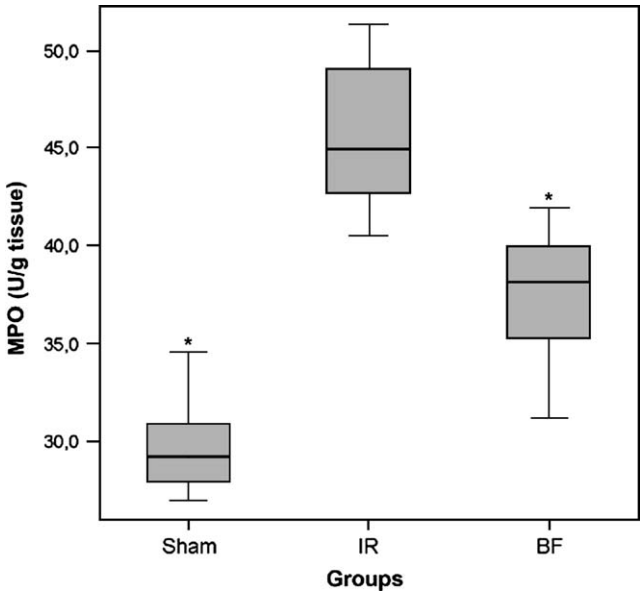

Fig. 4. Myeloperoxidase (MPO) levels in the experimental groups. *$P < 0.001$ versus ischaemic reperfusion (IR). BF, bencyclane fumarate.

in the rat is not a reliable model for mesenteric ischaemia. However, SMA occlusion with collateral ligation produces more severe and reproducible ischaemia with greater mortality than does SMA occlusion alone.[23] In our experimental model, collateral arcades from the right colic artery and the jejunal arteries proximal to the site of occlusion were ligated to avoid the variable contribution of collateral circulation to the distal ileum.

In conclusion, pretreatment of rats with BF before intestinal ischaemia reduces, but not completely prevents, the degree of histopathological damage from IR possibly by inhibiting LP, neutrophil accumulation and antioxidant activity in the intestinal mucosal tissue.

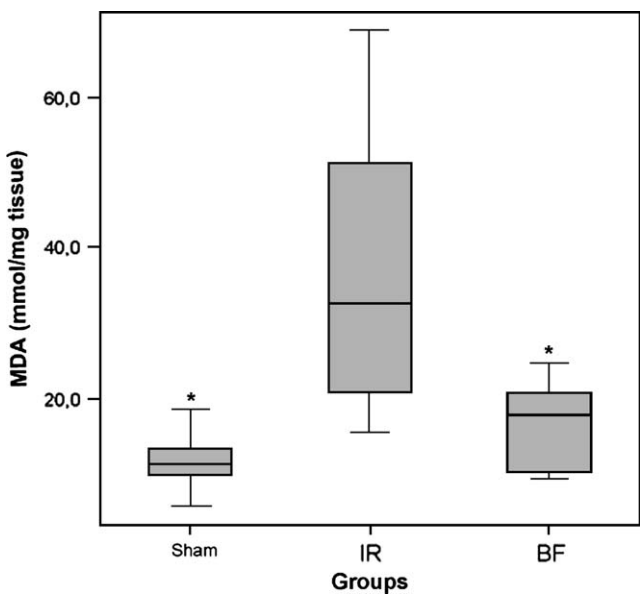

Fig. 3. Malondialdehyde (MDA) levels in the experimental groups. *$P < 0.001$ versus ischaemic reperfusion (IR).

ACKNOWLEDGEMENTS

The authors wish to thank Mustafa Ozturk, MD, working at the department of Public Health, Suleyman Demirel University, Medical School, for his assistance with the statistical analysis. We also want to thank to Omer Sekerci, PhD, working at the Department of English Language and Literature at the faculty of Science and the Letters, Suleyman Demirel University, for his help in the correction of the language of the article.

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