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The effect of heparin on trinitrobenzene sulphonic acid-induced colitis in the rat

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SUMMARY

Background: Reduced blood coagulability seems to protect against inflammatory bowel disease; pilot studies using heparin in patients with inflammatory bowel disease have reported positive results.

Aim: To evaluate the effects of heparin treatment on microangiographic and on inflammatory parameters in experimental colitis, induced by trinitrobenzene sulphonic acid (TNBS)-ethanol.

Methods: Four groups of rats: (i) controls (saline enema), TNBS-induced colitis with (ii) sham treatment (saline, s.c.), (iii) dexamethasone (0.25 mg/kg/day s.c.) and (iv) heparin (500 U/kg t.d.s., s.c.). Microangiography was performed 2 and 4 days after colitis induction. Partial thromboplastin time, colonic wet weight, macroscopic damage score and mucosal myeloperoxidase (MPO) activity were determined at day 4.

Results: TNBS-induced colitis caused a reduction in visible bowel wall vessels, which was prevented by heparin ($P < 0.05$) but not by steroids. The macroscopic damage scores and colon wet weights were similar in all colitis groups. Compared to untreated colitis the MPO activity in heparin-treated animals was of borderline significance.

Conclusions: Heparin treatment improved microangiographic features and reduced inflammation to a certain degree. Steroids delayed development of colon hypoperfusion, but were ineffective on MPO activity. It remains to be determined if the observed effects are due to the antithrombotic activity of heparin or to an anti-inflammatory action.

INTRODUCTION

Patients with inflammatory bowel disease are at risk of thromboembolism.[1] The reasons for this risk are not yet entirely understood, but it is well-known that disease activity is characterized by an increased number of platelets, increased circulating levels of serum fibrinogen, and other factors of coagulation, together with an inhibition of the fibrinolytic system.[2–4] On the other hand, prothrombotic factors unrelated to disease activity such as enhanced spontaneous and induced aggregation of platelets have been found in both Crohn's disease (CD) and ulcerative colitis (UC),[5] and the expression of P-selectins appears to be upregulated in peripheral venous blood of inflammatory bowel disease patients.[6, 7] The presence of granulomatous vasculitis in CD and the identification of microthrombi in normal mucosa of patients with UC have led to the hypothesis that blood hypercoagulability inducing gut ischaemia and infarction may constitute a main pathogenic event in inflammatory bowel disease.[8, 9] The protective effect of inherited defects of coagulation, i.e. haemophilia and von Willebrand's disease, against the occurrence of inflammatory bowel disease has recently been reported in a study of 9000 patients with bleeding disorders. The incidence of both CD and UC was significantly reduced

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in these patients, emphasizing the importance of thrombosis and vascular occlusion in the pathogenesis of inflammatory bowel disease.[10]

Recently a beneficial effect of heparin has been reported in UC[11] and, subsequently, also in CD.[12, 13] These studies, however, were open studies carried out in small numbers of patients. As well as its actions as an anticoagulant, heparin possesses a wide range of anti-inflammatory properties, mainly due to its polyanionic groups.[14]

We have previously shown that experimental colitis induced by trinitrobenzenesulphonic acid (TNBS) and alcohol induces a long-lasting significant bowel wall hypoperfusion assessed by microradiography.[15] The purpose of the present study was to investigate the effect of heparin treatment on tissue perfusion and on inflammation of TNBS-induced colitis, comparing its effects to corticosteroid treatment.

MATERIALS AND METHODS

Experimental groups

Male Sprague–Dawley rats (Charles-River; Calco, CO, Italy), weighing 175–200 g, were kept in pairs under controlled environmental conditions (12 h–12 h light–dark cycle, 22 °C, 55–65% humidity) with free access to tap water and pelleted standard chow throughout the experimental period; the rats were allowed to acclimatize for 1 week after purchase. The study protocol was approved by the governmental commission for animal protection.

One hour before colitis induction the animals were treated as follows: colitis controls (TNBS-C; $n = 14$): s.c. administration of saline (0.2 mL); colitis steroids (TNBS-S; $n = 14$): s.c. administration of 0.25 mg/kg dexamethasone (Decadron; M.S.D., Milan, Italy) and colitis heparin (TNBS-H; $n = 14$): s.c. administration of 500 U/kg heparin sodium (Liquemin; Roche, Milan, Italy). Colitis was induced under ether anaesthesia by intrarectal instillation of 25 mg TNBS (Sigma, Milan, Italy) in 50% ethanol (total volume 0.8 mL). Treatment was continued with the following protocol—TNBS-C: 0.2 mL saline s.c. every 8 h; TNBS-S: one injection of dexamethasone/morning followed by two saline injections 8 and 16 h later; TNBS-H: 500 U/kg heparin sodium s.c. every 8 h. In 10 control animals (controls) the same volume of saline was administered intrarectally and sham treatment was performed three times a day with s.c. saline injections.

Microradiography

Two and 4 days after colitis induction four rats/group were sacrificed for microangiographic investigations. Technical details on microradiography are described elsewhere.[16] Briefly, the abdomen was opened under anaesthesia (chloralium hydrate, 400 mg/kg i.p.) and the abdominal aorta was canulated distally to the renal arteries with a 20G teflon canula. The aorta was ligated proximally to the origin of the celiac trunk. The intestine was flushed with 100 mL warm saline (37 °C) after an incision on the portal vein, in order to avoid artefacts due to pressure overload. Subsequently 10 mL of 50% w/v micronized barium sulphate were injected through the same canula using a infusion pump (Braun, Milan, Italy) with a flux of 1.5 mL/min. The microradiologic examination (Gilardoni Radiolight, Milan, Italy) was carried out on the explanted colon opened along the antimesenteric border using a focal spot of 0.3 mm, a focus-film distance of 70 cm with an exposure time of 1–2 min at 18–22 kV, 5 mA.

The radiographs were examined with low magnification (10 ×) and the number of arteriae rectae, collateral arteries and terminal arteriolae (expressed per square cm of diseased area) was assessed.

Determination of blood cell counts, partial thromboplastin time (aPTT) and of colonic damage

The remaining rats were killed 4 days after colitis induction. Four hours after the last s.c. injection (heparin, dexamethasone or saline) blood was drawn from anaesthetized animals from the abdominal aorta for the determination of blood cell counts and aPTT (Instrumentation Lab., Milan, Italy; reactant: Actin FS; Düdingen, Switzerland). The colon was excised, opened, rinsed with cold saline, weighed, and the intestinal damage was assessed macroscopically according to Wallace *et al.*[17] Mucosal samples were immediately weighed, frozen in liquid nitrogen and stored at −80 °C until determination of MPO (within 2 weeks). MPO activity was assessed by the dianisidine–H_2O_2 assay.[18] Results are given as MPO units per mg tissue weight.

Data presentation and statistics

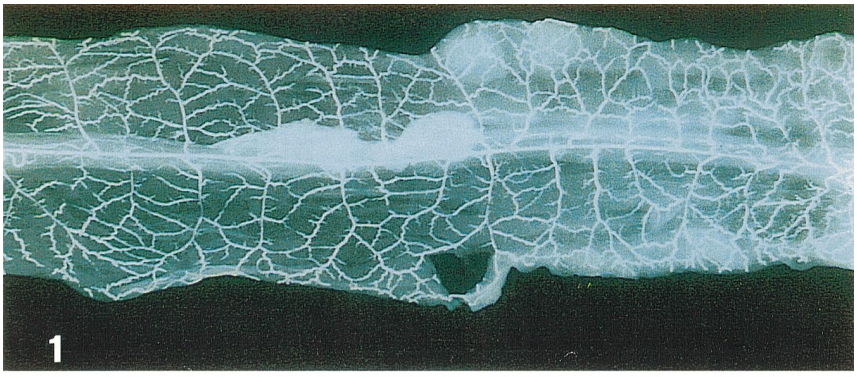
All data are given as mean values ± standard error (S.E.M.). Statistical analysis was performed by ANOVA followed by the unpaired *t*-test with Bonferroni's correction. Significance was set at $P < 0.05$.

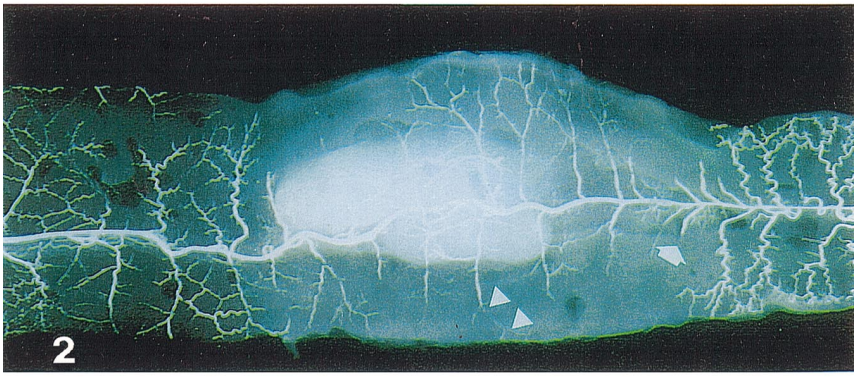
RESULTS

Microradiography

In control animals (Figure 1) barium-filled arteries were evidenced as previously described.[16] Two days after colitis induction there was no statistical change in the number of arteriae rectae and collateral arteries, although the number of arteria rectae was roughly doubled in colitic animals (Table 1). It is likely that this change is due to colonic contraction after colitis induction. The most important changes were observed in the number of terminal arteriolae which were significantly reduced ($P < 0.05$) in untreated colitis compared to controls, whereas the reduction in steroid-treated animals did not achieve significance. In the heparin-treated group the number of terminal arteriolae remained virtually unchanged.

Four days after the TNBS/ethanol challenge the colon persisted in contraction, as evidenced by the still doubled number of arteriae rectae in all groups with colitis. The decrease in collateral arteries filled with contrast material in untreated and in steroid-treated animals compared to controls did not reach significance. No change was observed in heparin-treated colitis. Contrast-filled terminal arteriolae were significantly reduced in untreated and in steroid-treated colitis ($P < 0.05$, both) compared to controls but not in the heparin-treated group. Heparin increased significantly





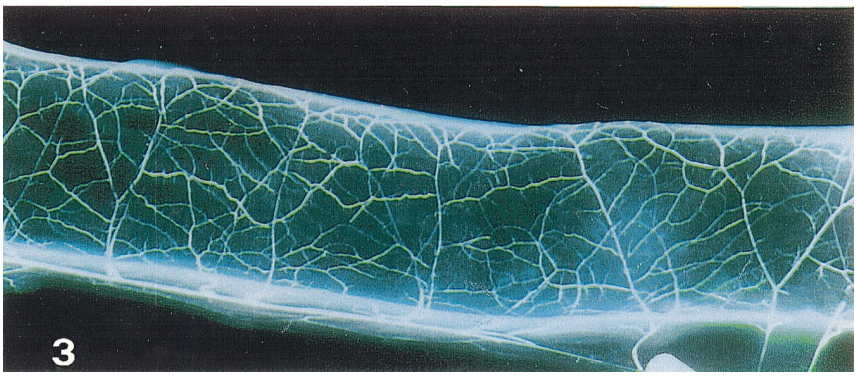


Figure 1. Microradiograph of the distal colon opened along the antimesenteric border showing the normal vascular pattern.

Figure 2. Microradiograph of the distal colon opened along the antimesenteric border in saline-treated TNBS-induced colitis 4 days after induction; a central hypoperfused lesion with rectilinear arteries showing a progressive thinning (arrowheads) or sudden complete interruption (arrow).

Figure 3. Microradiograph of the distal colon filled with air in heparin-treated colitis 4 days after induction; the vascular pattern is entirely conserved.

Table 1. Microangiographic features in healthy control rats and in TNBS-induced colitis, 2 and 4 days after colitis induction; TNBS-C: sham treatment (saline 0.25 mL, t.d.s., s.c.), TNBS-S: treated with dexamethasone (0.25 mg/kg/day, s.c.), TNBS-H: treated with heparin (100 U/kg, t.d.s., s.c.); data are mean values ± S.E.M.; *n*: number of observations per group; ANOVA and Bonferroni's *t*-test; [a]$P < 0.05$ vs. controls, [b]$P < 0.05$ vs. TNBS-C and TNBS-S

	Controls ($n = 4$)	TNBS-C ($n = 4$)	TNBS-S ($n = 4$)	TNBS-H ($n = 4$)	*P*-value
at 48 hours					
Arteriae rectae/cm^2	2.9 ± 0.6	4.2 ± 0.8	4.4 ± 0.7	4.9 ± 0.9	0.331
Collateral arteries/cm^2	36 ± 5	22 ± 9	41 ± 16	51 ± 16	0.450
Terminal arteriolae/cm^2	211 ± 30	25 ± 14[a]	122 ± 59	185 ± 53	0.043
at 96 hours					
Arteriae rectae/cm^2	—	4.1 ± 0.8	4.7 ± 2.9	3.9 ± 1.2	0.331
Collateral arteries/cm^2	—	21 ± 6	18 ± 7	45 ± 30	0.603
Terminal arteriolae/cm^2	—	27 ± 9[a]	28 ± 13[a]	259 ± 54[b]	0.001

($P < 0.05$) the number of terminal arteriolae compared to untreated and to steroid-treated colitis.

In untreated and in steroid-treated colitis the collateral arteries and the arteriae rectae frequently appeared truncated, while a progressive thinning of the vessels was observed less frequently (Figure 2). In heparin-treated animals these changes were not observed (Figure 3), despite a radiologically evident thickening of the bowel wall.

General outcome and colonic damage

Four days after colitis induction all animals with colitis showed a significant ($P < 0.001$) weight loss that was more pronounced in steroid-treated and sham-treated animals (Table 2). One animal of the steroid-treated group died on day 3. Colonic wet weight was significantly increased ($P < 0.001$) in colitic animals compared to controls. No difference in colonic wet weight was found between the groups with colitis. The macroscopic damage score (Figure 4) was significantly increased ($P < 0.002$) in all animals with colitis compared to untreated controls and no difference was observed between the three colitis groups, although heparin- and steroid-treated animals had lower scores than untreated colitis (Controls, 0.4 ± 0.2; TNBS-C, 8.3 ± 1.5; TNBS-S, 6.8 ± 1.5; TNBS-H, 6.6 ± 1.6). Compared to controls no difference was found between MPO activity in heparin-treated animals, whereas activity was increased in untreated colitis and in steroid-treated animals ($P < 0.001$) (Controls, 0.5 U/mg ± 0.1; TNBS-C, 15.2 ± 2.2; TNBS-S, 10.6 ± 2.3; TNBS-H, 8.2 ± 2.4) (Figure 5). MPO activity in heparin-treated animals, although reduced, failed to achieve significance by comparison with untreated ($P < 0.057$) or steroid-treated rats ($P < 0.494$). Heparin treatment significantly ($P < 0.001$) prolonged aPTT compared to controls or other colitis groups. All

Table 2. Body weight, colon wet weight, blood cell counts and partial thromboplastin time (aPTT) in healthy control rats and in TNBS-induced colitis, 4 days after colitis induction; TNBS-C: sham treatment (saline 0.25 mL, t.d.s., s.c.), TNBS-S: treated with dexamethasone (0.25 mg/kg/day, s.c.), TNBS-H: treated with heparin (100 U/kg, t.d.s., s.c.); *n*: number of observations per group; WBC: white blood cells; RBC red blood cells; Plts: platelets; data are mean values ± S.E.M.; ANOVA and Bonferroni's *t*-test. [a]$P < 0.05$ or less vs. controls; [b]$P < 0.001$ vs. TNBS-H.

	Controls ($n = 6$)	TNBS-C ($n = 6$)	TNBS-S ($n = 5$)	TNBS-H ($n = 6$)	*P*-value
Δ body weight; g	+20 ± 2	−15 ± 8[a]	−16 ± 4[a]	−4 ± 4[a]	0.001
colon wet weight; g	0.8 ± 0.1	2.1 ± 0.1[a]	1.7 ± 0.2[a]	1.8 ± 0.1[a]	0.001
Blood cells					
WBC × $10^3/\mu$L	4.2 ± 0.3	12.8 ± 1.2[a]	13.4 ± 1.5[a]	12.0 ± 1.3[a]	0.001
RBC × $10^6/\mu$L	6.5 ± 0.5	6.6 ± 0.9	6.3 ± 1.0	5.9 ± 1.4	0.957
Plts × $10^3/\mu$L	942 ± 24	1282 ± 154	1307 ± 182	1252 ± 136	0.208
Coagulation					
aPTT; s	11 ± 2[b]	12 ± 1[b]	11 ± 1[b]	52 ± 8	0.001

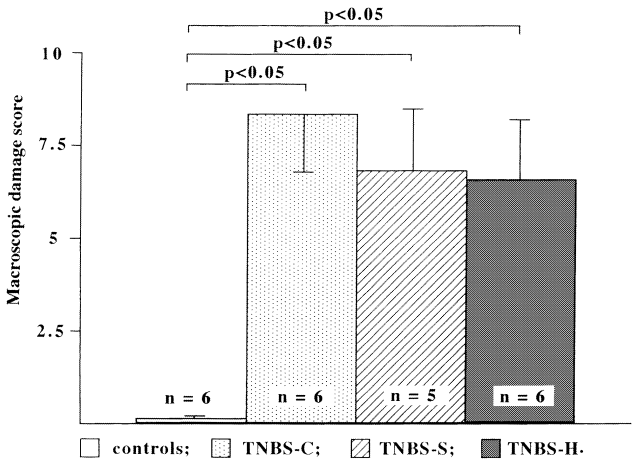


Figure 4. Macroscopic damage score in healthy control rats and in TNBS-induced colitis, 4 days after colitis induction; TNBS-C: sham treatment (saline 0.25 mL, t.d.s., s.c.), TNBS-S: treated with dexamethasone (0.25 mg/kg/day, s.c.), TNBS-H: treated with heparin (100 I.U./kg, t.d.s., s.c.); *n*: number of observations per group; data are mean values ± S.E.M.; Bonferroni's *t*-test.

colitis groups, independently of treatment, showed leucocytosis ($P < 0.05$) compared to controls. No statistical differences were found for red cell counts and for platelets.

DISCUSSION

Two and 4 days after colitis induction with TNBS/ethanol we found a significant reduction in the colonic vascular pattern assessed by microradiography. Steroid treatment seemed to delay development of bowel wall hypoperfusion but was not successful in its prevention. These microangiographic features, although not a true reflection of a reduced tissue oxygenation, suggest ischaemia of the bowel wall. Heparin completely abolished the observed angiographic alterations and reduced inflammation, as evidenced by the MPO activity in this latter group. Contraction and thus shortening of the distal colon due to inflammation may explain the almost doubled number of rectilinear arteries in all colitis groups. This may have led to an overestimation of the collateral arteries in saline-treated colitis at 2 days and in both saline- and steroid-treated colitis at 4 days, because the number of vessels was expressed per square centimetre. At the same time-points heparin treatment increased the number of these second-order arteries by 42% and 22%, respectively. These considerations were even more important for the terminal arteriolae, significantly reduced only in saline-treated colitis at 2 days, but reduced by 42% in steroid-treated animals. Heparin-treated colitis induced a 12% decrease at 2 days, but a 23% increase of terminal arteriolae at 4 days. This may indicate that in the heparin-treated group a transient tissue hypoperfusion might also be present.

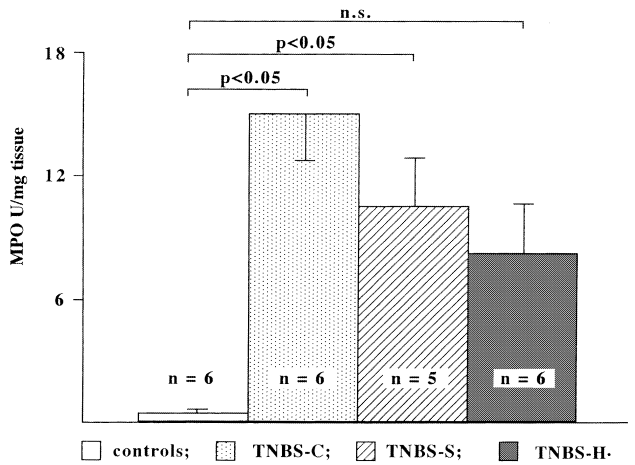


Figure 5. Myeloperoxidase activity in healthy control rats and in TNBS-induced colitis, 4 days after colitis induction; TNBS-C: sham treatment (saline 0.25 mL, t.d.s., s.c.), TNBS-S: treated with dexamethasone (0.25 mg/kg/day, s.c.), TNBS-H: treated with heparin (100 I.U./kg, t.d.s., s.c.); *n*: number of observations per group; data are mean values ± S.E.M.; n.s.: not significant; Bonferroni's *t*-test.

Similar data have been reported recently by Dobosz *et al.* in the acetic acid model of experimental colitis.[19] In that study heparin treatment, started 24 h after colitis induction, was associated with a preservation of colonic microcirculation, assessed by laser Doppler flowmetry, at 48 h. Moreover, compared to untreated colitis, anticoagulant therapy significantly reduced circulating interleukin-6 levels despite similar microscopic damage scores. Important long-lasting changes in microcirculation have also been described in TNBS/ethanol-induced chronic ileitis, assessed by laser Doppler flowmetry, with the resolution of mucosal ischaemia within 3 weeks.[20] These data are in agreement with our own findings extending observations up to 5 weeks (unpublished data).

In the acetic acid model a short, transient ischaemia (lasting for 15 min) has been described immediately after administration of acetic acid.[21] In this latter paper the authors hypothesized that colon damage was enhanced by the generation of free oxygen radicals induced by this kind of ischaemia-reperfusion. Observations on microcirculation at later time-points, however, have not been carried out by this group. This pathogenic mechanism appears not to apply for TNBS-induced

colitis, because ischaemia is long-lasting and reperfusion of the colon is a relatively late phenomenon and occurs through neovascularization starting 10 days after colitis induction. Generation of free radicals during TNBS metabolism in cultured colonocytes has been described,[22] but occurs very early, within a few minutes of contact, and seems to be independent of vascular supply.

Tissue ischaemia in TNBS/ethanol-induced colitis may have several mechanisms. First, deep caustic injury may directly induce vascular lesions. Second, oedema of the bowel wall and inflammatory infiltrate may lead to vascular compression and, finally, thrombotic occlusion of arteries may occur. If vascular lesions were present, a spread of contrast medium and clinically evident bleeding would have been present, but were not observed in our study. Vascular compression is likely, as evidenced by a progressive reduction of the vessel diameters in some radiographs. Thrombotic occlusions of small vessels have been described in this model of experimental colitis[23, 24] and were demonstrated in the present study microangiographically by the sudden interruption of normally barium-filled arteries in saline-treated as well as in steroid-treated colitis. Besides thrombosis, the formation of granulomata may contribute to vascular occlusion, at least at later time-points, as in the present study. In heparin-treated animals these radiological patterns were not observed.

Corticosteroids are known to reduce the production of the chemotactic leukotriene B4 and other eicosanoids and may have some effects on blood coagulation through inhibition of thromboxane A2 synthesis, reducing activation of platelets and vasoconstriction. However, in the model of indomethacin-induced enteritis, dexamethasone was shown to promote jejunal ulcer 'plugging', preventing blood loss in these animals.[25] Moreover, corticosteroids reduce the expression of several proinflammatory cytokines and adhesion molecules.[26] In experimental colitis the effects of steroids are controversial, with varying reports from accelerated healing to no effect,[22, 27, 28] despite some measurable effects on eicosanoid synthesis.

Besides its actions on blood coagulation heparin has been found to possess a wide range of anti-inflammatory properties. Binding to L- and P-selectins,[29] adhesion molecules that intervene in the interaction between leucocytes and the vascular endothelium, to interferon-γ,[30] tumour necrosis factor-α,[31] and chemokines[32] have all been described, with the consequent reduction of leucocyte chemotaxis, rolling, margination and diapedesis. In experimental colitis the administration of antibodies directed against adhesion molecules and thus blocking adhesion of leucocytes was reported to abolish almost completely the infiltration of neutrophils and to reduce MPO activity by 80%,[33] suggesting that by interfering in very early stages of inflammation, tissue damage can be prevented. Finally, heparin has an inhibitory effect on the biosynthesis and the release of endothelin-1, a potent vasoconstrictor,[34] found to be increased in the *lamina propria* and submucosa of inflammatory bowel disease patients.[35]

In the present study both treatments, corticosteroids and heparin, were started prior to colitis induction. While heparin significantly improved colonic blood supply and mucosal inflammation, dexamethasone appeared to only delay the occurrence of gut ischaemia, without affecting MPO activity. Although the TNBS/ethanol-induced colitis is not an aetiological model for human inflammatory bowel disease, our findings may allow the formulation of a hypothesis to explain the frequently partial success rates with systemic therapies in clinical trials.[36, 37] Extrapolating our experimental data on human inflammatory bowel disease, once mucosal inflammation is fully established, i.e. occlusion of small vessels has occurred, active principles like corticosteroids may not reach the diseased areas. This consideration may justify the use of heparin in addition to conventional medical therapy. Although in the present study no differences were found between the number of erythrocytes in heparin-treated animals compared to the other groups, one of the major drawbacks of a clinical use of heparin is the risk of bleeding, especially in ulcerative colitis where macroscopic rectal blood loss is frequently observed. In previous clinical reports, heparin treatment had to be discontinued, at least temporarily, in up to 14% of patients;[12, 13] one patient required blood transfusions. This, together with the i.v. administration route, implies that hospitalization of the patients for the first 10 days of therapy is necessary. Prevention of relapse in patients has so far not been investigated.

In conclusion, heparin treatment prevented tissue hypoperfusion and reduced tissue MPO activity to a certain degree, but did not prevent extension of the damaged area in experimental colitis, whereas dexamethasone only delayed the development of vascular alterations. These findings suggest that heparin, alone or in addition to conventional therapeutic approaches,

may be useful in the treatment of active inflammatory bowel disease or in the prevention of its relapses. Further studies are warranted to elucidate the exact mechanism of heparin action (anti-inflammatory, antithrombotic, or a combination of both) and to estimate the risk of intestinal bleeding.

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