Effect of Hyperbaric Oxygen on Experimental Acute Distal Colitis

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Summary
It has been demonstrated that hyperbaric oxygen (HBO) is useful as an adjunctive therapy for Crohn's disease. However, its effects on ulcerative colitis have not been investigated. In the present study, HBO was tested for acetic acid-induced colitis, and antioxidant systems were evaluated to clarify its possible mode of action. Thirty-six Sprague-Dawley rats were randomly divided into three groups: sham control (Group I), colitis induced by acetic acid without any therapy (Group II), colitis induced by acetic acid and treated with HBO (Group III). HBO was given for 5 days, 2 sessions per day at 2.5-fold absolute atmosphere pressure (ATA) for a period of 90 min in rats in which colitis had been induced (Group III). Rats were sacrificed on the 5th day after the procedure. Superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione peroxidase (GSH Px) activity were measured in the intestinal tissue and erythrocyte lysate. MDA and GSH Px were also determined in the plasma. Whereas MDA levels in erythrocyte, plasma and intestinal tissue were decreased, the levels of GSH Px and SOD were significantly increased in Group III compared to those of Group II. The results of our study suggest that hyperbaric oxygen therapy has beneficial effects on the course of experimental distal colitis and that antioxidant systems may be involved in its mode of action.

Key words
Distal colitis • Antioxidant enzymes • Hyperbaric oxygen

Introduction
Ulcerative colitis comprises a series of idiopathic and heterogeneous inflammatory disorders resulting from chronic up-regulation of the enteric mucosal immune system (Fox 1993, Fiocchi 1998). Although the exact etiopathogenesis of inflammatory bowel disease (IBD) is still not clear, it appears that there is chronic activation of the immunoinflammatory cascade in genetically susceptible individuals resulting in chronic mucosal damage. A small degree of physiological inflammation is present in the lamina propria of the healthy intestine, perhaps in response to a constant flux of antigenic substances encountered within the lumen. The intense inflammatory infiltrate seen in IBD represents a large influx of immune and inflammatory cells (Burgio et al. 1995). Recruitment occurs by a series of events beginning...
with transient tethering of leukocytes to the endothelium and culminating in diapedesis across the endothelium and into the mucosa (Salmi and Jalkanen 1998).

Cells recruited into the mucosa release a vast number of substances with inflammatory and directly damaging properties. These include arachidonic acid metabolites (prostaglandins, thromboxanes, leukotrienes), free radicals (reactive oxygen metabolites and nitric oxide), platelet activating factor (Fiocchi 1998), and various proteases (MacDonald and Pender 1998). Although the therapeutic impact of blocking one of many redundant inflammatory mediators may be limited, a number of candidates have been targeted.

Hyperbaric oxygen (HBO) has been described as an adjunctive therapy for healing perianal manifestations of Crohn's disease (Brady et al. 1989, Nelson et al. 1990, Lavy et al. 1994, Colombel et al. 1995). Possible modes of action include decreased activity of nitric oxide synthase (Rachmilewitz et al. 1998) and inhibition of proinflammatory cytokines (Weisz et al. 1997). The increase in concentration and partial pressure of oxygen during HBO therapy provides more oxygenation in the whole body. The increased tissue oxygen enhances the growth of fibroblasts, formation of collagen, angiogenesis, and phagocytic capabilities of the hypoxic leukocytes, so it has beneficial effects on wound healing. Recently, it has been revealed that much of the damage associated with reperfusion is brought about by inappropriate activation of leukocytes. Following an ischemic interval, the total injury pattern is the result of two components: a direct irreversible injury component from hypoxia, and an indirect injury, which is largely mediated by the inappropriate activation of leukocytes. HBO reduces the indirect component of injury by preventing such activation. The net effect is the preservation of marginal tissues that may otherwise be lost to ischemia-reperfusion injury (Jain 1996, Oriani et al. 1996).

HBO has been suggested to be beneficial in inflammatory bowel disease, but the mechanisms responsible for its therapeutic effects have not been elucidated. This study was designed to investigate HBO therapy involved in the evaluation of a variety of antioxidant enzymes as treatment for managing animals with induced acute colitis.

**Methods**

Thirty-six male Sprague-Dawley rats weighing 320-380 g were obtained from Gülhane School of Medicine Research Center. Before the experiment, the animals were fed a standard rat chow and water *ad libitum* and housed in metabolic cages with controlled temperature and 12-hour light/dark cycles for at least one week. The study was approved by the Institutional Animal Care and Use Committee, and all experiments were done in accordance with the National Institutes of Health guidelines.

The rats were randomly divided into three groups: Group I: sham control group (n=12), Group II: colitis group (n=12), Group III: colitis treated with HBO (n=12).

**Induction of colitis**

Distal colitis was induced by intracolonic instillation of 4 % acetic acid. This model, first described by MacPherson and Pfeiffer (1978), has been extensively used to investigate the acute phase of inflammation (Fabia et al. 1992, Yamada et al. 1992, Bell et al. 1995, Myers et al. 1997). After an overnight fast, each rat was lightly anesthetized with sevoflurane and polyethylene cannula was inserted into the lumen of the colon via the anus. The cannula was advanced so that the tip was 5 cm proximal to the anus. Initially, each rat received a 1-ml saline (0.9 %) flush followed by manual palpation of the abdomen to remove any feces. Then, 1 ml of acetic acid (4 % vol/vol in 0.9 % saline) was slowly infused into the distal colon and the rat was maintained in a head-down position for 30 s to limit expulsion of the solution. Finally, each rat received a 1 ml colonic wash containing phosphate-buffered saline (pH 7.4). Sham control rats were treated identically but instead of 4 % acetic acid they received 1 ml 0.9 % saline infusion.

HBO was performed after induction of colitis in Group III rats in the animal hyperbaric chamber for 5 days, 2 sessions per day at 2.5 ATA for a period of 90 min. On the 5th day, surviving animals were killed by an intracardiac injection of pentobarbital (200 mg/kg) following blood samples were withdrawn into the tubes containing EDTA.

A laparotomy was performed in order to obtain a segment of distal colon 5 cm in length. The lumen of resected specimen was irrigated with 0.9 % NaCl at 4 °C and weighed. Then the specimen was split longitudinally into two pieces and reserved for histological and biochemical analysis. Colonic tissue samples for biochemical analysis were homogenized in cold KCl solution (1.15 %) in a glass homogenizer on ice. These samples were then centrifuged and supernatant was used for following biochemical determinations.
Each blood sample was centrifuged for 10 min. at 4000 \( \times \) g and 4 °C. After removal of the plasma and buffy coats, erythrocytes were washed three times with two volumes of isotonic saline. Then, erythrocytes were lysed with cold distilled water (1:4), stored in a refrigerator at 4 °C for 15 min and the cell debris were removed by centrifugation (2000 \( \times \) g for 10 min).

Plasma, erythrocyte lysates and homogenized colonic tissue samples were stored at –70 °C for further laboratory analysis.

**SOD activity in erythrocyte lysate and plasma**

CuZn-SOD activity in erythrocyte lysate was measured as described earlier (Bulucu et al. 2000, Orhan et al. 1999, Mates et al. 1999). Each hemolysate was diluted 1:400 with 10 mM phosphate buffer, pH 7.00. 25 \( \mu \)l of diluted hemolysate was mixed with 850 \( \mu \)l of substrate solution containing 0.05 mmol/l xanthine sodium and 0.025 mmol/l 2-(4-iodophenyl)-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) in a buffer solution containing 50 mmol/l CAPS and 0.94 mmol/l EDTA, pH 10.2. Then, 125 \( \mu \)l of xanthine oxidase (80 U/l) were added to the mixture and absorbance was followed at 505 nm for 3 min against air. Twenty-five \( \mu \)l of phosphate buffer or 25 \( \mu \)l of various standard concentration in place of the sample were used as blank or standard determinations. CuZn-SOD activity was referred as U/ml in erythrocyte lysate.

**SOD activity measurements in colonic tissue**

SOD activity measurement was based on the generation of superoxide radicals produced by xanthine-xanthine oxidase system that reacts with 2-(4-ido-phenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form the red formosan dye. The SOD activity is then measured by the degree of inhibition of this reaction. The SOD activity was expressed as U/g tissue (Aydın et al. 2001).

**GSH Px activity in erythrocyte lysate and plasma**

GSH Px activity was measured by the method described in detail elsewhere (Bulucu et al. 2000, Orhan et al. 1999, Mates et al. 1999). The reaction mixture was 50 mmol/l Tris buffer, pH 7.6 containing 1 mmol/l of Na2EDTA, 2 mmol/l of reduced glutathione (GSH), 0.2 mmol/l of NADPH (nicotinamide adenine dinucleotide phosphate), 4 mmol/l of sodium azide and 1000 U of glutathione reductase (GR). Fifty \( \mu \)l of plasma 950 \( \mu \)l of reaction mixture, or 20 \( \mu \)l of erythrocyte lysate and 980 \( \mu \)l of reaction mixture were mixed and incubated for 5 min. at 37 °C. Then the reaction was initiated with 8.8 mmol/l H2O2 and the decrease in NADPH absorbance was followed at 340 nm for 3 min. Enzyme activities were reported as U/ml in erythrocyte lysate and plasma.

**GSH Px activity measurements in colonic tissue**

GSH Px activity measurement is based on the GSH Px-mediated catalysis of the oxidation of glutathione by tertbutyl hydroperoxide. In the presence of glutathione reductase and reduced nicotinamide adenine dinucleotide phosphate (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 at 340 nm was measured. GSH Px activity was expressed as U/g tissue (Paglia and Valentine 1967).

**Thiobarbituric acid reactive substance (TBARS) in erythrocyte lysate and plasma**

TBARS levels in both erythrocyte lysate and plasma were measured by the method described previously (Orhan et al. 1999, Mates et al. 1999). After the reaction of thiobarbituric acid with MDA, the reaction product was extracted in n-butanol and was measured spectrofluorometrically (excitation: 532 nm, emission: 553 nm, slit 10 nm). Erythrocyte TBARS levels were determined in erythrocyte lysates obtained after centrifugation. After the reaction of thiobarbituric acid with MDA, the reaction product was measured spectrophotometrically. Tetramethoxy propane solution was used as standard. TBARS levels of plasma and erythrocytes were expressed as nmol/ml.

**TBARS measurements in colonic tissue**

Tissue TBARS concentrations were estimated by the method of Ohkawa et al. (1979). The supernatant was resuspended in 4 ml water, 0.5 ml glacial acid and 0.5 ml 0.33 % aqueous thiobarbituric acid solution. The mixture was heated for 60 min in a boiling water bath. After cooling of the samples, the formed complex by thiobarbituric acid reactant substances was extracted into n-butanol and the formed chromogen was measured at 532 nm by a spectrophotometer. A standard absorption curve for TBARS was prepared using tetramethoxy propane. TBARS level was expressed as nmol/g tissue.

**Histopathological analysis**

Histopathological analysis was performed with the scale previously used for experimental colitis by
A pathologist without knowledge about the treatment protocols examined the distal colon segment 5 cm long immediately after laparotomy whether there was any focal, multifocal or diffuse ulcer and necrosis. This macroscopic scoring was performed in each rat. Then the specimen was fixed in 10 % neutral buffered formalin and embedded in paraffin for further histologic analysis. The pathologist blinded to the treatment groups examined the 4 \( \mu \)m slides stained with hematoxylin and eosin and scored the tissue for edema, inflammatory infiltration in each layer of the bowel wall and necrosis. The injury of the tissue was evaluated by histopathologic scoring (minimum 0, maximum 6) which estimated the sum of macroscopic and microscopic scores.

### Table 1. The scale for histopathologic (macroscopic and microscopic) scoring.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal appearance</td>
<td>0</td>
</tr>
<tr>
<td>Focal ulcer</td>
<td>1</td>
</tr>
<tr>
<td>Multifocal ulcer</td>
<td>2</td>
</tr>
<tr>
<td>Diffuse ulcer and necrosis</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal histologic appearance</td>
<td>0</td>
</tr>
<tr>
<td>Inflammation on mucosa and submucosa</td>
<td>1</td>
</tr>
<tr>
<td>Inflammation on the entire wall of bowel</td>
<td>2</td>
</tr>
<tr>
<td>Ulcer and necrosis on the entire wall</td>
<td>3</td>
</tr>
</tbody>
</table>

Statistical analyses

All results were represented as means ± S.E.M. Statistical significance of data was determined by Mann Whitney-U test. P<0.05 values were considered significant.

Results

Intrarectal administration of 4 % acetic acid in the distal colon of rats produced an inflammatory response in all experimental animals (n=24) as evidenced by macroscopic inspection of the colon. All rats with colitis developed diarrhea. The presence of diarrhea did not have a significant effect on body weight. No macroscopic evidence of inflammation or diarrhea was found in the sham control animals (n=12). No complication was detected related to the acetic acid instillation procedure and HBO. However, two rats in group II died following colitis induction on the third day.

TBARS level measurements

There was an increase in the levels of MDA in erythrocytes and plasma of group II compared to group I (p<0.05), whereas MDA levels were decreased in erythrocytes and the plasma of group III compared to group II (p<0.05) (Table 3). In colonic tissue, there was a decrease in the level of MDA of group III compared to group II in which the level of MDA was increased compared to group I (p<0.05) (Table 1).

GSH Px activity measurements

There was a decrease in the levels of GSH Px of group II compared to group I and there was an increase in the level of GSH Px of group III compared to the group II in colonic tissue, erythrocytes and the plasma (p<0.05) (Tables 2 and 3).

SOD activity measurements

SOD activity was significantly decreased in both colonic tissue and erythrocytes of group II compared to group I and there was an increase in the level of GSH Px of group III compared to the group II in colonic tissue, erythrocytes and the plasma (p<0.05) (Tables 2 and 3).

Histopathological findings

The average weight of 5 cm segment of distal colon on day 5 in groups I, II and III was 0.33±0.01 g, 0.93±0.05 g and 0.40±0.03 g, respectively (p<0.05). Almost normal histological appearance, which corresponded to histopathological score of 0, was found in group I. Following acetic acid instillation, various degrees of multifocal large mucosal ulcerations besides prominent edema and congestion of submucosa and polymorphonuclear leukocyte infiltration of the whole bowel wall were present in group II. Two rats died due to perforation of the colon because of ulceration involving the entire wall of the bowel in group II. In contrast to group II, only superficial and mostly focal ulcers were present besides mild edema of submucosa and inflammatory infiltration of mucosa and submucosa in HBO therapy group. The mean histopathological scores in groups II and III were 3.5±0.5 and 1.8±0.7, respectively (p<0.05).
Table 2. Malondialdehyde (MDA), glutathione peroxidase (GSH Px), and superoxide dismutase (SOD) in colonic tissue.

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (nmol/g)</th>
<th>GSH Px (U/g)</th>
<th>SOD (U/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (n=12)</td>
<td>8.6 ± 2.9</td>
<td>298.8 ± 44.6</td>
<td>561.8 ± 63.1</td>
</tr>
<tr>
<td>Group II (n=10)</td>
<td>12.3 ± 2.8</td>
<td>239.5 ± 31.4</td>
<td>322.3 ± 38.3</td>
</tr>
<tr>
<td>Group III (n=12)</td>
<td>2.7 ± 0.3</td>
<td>298.2 ± 41.2</td>
<td>455.2 ± 14.2</td>
</tr>
<tr>
<td>P (I vs. II)</td>
<td>P&lt;0.01</td>
<td>P&lt;0.005</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>P (I vs. III)</td>
<td>P&lt;0.0001</td>
<td>NS</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>P (II vs. III)</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.004</td>
<td>P&lt;0.0001</td>
</tr>
</tbody>
</table>

Data are means ± S.D., NS - not statistically significant (P>0.05).

Table 3. MDA, GSH Px, and SOD in erythrocytes and plasma.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Erythrocytes</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDA nmol/ml</td>
<td>GSH Px U/ml</td>
</tr>
<tr>
<td>Group I (n=12)</td>
<td>1.8 ± 0.6</td>
<td>77.3 ± 17.1</td>
</tr>
<tr>
<td>Group II (n=10)</td>
<td>9.5 ± 1.4</td>
<td>42.3 ± 7.7</td>
</tr>
<tr>
<td>Group III (n=12)</td>
<td>4.6 ± 0.9</td>
<td>70.4 ± 16.3</td>
</tr>
<tr>
<td>P (I vs. II)</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>P (I vs. III)</td>
<td>P&lt;0.0001</td>
<td>NS</td>
</tr>
<tr>
<td>P (II vs. III)</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
</tr>
</tbody>
</table>

Data are means ± S.D., NS - not statistically significant (P>0.05).

Discussion

In the present study, we used the acetic acid-induced model of experimental colitis in rats to determine the effects of HBO on distal colitis. Inflammation is usually limited to mucosa and submucosa in human ulcerative colitis. The most important microscopic findings in human inflammatory bowel disease are the loss of mucus (Surawicz 1992), crypt abscess (Le Berre et al. 1995) and glandular distortion (Seldenrijk et al. 1991). Inflammation may extend to the serosa due to intestinal susceptibility and the acid concentration when colitis is induced with acetic acid. As the glandular distortion is a sign of chronic disease, it is unusual to see this change in acute colitis induced by acetic acid. Rarely, the crypt abscess is present on histological examination. HBO has been suggested as an adjunctive therapy for healing perianal manifestations of Crohn's disease (Brady et al. 1989, Nelson et al. 1990, Lavy et al. 1994, Colombel et al. 1995). Possible modes of action include decreased activity of nitric oxide synthase (Rachmilewitz et al. 1998) and inhibition of proinflammatory cytokines (Weisz et al. 1997). We found that HBO increased the levels of GSH Px, an enzyme that controls the cellular defense mechanism, in colonic tissues, plasma and erythrocytes. On the other hand, SOD, another enzyme that controls the cellular defense mechanism, did not increase. The levels of MDA, one of the reduction products of oxygen metabolism, decreased in erythrocytes, plasma, and colonic tissue in the HBO group as compared to the control group. We found that oxidative stress and tissue damage was decreased because of HBO therapy in colitis.

Oxidative stress and resultant tissue damage are the hallmark of cell death. There is increasing evidence that, in certain pathologic states, the increased production and/or ineffective scavenging of such reactive oxygen species may play a crucial role in determining tissue injury. The levels of intermediate reduction products of oxygen metabolism (i.e. superoxide, hydroxyl radicals, and H2O2) are controlled by various cellular defense mechanisms comprising enzymatic (SOD, catalase, GSH Px) and non-enzymatic scavenger components (Baynes and Thorpe 1999, Wohaieb and Godin 1987).

In samples from HBO-treated group there were usually focal ulcers and less inflammatory infiltration,
necrosis, edema and hemorrhage than in samples from control colitis group. In this study, HBO apparently decreased the tissue damage due to acetic acid as seen by both macroscopic and microscopic evaluation on day 5 following acetic acid-induced colitis. In a previous study, Rachmilewitz et al. (1998) evaluated the specimen on day 1 following acetic acid-induced colitis and HBO therapy and observed a lack of correlation between macroscopy and histology. This difference may be related to elapsed time for evaluation besides the duration of HBO therapy (5 days vs. 1 day). Various rates of beneficial effect observed histologically were reported in experimental colitis treated by HBO. The weight of colonic segment in HBO therapy group was relatively lower than in the colitis group and this is well accepted as an indicator of less edema and inflammation. We observed that, acetic acid-induced colitis was ameliorated by adding HBO.

In a previous study, Rachmilewitz et al. (1998) assessed the effect of HBO on colonic damage in two models of experimental colitis and examined whether this effect is mediated by modulation of nitric oxide synthase (NOS). They found that HBO reduced the extent of the injury induced by acetic acid and trinitrobenzoic acid (TNB) by 51 and 62 %, respectively. The protection provided by HBO was accompanied by a significant decrease in colonic weight, prostaglandin E2 generation, myeloperoxidase, and NOS activities. In acetic acid-induced colitis, leukotriene B4 generation was also significantly decreased. They concluded that HBO effectively ameliorated colitis induced by acetic acid and TNB. The decreased NOS activity induced by HBO suggested that reduction in NO generation may be among the mechanisms responsible for the anti-inflammatory effect of HBO. Thus, HBO may be considered for the treatment of patients with refractory inflammatory bowel disease (Rachmilewitz et al. 1998). Lavy et al. (1994) investigated the effect of HBO on refractory perianal Crohn’s disease in ten patients. They found that complete healing occurred in 5 patients after one to two courses. In additional 2 cases, after three courses, 1 patient improved but did not heal, and 2 did not improve. No adverse effects were noted in any of the 10 patients. Follow-up for 18 months did not reveal any recurrence. These preliminary results confirm that HBO is a safe and efficient therapeutic option for perianal Crohn’s disease.

In conclusion, HBO can be used in distal colitis management. The nature and mechanism of the HBO influence in distal colitis remain to be investigated.

Acknowledgements

References


Reprint requests
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