Hyperbaric oxygen improves healing in experimental rat colitis

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\textsuperscript{1}Gulhane Military Medical Academy, Marmara University School of Medicine, Department of General Surgery\textsuperscript{2}, Istanbul, Turkey, Haydarpasa Teaching Hospital, Departments of General Surgery, \textsuperscript{3}Underwater Medicine and \textsuperscript{4}Pathology

Akin, ML, Gulluoglu BM, Uluutku H, Erenoglu C, Elbuken E, Yildirim S, Celenk T, Hyperbaric oxygen improves healing in experimental rat colitis. Undersea Hyperb Med 2002; 29(4):279-285 - This study was designed to investigate therapeutic effects of hyperbaric oxygen on experimentally induced colitis in rats by assessing oxidative tissue damage, neutrophil accumulation and histological changes. Six groups of animals were used. No procedures were done in the sham group. In the vehicle group, 50\% ethanol-induced colitis, and in four other groups, 2,4,6-trinitrobenzene sulphonic acid-induced colonic inflammation was achieved. In acute and chronic colitis non-treatment groups, no other procedure was done. In acute and chronic colitis hyperbaric oxygen treatment groups, rats underwent hyperbaric oxygen treatment for two or fourteen days. On the third and fifteenth days respectively tissue and blood samples were taken for microscopic and macroscopic damage assessment, myeloperoxidase activity and serum carbonyl content measurements. There was significant colonic tissue damage in non-treatment groups at 48 hours and 14 days. Hyperbaric oxygen treatment ameliorated the macroscopic damage significantly in chronic colitis. Amelioration of microscopic changes was not significant in each hyperbaric oxygen-treated group. Hyperbaric oxygen treatment significantly reduced tissue myeloperoxidase activity in acute colitis and decreased plasma carbonyl content in chronic colitis. In the present study, hyperbaric oxygen treatment significantly ameliorated trinitrobenzene sulphonic acid-induced chronic colitis in rats.

Experimental colitis, hyperbaric oxygen, protein oxidation, myeloperoxidase.

INTRODUCTION

Inflammatory bowel diseases (IBDs) such as ulcerative colitis and Crohn’s disease affect the mucosa of the luminal organs of the alimentary tract, mostly small and large bowel. They are multifactorial disorders and their etiology remains unknown, but the affected tissues show infiltration of inflammatory cells and ulcer formation (1,2). Genetic susceptibility, environmental factors, innate immunity, and acquired immunity are among the factors believed to contribute to the etiopathogenesis of IBD (1). Various mediators such as arachidonic acid metabolites, interleukins, and nitric oxide also have been proposed to be involved in the mechanism of colonic inflammation (3-5). IBDs cause significant morbidity and mortality, and no definitive method of treatment has been described to eradicate the disease and augment the patients’ quality of life. However, some novel modalities have been reported recently to treat IBDs (5-7).
Hyperbaric oxygen (HBO₂), 100% oxygen at 2-3 atmospheres absolute pressure, results a significant increase in arterial oxygen tension, sometimes up to 2000 mmHg, and oxygen tension of tissues as much as 400 mmHg (8). HBO₂ has various beneficial physiological, cellular and biochemical effects. HBO₂ has been used successfully to treat decompression sickness, air embolism and carbon monoxide poisoning (7,8). HBO₂ also restores decreased neutrophil-mediated killing of bacteria due to local hypoxia by augmenting free radical generation and increases defense against infection by enhancing the rate of killing of bacteria by phagocytes (9). HBO₂ has also been successful to treat refractory skin wounds, burn wounds and perianal Crohn’s disease (10-12).

The aim of this study is to investigate the therapeutic effects of hyperbaric oxygen on experimentally induced colitis in rats by assessing the macroscopic and microscopic features and measuring tissue myeloperoxidase (MPO) activity and protein oxidation (PO).

METHODS

Animals
Forty-two male Wistar-Albino rats (200 and 260 g) were used. Rats were kept at constant temperature (20±2°C) and light-dark cycles (12-h/12-h). Animals were fed with regular rat chow and tap water. The experimental protocol was designed according to the ethical standards for animal use. Principles in the care and use of animals approved by the Council of the American Physiological Society were followed.

Induction of Colitis
The rats were anaesthetized with ether and colitis was induced using the method described by Morris et al (13). A single intracolonic dose of 30 mg of 2,4,6-trinitrobenzene sulphonic acid (TNBS; Sigma Chemical Co., St.Louis, MO, USA) in 1 ml of 50% ethanol (vol/vol) was administrated through a 5 French catheter placed 8 cm from the anal verge.

Hyperbaric Oxygen Administration
Taskizak Navy Dockyard produced the hyperbaric oxygen chamber in 1988 of a special design for animal studies (14). HBO₂ treatment was applied as 100% oxygen at 2-ATA for 2 hours daily. Rats were placed in the chamber with their wire-cages. All same-group rats underwent HBO₂ treatment simultaneously.

Experimental Protocol
Rats were separated into SHAM (n=4), vehicle (VHC, n=6), acute colitis with no treatment (ACNTR, n=8), chronic colitis with no treatment (CCNTR, n=8), acute colitis with HBO₂ treatment (ACHBO₂, n=8), and chronic colitis with HBO₂ treatment (CCHBO₂, n=8) groups. Neither intracolonic instillation nor HBO₂ treatment was given to animals in the SHAM group. Colitis was induced in other groups after an overnight fast. In the VHC group, only a single intracolonic dose of 1 ml of 50% ethanol was administrated as described above. In ACNTR, CCNTR, ACHBO₂, and CCHBO₂ groups, TNBS-ethanol was administered in order to induce colitis. In ACHBO₂ and CCHBO₂ groups, rats underwent HBO₂ treatment, commencing at the day of colitis induction, for 3 and 15 sessions, respectively (6). Animals in ACNTR and CCNTR were kept alive under normal laboratory conditions without any treatment until their sampling date, which were third and 15th days after the colitis induction, respectively.

The rats in ACNTR and ACHBO₂ groups were autopsied on day 3, where in CCNTR and CCHBO₂ groups, sampling took place on day 15. A midline abdominal incision was made under ether anesthesia and 1 ml of blood was obtained from the portal vein in order to measure PO.
Afterwards, the entire colon was resected and incised along its anterior longitudinal axis. The interior aspect of distal 8 cm of colon was inspected to score macroscopic damage (MD) according to the criteria of Wallace et al (15; Table 1). Then this 8 cm long–colon was divided into full thickness segments of 1 cm on its longitudinal axis. In each animal, the single most distal segment was selected and placed in 10% formalin solution for further blocking in paraffin. The one proximal to this segment was kept at −70°C for measurement of MPO activity. The animals were decapitated to terminate life.

**Table 1. Macroscopic damage scoring system**

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>No damage</td>
</tr>
<tr>
<td>1</td>
<td>Hyperaemia, no ulcers</td>
</tr>
<tr>
<td>2</td>
<td>Hyperaemia and thickening of the bowel wall, no ulcers</td>
</tr>
<tr>
<td>3</td>
<td>One ulcer without thickening of the bowel wall</td>
</tr>
<tr>
<td>4</td>
<td>Two or more sites of ulceration/inflammation</td>
</tr>
<tr>
<td>5</td>
<td>Two or more major sites of ulceration and inflammation or one site of ulceration/inflammation extended &gt;1 cm along the length of the colon</td>
</tr>
<tr>
<td>6-10</td>
<td>If the damage covered &gt;2 cm along the length of the colon, the score was increased by 1 for each additional cm of involvement</td>
</tr>
</tbody>
</table>

The tissues fixed in 10% formalin solution were embedded in paraffin, and routine 5 µm serial sections prepared on glass slides. Sections were stained with haematoxylin & eosin and examined blindly under light microscopy at x40, x100 and x400 magnifications by the same observer. Blood samples taken from the portal vein were centrifuged for 15 minutes at 3,000 g and plasma frozen at −70°C for determination of PO.

**Determination of Myeloperoxidase Activity**

Tissue MPO activity was utilized as an index of tissue accumulation of polymorphonuclear (PMNs) (16). The tissue samples (0.2 to 0.5 g) taken from distal colon were homogenized in 10 vol of ice-cold potassium phosphate buffer (20 mM \( \text{K}_2\text{HPO}_4 \), pH 7.4). The homogenate was centrifuged at 10,000 rpm for 20 minutes at 4°C. The pellet was homogenized with an equivalent volume of 50 mM acetic acid (pH 6.0) containing 0.5% (w/v) hexadecyltrimethylammonium hydroxide. MPO activity was evaluated by measuring the \( \text{H}_2\text{O}_2^- \) dependent oxidation of 3,3',5,5'-tetramethylbenzidine (Sigma). One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance of 1.0/minute at 655 nm and 37°C (17).

**Determination of Protein Oxidation (Carbonyl Content)**

Plasma carbonyl molecule formation was evaluated to reflect PO as a biochemical marker of oxidative damage in tissue samples (18). PO was quantified using the interaction between 2,4-dinitrophenylhydrazine (DPN; Sigma) and the carbonyls generated from the peroxyl-mediated oxidation of proteins to yield a chromophore that absorbs strongly at 380 nm (19). A 0.5 ml aliquot of plasma obtained from portal vein were precipitated by addition of 20% trichloroacetic acid (TCA). Precipitated protein was collected by centrifugation and suspended in 0.5 ml of 10 mM DNP dissolved in 2 M HCl. The samples were incubated for 1 hour at 37°C with occasional mixing. Then, protein was precipitated by addition of 20% TCA, collected by centrifugation, and the pellet washed three times with 1 ml of an ethanol: ethyl acetate (1:1) solution to remove
unreacted DNP. The protein precipitate was solubilized by addition of 1 ml of 1 N NaOH, and the absorbance was determined at 380 nm. The carbonyl content was calculated assuming a molar extinction coefficient of 22.000.

**Statistical Analysis**

All data are expressed as the mean ± S.E.M. *Multiple analysis of variance (ANOVA)* and *unpaired student’s t-test* were used for statistical analysis to determine significance among the variances. Differences were considered significant when *p<0.05*.

**RESULTS**

The main results of the study were summarized in Table 2.

**Table 2. Summary of results (mean ± SEM)**

<table>
<thead>
<tr>
<th>Value</th>
<th>SHAM</th>
<th>VHC</th>
<th>ACNTR</th>
<th>ACHBO₂</th>
<th>CCNTR</th>
<th>CCHBO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD score</td>
<td>0.0 ± 0.0</td>
<td>1.7 ± 0.2</td>
<td>4.5 ± 0.9*</td>
<td>2.8 ± 0.5#</td>
<td>3.7 ± 0.4</td>
<td>2.8 ± 0.3*</td>
</tr>
<tr>
<td>MPO (u/g)</td>
<td>125 ± 47</td>
<td>99 ± 20</td>
<td>230 ± 61</td>
<td>86 ± 21*</td>
<td>98 ± 13</td>
<td>95 ± 18</td>
</tr>
<tr>
<td>PO (nmol/ml)</td>
<td>376 ± 54</td>
<td>388 ± 43</td>
<td>428 ± 21</td>
<td>456 ± 61</td>
<td>487 ± 80</td>
<td>326 ± 51#</td>
</tr>
</tbody>
</table>

SEM= standard error of mean; MD= macroscopic damage; *= p<0.05;#= p=0.05; VHC= vehicle; ACNTR= acute colitis with no treatment; ACHBO₂= acute colitis with hyperbaric oxygen treatment; CCNTR= chronic colitis with no treatment; CCHBO₂= chronic colitis with hyperbaric oxygen treatment; MPO= myeloperoxidase; PO= protein oxidation.

**MD Scoring**

MD score of SHAM was nil. MD score of CCNTR group (3.7± 0.4) was increased compared to the VHC group (1.7± 0.2) where the difference was found to be significant (*p<0.001*). Although there was a decrease of MD score in ACHBO₂ group (2.8± 0.5) when compared to MD score of ACNTR group (4.5± 0.9), it was not significant. There was a significant difference between CCNTR and CCHBO₂ groups, where the high MD score determined in CCNTR group decreased in CCHBO₂ group (2.8± 0.3) after receiving HBO₂ (*p<0.05*). There was no difference between ACNTR and CCNTR groups (Table 2).

**Histopathology**

The appearance of colon was normal in SHAM group. In VHC group, mild inflammatory changes such as neutrophil infiltration limited to mucosa and submucosa as well as disruption of glandular structures was apparent. Significant acute inflammatory cell infiltration and extensive destruction of colonic layers with wide ulceration was observed in the ACNTR group. Almost the same features of acute inflammatory changes were apparent in ACHBO₂ group, but to a lesser extent. In the CCNTR group, chronic inflammatory changes such as mononuclear cell infiltration dominate the histopathology with scarce transmural ulcer formation. Distinct features of regeneration such as reorganization of superficial epithelium and reactive lymphoid structure formation were observed in the CCHBO₂ group.
Myeloperoxidase Activity

The MPO activity in ACNTR group (230±61 u/g wet weight [ww]) was higher than that of SHAM group (125±47 u/g ww), but the difference was not significant statistically. There was also no difference between MPO activities of SHAM and CCNTR groups (98±13 u/g ww), or between the VHC (99±20 u/g ww) and CCNTR groups. When the ACNTR group was compared to the CCNTR group, there was a significant decrease of MPO activity in CCNTR group (p<0.05). The difference between MPO activities of the CCNTR and CCHBO2 (95±18 u/g ww) groups were not found to be significant, whereas MPO activity was significantly lower in the ACHBO2 (86±21 u/g ww) group than in the ACNTR group (p<0.01) (Table 2).

Protein Oxidation (Carbonyl Content)

There were no significant differences in carbonyl contents of SHAM (376±54 nmol/ml), VHC (388±43 nmol/ml), ACNTR (428±21 nmol/ml) and CCNTR (487±80 nmol/ml) groups. There was also no significance difference between carbonyl contents of the ACNTR and ACHBO2 (456±61 nmol/ml) groups. The carbonyl content of CCHBO2 group (326±51 nmol/ml) was significantly lower than that of CCNTR group (p<0.05) (Table 2).

DISCUSSION

In the present study, HBO2 significantly improved healing of chronic colonic damage induced by TNBS-ethanol. Macroscopic and microscopic assessment criteria as well as PO levels clearly showed that HBO2 ameliorated IBD resembling colitis in rats. Since IBD affects the bowel transmurally, chronic colitis in rats is more likely to mimic human disease (1). The findings of a therapeutic role in colitis for HBO2 were more obvious in this group. Improved healing was not pronounced when considering HBO2’s effect on the acute colitis group. The data reflected a decrease in inflammation but not the cellular damage occurring in the acute period. Since MPO is a marker of neutrophilic inflammation (16), and HBO2 diminished the MPO levels, inflammation was significantly less in the acute phase. Thus HBO2 is effective in decreasing the inflammation but could not promote healing in the short interval between induction of colitis and sampling time in the acute colitis group. Prolonged exposure to HBO2 may have cumulative effects, which also improve healing. In addition, the interval necessary for effectiveness probably had been achieved by 14 days. Microscopic findings revealed the regenerative changes by HBO2 in chronic colitis, such as re-epithelialization, which was not apparent in the acute phase.

We chose the TNBS-ethanol method to induce the colitis because TNBS is a contact sensitizing allergen, and the ethanol breaks the mucosal barrier and allows penetration of TNBS into the bowel wall (1,13). Since TNBS is a covalently reactive compound, its administration results in acute transmural necrosis, which is likely, caused by oxidative damage (20). Results of macroscopic damage in the present study showed that colonic inflammation was due to TNBS, not to ethanol only. Although TNBS model of experimental colitis differs from human IBD, the inflammatory response resembles the events in human IBD. There was significant colonic tissue damage resembling human IBD at 14 days following TNBS-ethanol induction in the present study. We determined carbonyl contents in portal vein in order to assess PO. This provided more objective criteria to evaluate the colonic damage (18) than MPO determination, which is a method of assessing neutrophil inflammation. Since a widely acknowledged objective scoring system for microscopic evaluation is not available, we commented on the microscopic appearance according to the main features of inflammation and tissue damage. Microscopic
assessment and macroscopic damage scoring as well as carbonyl content determination provided additional objective information.

The experimental data in this study, although encouraging, have certain limitations. The differences between normal rats and colitis groups were not significant in comparisons of MPO and PO measurements. These findings were not concordant to the MD scoring and microscopic features supporting damage due to TNBS-ethanol enema administration. This discrepancy could be the result of the relatively small sample number in each group and/or the qualitative nature of the histology assessment.

Since various mediators such as arachidonic acid metabolites, interleukins and nitric oxide have been proposed to be involved in colonic inflammation (3-5), some novel modalities such as bombesin (6), N\textsuperscript{G}-nitro-L-arginine methyl ester (5,21), bosenthan (22) and rebamipide (23) have been proposed for the treatment of experimental colitis. In a recent study, Rachmilewitz et al (7) found that HBO\textsubscript{2} treatment effectively decreased tissue damage in both TNBS-ethanol and acetic acid induced colitis. In their study, they assessed the colonic injury on day 1 and 7, which is rather a short time for establishing chronic colitis. They found that exposure to HBO\textsubscript{2} significantly decreased NOS activity, MPO level and eicosanoid production, as well as macroscopic damage in rats with colitis within 7 days. They proposed a mechanism for this effect in which HBO\textsubscript{2} either decreased the production of nitric oxide synthase (NOS) or oxygen radicals (OR).

HBO\textsubscript{2} treatment has been used effectively in various experimental and clinical conditions without major side effects. The therapeutic effects of HBO\textsubscript{2} have been proposed to relate to the mechanical effects of increased pressure and physiological effects of hyperoxia (24). It has also been reported to be effective treatment for experimental small bowel ischemia-reperfusion injury (25) and severe perianal Crohn’s disease (12). There are various explanations for HBO\textsubscript{2}’s local and systemic effects on tissues against these disorders. The most widely acknowledged characteristic of HBO\textsubscript{2} is that it significantly increases systemic and local arterial oxygen pressures, which relieves oxygen debt in tissues (8). Undeniably, this results in acceleration of the healing process.

In the present study, exposure to HBO\textsubscript{2} apparently decreased the inflammation in the acute phase and ameliorated the colonic damage in chronic phase of TNBS-ethanol induced rat colitis. We attributed these results to HBO\textsubscript{2}’s anti-inflammatory effects for which the exact mechanisms still remain to be clarified. In fact, increasing oxygen delivery to the inflamed area by HBO\textsubscript{2} exposure would have helped restore cellular oxygen utilization, further resulting accelerated tissue repair. Future studies must be planned to clarify the exact roles of NOS, ORs and the interaction between each other in the pathogenesis of colitis. Finally, controlled human studies should be designed to evaluate the role of HBO\textsubscript{2} in IBD treatment, which may have clinical promise.

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REFERENCES