Protective effect of hyperbaric oxygen treatment on rat intestinal mucosa after mesenteric ischaemia and reperfusion

Kurtuluş Açiksari¹, Seracettin Eğin², Gülçin Hepgül³, Gamze Tanriverdi⁵, Devrim S Kanber⁶, Sibel Demirci⁵, Halil Doğan⁷, Doğanç N Özüçelik⁸, Akın S Toklu⁴, İsmail Seçkin⁵, Hakan T Yanar⁹

¹Department of Emergency Medicine, Faculty of Medicine, Istanbul Medeniyet University, Istanbul, Turkey
²Department of General Surgery, MoH Okmeydanı Training and Research Hospital, Istanbul, Turkey
³Department of General Surgery, Bagcilar Training and Research Hospital, Istanbul, Turkey
⁴Department of Underwater and Hyperbaric Medicine, Faculty of Medicine, Istanbul University, Istanbul, Turkey
⁵Department of Histology and Embryology, Cerrahpasa Faculty of Medicine, Istanbul University, Istanbul, Turkey
⁶Department of Biophysics, Cerrahpasa Faculty of Medicine, Istanbul University, Istanbul, Turkey
⁷Department of Emergency Medicine, MoH Bakirkoy Dr. Sadi Konuk Training and Research Hospital, Istanbul, Turkey
⁸Health Sciences Faculty, Istanbul University, Istanbul, Turkey
⁹Department of General Surgery, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkey

Corresponding author: Assistant Professor Kurtuluş Açiksari, Department of Emergency Medicine, Faculty of Medicine, Istanbul Medeniyet University, Istanbul, Turkey. kurtulus.aciksari@medeniyet.edu.tr

Key words
Mesenteric ischaemia; Ischaemia-reperfusion injury; Histology; Experimental study

Abstract

Introduction: Mesenteric ischaemia results from a lack of adequate blood flow and oxygenation through the mesentery and intestines. The aim of the present study was to evaluate the effect of hyperbaric oxygen treatment (HBOT) on the healing process in intestinal mucosa of rats undergoing mesenteric ischaemia and reperfusion.

Methods: Thirty-two Wistar-Albino rats were divided into four groups of eight: 1) ischaemia/reperfusion (I/R); 2) sham operation; 3) I/R+HBOT started 6 hours after reperfusion; 4) I/R+HBOT started 12 hours after reperfusion. In the I/R groups, a vascular clamp was placed across the superior mesenteric artery to occlude arterial circulation for 60 minutes, followed by reperfusion. A dose of HBOT consisted of 100% oxygen breathing for 90 minutes at 2.5 atmospheres absolute pressure. Thirteen doses of HBOT were administered after ischaemia. The rats were sacrificed on the eighth day, and their intestinal tissues were harvested for histopathologic analysis. The tissue levels of catalase, malondialdehyde, and glutathione were determined.

Results: The histopathological scores (HSCORE) were consistent with macroscopic examinations. The scores were significantly higher (worse) in Group 1 compared to Group 2, Group 3, and Group 4 (for all comparisons, P < 0.05). Group 4’s HSCORE was significantly higher than those of Group 2 and Group 3 (for both comparisons P < 0.05). Group 3’s HSCOREs were only marginally higher than Group 2. Group 3 exhibited higher glutathione levels than Group 1 (P < 0.05). There were no significant differences across the groups with respect to malondialdehdye and catalase levels.

Conclusion: A beneficial effect of HBOT was observed on oxidative stress and inflammation in acute mesenteric ischaemia-reperfusion.

Introduction

Mesenteric ischaemia is caused by an insufficient blood flow and oxygenation through the mesentery and intestines. Acute mesenteric ischaemia (AMI) followed by reperfusion leads to ischemia reperfusion (I/R) injury and is a very serious life-threatening syndrome that can cause disseminated tissue injury or multi-organ failure.

One per thousand cases seen at emergency departments in Europe and the USA are AMI events. The occurrence of AMI is increasing in parallel to the increase in the prevalence of co-morbid diseases in an aging population. Pre-existing diseases worsen the prognosis in intestinal necrosis. If AMI is not treated, it may result in infarction of the mesenteric region, intestinal necrosis, augmented inflammatory responses, and death. Through early intervention this process
might be stopped and reversed, though reperfusion itself can be associated with injury. The diagnosis of AMI is difficult and failure to diagnose it before the development of intestinal necrosis is responsible for its high mortality rate.\textsuperscript{1-5}

Hyperbaric oxygen treatment (HBOT) is used for the treatment of various diseases such as carbon monoxide poisoning, decompression illness, osteomyelitis, and diabetic foot wounds.\textsuperscript{6} HBOT entails 100% oxygen inhalation for periods of 1–2 hours intermittently under a higher than normal ambient pressure. This increases the dissolved oxygen concentration within arterial blood and, hence, increases oxygen diffusion rates in tissues with poor perfusion.\textsuperscript{7} Recently, it has been found that it may have a protective effect in central nervous system ischaemic conditions such as stroke,\textsuperscript{8} acute cerebral ischaemia,\textsuperscript{9} and in cardiovascular ischaemic events.\textsuperscript{10,11}

The aim of this study was to evaluate the effect of HBOT on the healing process of intestinal mucosa of rats undergoing mesenteric ischaemia and reperfusion procedures.

Materials and methods

STUDY DESIGN AND SETTINGS

This study was conducted at the Experimental Medical Center of Istanbul University after approval from the local ethical committee for animal studies of the same institution (Process number: 96, 2013). HBOT was conducted under the supervision of an underwater medicine expert from Istanbul University’s Underwater Medicine Department.

EXPERIMENTAL ANIMALS

Thirty-two male Wistar-Albino rats, each weighing between 250 and 300 g, were included. They were housed in stainless steel cages at a constant temperature (22°C) and a 12-h day/night cycle. The rats were fasted but had free access to water the night before the experiment. They were fed commercial rat chow and accessed water ad libitum at other times.

INTESTINAL ISCHAEMIA / REPERFUSION (I/R) MODEL

Rats were anaesthetized using 0.1 ml·100g\textsuperscript{-1} intraperitoneal ketamine hydrochloride 50 mg·ml\textsuperscript{-1} and xylazine hydrochloride 20 mg·ml\textsuperscript{-1} mixed at a ratio of 2:1 respectively. After anaesthesia a 4 cm median laparotomy incision was made aseptically. The small intestine was exposed and the mesenteric artery was located and isolated without harming the mesenteric vein. The mesenteric artery was obstructed with a vascular clamp (ischaemic process). After clamping the artery, the small intestine was placed into the abdominal cavity again for 60 minutes and the surgical wound was stitched using 4-0 monofibre nylon stitches. After 60 minutes of ischaemia the abdominal cavity was reopened, and the vascular clamp was removed, starting the reperfusion process. At this time, the abdomen was again closed with 4-0 monofibre nylon until the experiment was completed.

HYPERBARIC OXYGEN TREATMENT

HBOT was administered in an experimental hyperbaric chamber as follows. The rats were pressurized over 15 minutes to 2.5 atmospheres absolute (atm abs) pressure. They breathed 100% oxygen at this pressure for 90 minutes before being decompressed back to 1 atm abs over 15 minutes. Every treatment commenced at the same hour in the morning (10:00 AM) in order to minimize any effect of biological rhythm changes. Rats receiving HBOT underwent a seven day course as follows; three sessions per day in the first two days (starting at six or 12 hours after reperfusion on day one as above); two sessions per day in the third and fourth days, and one session in each of the subsequent three days.

EXPERIMENTAL GROUPS

The animals were divided into four groups consisting of equal numbers of rats (n = 8).

Group 1, Ischaemia/reperfusion (I/R): Ischaemia and reperfusion procedures were performed without HBOT.

Group 2, Sham operation: Small intestines were exposed, and mesenteric arteries were located and dissected. When the process ended, the abdomen was stitched with 4-0 monofibre nylon stitches and remained stitched until the experiment was completed.

Group 3, I/R + HBOT started 6 h after reperfusion: HBOT was initiated 6 h after the beginning of reperfusion. All animals in this group received HBOT for seven days.

Group 4, I/R + HBOT started 12 h after reperfusion: HBOT was initiated 12 h after the beginning of reperfusion. All animals in this group also received HBOT for seven days.

COLLECTION OF SAMPLES

All rats were sacrificed on the eighth day by administering an excessive amount of an anaesthetic into the heart (2 ml of ketamine hydrochloride 50 mg·ml\textsuperscript{-1}). Ten centimetres of small intestine proximal to the ileocaecal area was removed for histopathological analysis.

HISTOLOGICAL PREPARATION

Histopathology was conducted with the laboratory staff blinded to the study groups. One centimetre segments from the third, fifth, and seventh cm of the 10 cm small intestine specimens were placed in 10% neutral buffered formalin for at least 24 h. Paraffin-embedded cross-sections (3 mm) were stained with hematoxylin-eosin (H + E) and Azan.
Table 1
Scoring system for I/R injury as described by Verhaegh et al.\textsuperscript{12}

<table>
<thead>
<tr>
<th>Grade</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal mucosa</td>
</tr>
<tr>
<td>1</td>
<td>Subepithelial Gruenhagen space capillary congestion</td>
</tr>
<tr>
<td>2</td>
<td>Extension of subepithelial space with moderate epithelial lifting</td>
</tr>
<tr>
<td>3</td>
<td>Massive epithelial lifting down the sides of villi, few tips denuded</td>
</tr>
<tr>
<td>4</td>
<td>Denuded villi</td>
</tr>
<tr>
<td>5</td>
<td>Loss (destruction) of villi, haemorrhage</td>
</tr>
<tr>
<td>6</td>
<td>Crypt layer injury</td>
</tr>
<tr>
<td>7</td>
<td>Transmucosal infarction</td>
</tr>
<tr>
<td>8</td>
<td>Transmural infarction</td>
</tr>
</tbody>
</table>

I/R INJURY HISTOLOGY

Specimens were evaluated and photographed by a bright field microscope (Olympus BX61, Tokyo, Japan) under magnifications of 10 or 20 times (see figure captions). Histopathologic changes were evaluated by a single independent assessor blinded to study group. The degree of I/R injury was scored on a scale from 0–8 as described by Verhaegh et al.\textsuperscript{12} (Table 1).

ANTIOXIDANT / OXIDATIVE STRESS MARKERS

Tissue specimens were weighed and homogenized in cold 0.1 M phosphate buffer (pH 7.4) using an automatic homogenizer. The homogenates were then centrifuged at 15,000 rpm at 4°C for 15 min. Clear supernatants were used for the catalase (CAT), glutathione (GSH), and malondialdehyde (MDA) assays. Tissue protein levels were also measured at this step using the method described by Lowry et al.\textsuperscript{13}

Catalase enzyme converts hydrogen peroxide to water and oxygen. Catalase activity was measured by the Aebi method.\textsuperscript{14} This method is based on the hydrolyzation of H\textsubscript{2}O\textsubscript{2} and reduced absorbance at 240 nm. The results are expressed as U·mg\textsuperscript{-1} of protein tissue.

Malondialdehyde (MDA) is a product of lipid peroxidation. Tissue MDA assays were performed according to the method described by Ohkawa et al.\textsuperscript{15} MDA reacts with thiobarbituric acid under acidic conditions at 95°C, forming a pink complex that absorbs at 532 nm. 1,1,3,3-tetraethoxypropane was used as the standard. The results are expressed in nmol·ml\textsuperscript{-1}.

Glutathione (GSH) levels were determined according to Beutler’s method using Ellman’s reagent.\textsuperscript{16} The procedure is based on the reduction of Ellman’s reagent by sulfhydryl groups to form 5,5’-dithiobis (2-nitrobenzoic acid) with an intense yellow colour, measured spectrophotometrically at 412 nm. The results were expressed as nmol·mg\textsuperscript{-1} of protein.

Table 2
Histopathological scoring of intestinal injuries by experimental group. Group 1 = I/R injury. Group 2 = sham operation. Group 3 = I/R + HBOT started at 6 hours after I/R. Group 4 = I/R + HBOT started at 12 hours after I/R. * = P < 0.005 compared to Group 1. # = P < 0.005 difference with Group 4

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean (SD)</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.0 (0.5)</td>
<td>7.1</td>
<td>6.4–7.8</td>
</tr>
<tr>
<td>2</td>
<td>0.3 (0.2)*#</td>
<td>0.3</td>
<td>0.0–0.6</td>
</tr>
<tr>
<td>3</td>
<td>0.4 (0.3)*#</td>
<td>0.3</td>
<td>0.0–1.0</td>
</tr>
<tr>
<td>4</td>
<td>1.1 (0.7)#</td>
<td>1.1</td>
<td>0.2–2.4</td>
</tr>
</tbody>
</table>

STATISTICAL ANALYSIS

The distribution of the variables was tested using the Kolmogorov Smirnov test. Descriptive statistics included mean, standard deviation, median, maximum and minimum values where appropriate. Kruskal-Wallis and Mann-Whitney U tests were used for the analysis of quantitative data. Sample size was determined based on an anticipated effect of HBOT derived from similar study by Bertoletto et al.\textsuperscript{17} With alpha set at 0.05 and with eight rats per group the study power was 86%. SPSS 22.0 software package (IBM, New York, USA) was used for all statistical analyses.

Results

HISTOPATHOLOGICAL FINDINGS

The histopathological scores (HSCORE) of the intestinal injuries are shown in Table 2. HSCOREs were significantly higher (worse) in Group 1 compared to Groups 2, 3, and 4 (P < 0.05). Group 4’s HSCORE was significantly higher than those of Group 2 and Group 3 (P < 0.05). There was a small but significant difference between Group 2 and 3 (the latter being higher) (P < 0.05). Initial HBOT at 6 h after I/R injury (Group 3) resulted in a better HSCORE when compared to HBOT started at 12 h (Group 4).

The histological examination of Group I (I/R) demonstrated injured intestinal mucosae with moderate epithelial lifting, destruction or loss of villi, haemorrhage and also crypt layer injuries (Figure 1 a, b, c, d). Normal histological structures were observed in Group II (sham operation) (Figure 2 a, b, c, d). Group III (I/R + HBO at 6 hours) (Figure 3 a, b, c, d), and Group IV (I/R + HBO at 12 hours) (Figure 4 a, b, c, d) exhibited near normal intestinal mucosae, intact villi and epithelial layers with the goblet cells between columnar epithelial cells.

BIOCHEMICAL ANALYSIS

Oxidative stress marker assays are presented in Table 3. There were no differences between the study groups with respect to MDA and CAT levels that reached statistical significance. A significant difference was found between Group 1 and Group 3 with respect to GSH levels (P < 0.05). In Groups 3 and 4 there were increases in GSH...
levels and CAT enzyme activity compared to the other groups (Group 3 > Group 4). The tissue MDA levels in Groups 3 and 4 were higher than the other groups. However, these differences did not reach statistical significance.

Discussion

HBOT significantly decreased intestinal damage in this animal model of I/R injury. In particular, HBOT initiated 6 h following the I/R injury markedly reduced histological damage scores compared to the untreated I/R group. In addition, there were increased levels of GSH and CAT enzyme activity in the HBOT groups, suggesting that HBOT positively affected antioxidant capacity, though at different levels.

It is thought that HBOT after I/R may ameliorate both ischaemia and the adverse effects of reperfusion. The beneficial effects of high oxygen concentration on ischaemic or I/R damage have inspired many studies over the years. In experimental studies, intraluminal oxygen administration was tried before the use of HBOT in this area. In a 1976 study using rats, it was found that mortality rates decreased by 50% when oxygen was administered into the lumen of ischaemic intestine. The authors suggested that the administration of intraluminal oxygen to protect the mucosal integrity until
sufficient blood flow was restored would increase survival rates in humans.19

A beneficial effect of HBOT in ischaemic or I/R injuries has been demonstrated in different tissues and organs.20–22 In our study, we found that HBOT initiated 6 h after reperfusion of ischaemic bowel was more protective than the same treatment initiated after 12 h. Whether even earlier initiation of HBOT after the injury might be more beneficial requires additional study.

Although there are small number of reports of a beneficial effect of HBOT in the treatment of ischaemia and ischaemic ulcers at colonic anastomoses following the resection of colon, data describing HBOT for the treatment of these conditions in humans are scarce.23,24 Thus, defining an HBOT paradigm for optimum benefit needs further study on different injury types and patient age groups, including patients with comorbidities. However, for the treatment of I/R injuries, HBOT might be an alternative or adjunct to conventional treatment choices.

The evaluation of the GSH and CAT levels of Group 3 suggested that HBOT at 6 h increased antioxidant levels/activity more than the same treatment administered at 12 h. It was thought that HBOT started after 12 h might have prevented the necessary responses from occurring since, by that time, the critical time necessary for activation of the antioxidant system passes. These results may be a guide for determining the timing of treatment after I/R.

As an end product of lipid peroxidation, MDA is regarded as an indicator of oxidative stress. Ilhan et al.25 found that HBOT administered prior to ischaemia elicited a beneficial effect on renal I/R by reducing oxygen radical peroxidation of lipid membranes. In our study, the MDA levels of the groups that received HBOT (Groups 3 and 4) were higher than those of the other groups, although this did not reach to statistically significant level. The rise of the MDA levels may be considered to be a sign of oxidative damage thought to develop as a result of increased oxygen in a cellular level after HBOT. However, the elevation of antioxidant levels in the same groups may have mitigated peroxidative damage, providing a balance between the oxidant and antioxidant systems.

This study has several limitations. Firstly, the follow-up time is relatively short. However, the histology in the HBO treated animals was little different compared to the surgical controls, so complications arising after longer follow-up seems unlikely. Secondly, the rats received HBOT based on only one dosing regimen. Other dosing regimens might yield more beneficial results, and whether the treatment used here was optimal for achieving maximum benefit from HBOT is unknown. Unfortunately, studies on the dose, duration, timing, and number of repetitions for HBOT are very limited in all its indications. Finally, observations gained from experimental animal models may not translate successfully to humans with similar clinical conditions.

The strengths of this study include being a very thoroughly planned and standardized experiment with blinded evaluation of eventual outcomes. Additionally, significant amelioration of tissue injury in our study group might add extra data to the literature on potential benefits of HBOT after I/R injury.

### Conclusion

Tissue histology and oxidative stress parameters demonstrated a protective effect of HBOT against mesenteric I/R injury in this rat model, especially when initiated at 6 h after reperfusion. HBOT may ameliorate tissue injury as a supplementary intervention in mesenteric I/R scenarios.

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A recurrent, ischaemic ileocolonic injury


Conflicts of interest and funding: nil

Submitted: 06 April 2019
Accepted after revision: 25 July 2019

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