Effects of hyperbaric oxygen therapy on acetaminophen induced nephrotoxicity and hepatotoxicity: the role of heme oxygenase-1

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Abstract
The aim of this study was to investigate the effects of hyperbaric oxygen (HBO) therapy on acetaminophen (APAP) induced renal and liver injury and the role of heme oxygenase-1 (HO-1) activation. Wistar-Albino rats were randomly assigned into four groups. Control group received no treatment. APAP (3gr/kg) was administered by gastric lavage in APAP group. Animals in the APAP+HBO and APAP+zinc protoporphyrin (ZnPP)+HBO groups received HBO therapy (90 min at 2.5 atm), starting 1 hour after APAP administration, for 2 consecutive days. HO-1 activity was inhibited by ZnPP. APAP+ZnPP+HBO group received intraperitoneal 50 µmol/kg ZnPP injection 30 minutes after APAP treatment and HBO therapy for 2 days. Serum and tissue samples were taken at 48 hours after APAP treatment. Renal and liver functions were evaluated by serum levels of urea, creatinine and transaminases. Lipid peroxidation and tissue levels of antioxidant enzymes were measure by ELISA. Tissue injury was evaluated by light microscopy. HO-1 level was determined by immunohistochemistry. HO-1 mRNA level was investigated by polymerase chain reaction (PCR). Serum transaminase levels significantly increased after APAP treatment (p<0.05) and severe tissue injury was detected on liver slides (p<0.05). HBO therapy both reduced transminase levels and alleviated liver injury (p<0.05). The inhibition of HO-1 by ZnPP aggravated liver injury (p<0.05). HBO therapy reduced lipid peroxidation (p<0.05) and increased the activity of superoxide dismutase (p<0.05). Tissue HO-1 level increased after APAP treatment (p<0.05). APAP nephrotoxicity was not observed in this model. In conclusion, HBO therapy ameliorates APAP induced liver injury by increasing liver HO-1 levels.

Introduction
Acetaminophen (APAP) is one of the most frequently used analgesics in the world. APAP overdose causes hepatotoxicity and nephrotoxicity in humans and animals (1,2). It is suggested APAP toxicity is caused by N-acetyl-p-benzoquinone imine (NAPQI), which depletes intracellular glutathione (GSH) levels (3). Thus, the metabolism of NAPQI is interrupted. NAPQI causes toxicity by binding intracellular proteins. Other mechanisms are also possible. APAP is metabolised by the cytochrome P-450 (CYP) system via reductive metabolism. CYP system uses oxygen to metabolise APAP and metabolism of large amount of APAP requires more oxygen. Therefore, Salhanick et al. suggested that hypoxia contributes to APAP toxicity (4). Hyperbaric oxygen (HBO) therapy, which increases tissue oxygen levels may be beneficial in APAP toxicity (4-6). Hem oxygenase-1 (HO-1), the rate limiting enzyme of heme catabolism, has antiapoptotic and antiinflammatory effects (7). Foud et al. showed that induction of HO-1 by hemin protects kidneys against APAP toxicity in rats (8). On the other hand, HBO therapy has been shown to increase tissue HO-1 levels in experimental models of renal I/R injury and sepsis (9-11).

The aim of this study was to investigate the effects
of hyperbaric oxygen (HBO) therapy on acetaminophen (APAP) induced renal and liver injury and the role of HO-1 activation.

Material and Methods

Animals

A total of 28 Wistar-Albino rats weighing 250-300 g were used in the study. Animal Ethical Committee of Gulhane Military Medical Academy (GMMA) approved the study protocol (#14/147). Rats were housed under standard laboratory conditions (22 ± 1°C, 12 h light/dark cycle) and fed with standard rat chow and tap water ad libitum.

Experimental Groups

Animal were randomly assigned into four groups: Control (n=4), APAP (n=8), APAP+HBO (n=8), APAP+Znpp+HBO (n=8). The animals in the control group received no treatment. APAP (3gr/kg) was administered by gastric lavage in APAP group. Animals in the APAP+HBO and APAP+zinc protoporphyrin (ZnPP)+HBO groups received HBO therapy (90 min at 2.5 atm), starting 1 hour after APAP administration, for 2 consecutive days in an experimental hyperbaric chamber. The chamber was compressed with 100% O2 at 2.5 ATA in 10 min. After 70 min at 2.4 ATA, the chamber was decompressed to normal atmospheric pressure in 10 min. HO-1 activity was inhibited by ZnPP. APAP+ZnPP+HBO group received intraperitoneal 50 µmol/kg ZnPP injection 30 minutes after APAP treatment. They received HBO therapy for 2 days.

Forty-eight hours after APAP administration, animals were anesthetized with intramuscular ketamine and chlorpromazine. Anterior abdominal laparotomy was performed and liver and both kidneys were removed. Then median sternotomy was applied and blood samples were collected using intracardiac puncture. Euthanasia was administered by aortic dissection.

Blood samples were centrifuged at 3000 rpm for 5 min to obtain plasma. Plasma samples were kept at -80 °C until being analyzed. Levels of aspartate amino transferase (AST), alanine amino transferase (ALT), urea, creatinine, Na+, K+ were measured with a Cobas 6000 biochemistry analyzer (Roche, Germany) using commercial kits.

Liver and kidney samples were divided into small pieces and put into formalin solution for histological and immunohistochemical examination. Other samples were frozen in liquid nitrogen, and preserved at -80°C until analysis of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and malonyl dialdehyde (MDA).

Preparation of tissue samples and determination of oxidative stress

Liver tissue samples were weighed and homogenized in 2 ml buffer (Tris (10 mM), diethylenetriaminepentaacetic acid (1 mM), phenylmethanesulphonyl fluoride (1 Mm; pH 7.4)] at +4°C by a homogenizer (Thermo scientific, USA). The tissue samples were diluted as a final concentration of 100 mg/ml. The diluted samples were centrifuged at 3000 rpm for 20 min (Heraeus Biofuge A, Germany).

SOD, CAT, GSH and MDA levels in tissue homogenates were measured by rat ELISA Kits (YH-Biosearch, Public Republic of China). The absorbances were read at 450 nm by an ELISA plate reader (Spectramax M2, Molecular Devices, USA).

The protein content of kidney homogenates was measured by using Biuret’s method.

Histology and immunohistochemistry

Kidney and liver tissues were fixed with formalin, embedded in paraffin, sectioned at 4 µm thickness and then stained with hematoxylen-eosin (H&E) and examined with a light microscope. Kidney tissue damage was assessed using a modified semi-quantitative. Kidney damage parameters as designated by examining proximal tubular structural changes, proximal tubular atrophy, loss of edge of tubular, tubular dilation, cast formation, vacuolization, mononuclear cell infiltration, erythrocyte extravasation, changing in renal corpuscle morphology and interstitial area. Similarly, liver damage were determined by centrilobular necrosis, sinusoidal dilatation and inflammation. Tissue damage score was obtained by calculating percentage of damaged structure in image area (0=normal; 1=0−25%; 2=25−50%; 3=50−75%; 4>75%).

Immunohistochemistry were carried out in formaldehyde fixed and, paraffin-embedded liver tissue sections mounted on poly L-lysine coated slides. The slides were deparaffinized and boiled in citrate buffer (pH=6) in a microwave oven for 20 minutes for antigen retrieval. The tissue sections were then incubated for 30 minutes with 3% H2O2 to block the endogenous peroxidase activity. After subsequent treatment with nor-
HBO therapy and APAP toxicity

Table 1. Biochemical and histological analysis.

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>APAP</th>
<th>APAP+HBO</th>
<th>APAP+ZnPP+HBO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Renal functions</strong></td>
<td></td>
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<tr>
<td>Na⁺</td>
<td>140.5±0.91</td>
<td>137.16±1.93</td>
<td>140.35±3.82</td>
<td>139.00±2.15</td>
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<tr>
<td>K⁺</td>
<td>5.86±0.15</td>
<td>5.78±0.63</td>
<td>6.13±0.83</td>
<td>5.63±0.53</td>
</tr>
<tr>
<td>Urea</td>
<td>56.00±3.56</td>
<td>41.38±5.40</td>
<td>49.25±15.99</td>
<td>50.63±7.09</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.26±0.03</td>
<td>0.35±0.06</td>
<td>0.37±0.03</td>
<td>0.37±0.06</td>
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<tr>
<td><strong>Liver functions</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>AST</td>
<td>102±8</td>
<td>1057±14a</td>
<td>201±54 b</td>
<td>2258±645 a,c</td>
</tr>
<tr>
<td>ALT</td>
<td>51±4</td>
<td>547±156a</td>
<td>124±40 b</td>
<td>1298±264 b,c</td>
</tr>
<tr>
<td><strong>Lipid peroxidation and antioxidant enzyme levels in liver tissue</strong></td>
<td></td>
<td></td>
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<tr>
<td>MDA</td>
<td>0.39±0.08</td>
<td>0.34±0.04</td>
<td>0.18±0.07 a,b</td>
<td>0.17±0.04 a,b</td>
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<tr>
<td>SOD</td>
<td>26.9±3.1</td>
<td>12.0±0.7 a</td>
<td>19.6±2.1 a,b</td>
<td>8.1±1.6 a,c</td>
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<tr>
<td>GPX</td>
<td>38.9±7.1</td>
<td>34.2±4.4</td>
<td>27.7±2.4</td>
<td>19.3±3.1 a,b</td>
</tr>
<tr>
<td>CAT</td>
<td>8.5±1.1</td>
<td>7.2±0.9</td>
<td>6.8±1.2</td>
<td>5.3±0.9</td>
</tr>
<tr>
<td><strong>Liver histology and HO-1 immunohistochemistry (IHC)</strong></td>
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<tr>
<td>Injury score</td>
<td>0±0</td>
<td>3.00±0.53 a</td>
<td>1.38±0.74 b</td>
<td>3.75±0.46 a,c</td>
</tr>
<tr>
<td>IHC score</td>
<td>1.00±0.0</td>
<td>1.87±0.35</td>
<td>2.25±0.46 a</td>
<td>1.71±0.95</td>
</tr>
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</table>

*p<0.05 vs. Control, a p<0.05 vs. APAP, b p<0.05 vs.APAP+HBO

Mal goat serum, sections were incubated at 37°C for 1 h with the primary anti-HO-1 antibody (1:200; AB1284; Millipore, Boston, MA, USA). Biotin-labeled goat antirabbit IgG secondary antibody (GGLH-15P; ICLllab, Newberg, OR, USA) was used, and dianinobenzidine was used for color development. Sections were counterstained with Mayer’s hematoxylin. The observer performed light microscopy and scored semiquantitatively the quantity of HO-1 staining in the whole section as 0 for negative, 1- for weak (+), 2 for moderate (++) and 3 for strong (+++) staining as described before.

**HO-1 mRNA polymerase chain reaction (PCR)**

RNA isolation from tissue samples were done with NucleoSpin RNA isolation kit (Machery-Nagel GmbH). cDNA was formed with SuperScript® III First-Strand Synthesis System (Invitrogen) kit. Primers for HO-1 mRNA were designed by using OligoYap 4.0 software. 18s rRNA was used as internal control. HO-1 Forward (5′-ACTCTGTCTCATGTAGCCTTCT-3′), HO-1 Reverse (5′-GCATCTCCATTCCTTTCA-3′), HO-1 Probe (5′-[TxRed] AGACAAAAGGAGAGACAGAGAAG[BHQ2]-3′), 18s Forward (5′-TGTCTCAAGAATTCGCGATCATGCAT), 18s Reverse (5′-AACATACCTGATAATGAGCCATTC), 18s Probe (5′-[FAM]TACGACGGCGCTAGACGTAAAC). QIAGEN OneStep RT-PCR Kit was used. Real-time PCR studies were done with Applied Biosystems 7500 Real-time PCR.

**Statistical analysis**

SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All data are presented as mean ± standard deviations (SD). The Kruskal-Wallis test was used for non-normally distributed data, and dual comparisons between groups were evaluated with Mann-Whitney U test with Bonferroni correction. A p-value less than 0.05 was considered as statistically significant.

**Results**

Renal functions were evaluated with serum levels of urea, creatinine, Na+ and K+ (Table 1). Serum urea and creatinine levels were in normal range in all groups. Renal functions did not change after APAP treatment. Liver function were evaluated with serum levels of AST and ALT (Table 1). APAP treatment increased transaminase levels, confirming the development of hepatotoxicity. HBO therapy reduced AST and ALT levels compared to APAP group. AST levels were significantly
higher in APAP+ZnPP+HBO group compared to Control and APAP+HBO groups. ALT levels were significantly higher in APAP+ZnPP+HBO group compared to Control, APAP and APAP+HBO groups.

MDA level in liver tissue decreased in APAP+HBO and APAP+ZnPP+HBO groups compared to Control and APAP groups (Table 1). SOD level significantly decreased after APAP therapy. HBO therapy increased SOD level. However, SOD level in APAP+ZnPP+HBO group was significantly lower than both APAP and APAP+HBO groups (Table 1). GPx level in APAP+ZnPP+HBO group was significantly lower than both Control and APAP groups (Table 1). CAT level were similar in all groups. Since nephrotoxicity was not observed in our model, we did not measure kidney MDA, SOD, GPx, and CAT levels.
Centrilobular necrosis was seen in APAP treated liver sections (Figure 1). Injury score was significantly higher in APAP group compared to Control group (Table 1). HBO therapy reduced tissue liver injury. ZnPP treatment increased tissue injury. Histological investigation of kidneys showed normal appearance in all groups.

HO-1 immunohistochemistry of liver samples showed increased HO-1 staining after APAP treatment (Figure 1). However, HO-1 staining scores were not significantly different in APAP group compared to Control group. HO-1 staining in APAP+HBO group was significantly higher than Control group. HO-1 staining reduced after ZnPP treatment (Table 1).

The PCR studies to detect HO-1 mRNA levels in liver tissues were unsuccesful.

Discussion

In this study we investigated the effects of HBO therapy on APAP induced nephrotoxicity and hepatotoxicity in rats. We could not evaluate renal effects of HBO therapy since nephrotoxicity was not observed. However, we found that HBO therapy reduces tissue injury and serum transaminase levels after APAP induced hepatotoxicity. Furthermore, we found that HBO therapy increased HO-1 levels in liver tissue. Inhibition of Ho-1 activity by ZnPP prevented beneficial effects of HBO therapy.

There is only one study that investigated the effects of HBO therapy on APAP induce nephrotoxicity. In this study Cermik et al. (5) combined HBO therapy with N-acetilsistein (NAC) in the treatment of APAP induced nephrotoxicity in rats. They found that HBO therapy reduced serum urea and creatinine levels as well as renal tissue injury by an antiinflammatory effect (5). Since nephrotoxicity was not developed in our study, we could not evaluate the effect of HBO therapy. Further studies are needed in this area.

The number of studies that investigated the effect of HBO therapy in APAP induced hepatotoxicity is limited. Marzella et al. (12) showed that HBO therapy (2.8 ATA, 90 minutes) starting right after APAP treatment increased serum transaminase levels measured at 24 hours. However, a longer HBO therapy (3-4 hours) reduced transaminase levels but increased mortality. Marzella et al. concluded that the deaths were related to toxic effects of prolonged HBO therapy protocols. They suggested early and short HBO therapy increases oxidative stress caused by biotransformation of drugs via CYP system but long HBO therapy protocols may be protective by non-specific inhibition of liver metabolism (12).

Salhanick et al. (4) investigated the effects of HBO therapy on APAP hepatotoxicity in rats. They showed that HBO therapy (2.5 ATA, 90 minutes) after APAP therapy reduces ALT levels measured at 4th and 6th hours. They also found that histologic scores were correlated with serum ALT levels. HBO therapy increases hypoxia inducible factor-1 (HIF-1) levels in HEP G2 hepatocytes (4). They suggested that beneficial effects of HBO therapy may be through HIF-1 transcription factor. But, HBO therapy did not increase HIF-1 transcription and DNA binding of HIF-1. Further studies are needed to shed light on this mechanism.

Taslipinar et al. (6) investigated the effects of HBO therapy in combination with NAC in a rat model of APAP hepatotoxicity. HBO therapy (2.8 ATA, 90 minutes) was started 24 hours after APAP treatment. HBO therapy was applied twice daily for 5 days. Serum transaminase levels were significantly lower in NAC and NAC+HBO groups. Additionally, histological injury scores were lower than control in NAC and NAC+HBO groups. However, both parameters were not significantly different in NAC and NAC+HBO groups. They found that HBO therapy does not have additional benefit when used in combination with NAC treatment. We think that since HBO therapy started very late compared to our study (24 h vs. 1 h), this may explain the lack of beneficial effects of HBO therapy in this study. HBO therapy should not be delayed for 24 hours after APAP toxicity.

To our best knowledge, our study is the first study that investigated the effects of HBO therapy on liver HO-1 levels after APAP toxicity. We found that HBO therapy increased liver HO-1 levels. On the other hand, inhibition of HO-1 by ZnPP increased serum transaminase levels and tissue injury. We think that beneficial effects of HBO therapy is mediated through activation of HO-1 levels in hepatocytes.

In conclusion, HBO therapy ameliorates APAP induced liver injury by increasing liver HO-1 levels. Further studies are needed to explore the beneficial mechanisms of HBO therapy.

Conflict of Interest

No conflict of interest was declared by the authors.
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References


