LABORATORY STUDY

The effect of hyperbaric oxygen therapy on rhabdomyolysis-induced myoglobinuric acute renal failure in rats

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ABSTRACT
Myoglobinuric acute renal failure (MARF) may develop after severe muscle injury. Heme oxygenase-1 (HO-1), a stress-response protein, has been implicated as a protective agent against MARF. We hypothesized that hyperbaric oxygen therapy (HBOT) may alleviate MARF by inducing renal HO-1 expression. Wistar-Albino rats were randomly assigned into three groups: Control (n = 4), MARF (n = 8), MARF + HBO (n = 8). MARF was induced by intramuscular glycerol (50%, 8 mL/kg) injection. Saline (8 mL/kg) was injected into the hind limb of the animals in the control group. Animals in the MARF + HBO group received two sessions of HBO therapy (90 min at 2.5 atm) 2 and 18 h after glycerol injection. Serum and tissue samples were taken at 24 h. Serum urea and creatinine levels increased in the MARF and MARF + HBO groups confirming the development of MARF. But, serum urea and creatinine levels were similar in MARF and MARF + HBO groups. Oxidative stress parameters were similar among all groups. Histological renal injury score was similar in MARF and MARF + HBO groups confirming the development of MARF. But, serum urea and creatinine levels were similar in MARF and MARF + HBO groups. Oxidative stress parameters were similar among all groups. Histological renal injury score was similar in MARF and MARF + HBO groups. HO-1 level, determined by immunohistochemistry, was significantly higher in MARF and MARF + HBO groups, compared to the control group. Although HO-1 level in MARF + HBO group was higher than MARF group, it was not statistically significant. We found that HBOT did not reduce renal injury in experimental MARF model. HBOT is used to reduce the muscle damage after crush injury, which may be accompanied by MARF. Therefore, more studies are needed to understand the effects of HBO treatment on renal functions after MARF.

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Introduction
Rhabdomyolysis, the breakdown of skeletal muscle cells, occurs due to traumatic and non-traumatic etiologies. The intracellular constituent of damaged muscle cells, which are released into systemic circulation, leads to several clinical manifestations. Myoglobinuric acute renal failure (MARF) is a frequent and severe complication of rhabdomyolysis. MARF develops in 7–33% of patients with rhabdomyolysis.1

The mechanisms involved in the pathogenesis of MARF are intrarenal vasoconstriction, tubular injury, and tubular obstruction.2,3 Myoglobin is a heme protein and contains iron in ferrous form (Fe⁺²), which is required for the binding of molecular oxygen. Myoglobin is freely filtered through the renal glomerulus and taken into tubular epithelial cells by endocytosis, where it is metabolized into three final products; bilirubin, carbon monoxide (CO), and Fe⁺². Fe⁺² lead to the free radical formation by Fenton and Haber–Weiss reaction. The role of oxidative stress and hydroxyl radicals are well-known in the pathogenesis of this disease. Reactive oxygen species result in oxidative stress that causes protein denaturation, DNA damage, and lipid peroxidation.4 Increased oxidative stress in MARF was shown by several studies.5–7 Hydroxyl radicals cause endothelial dysfunction and augment responses to renal sympathetic nerve stimulation in isolated perfused kidneys. These impaired vascular responses are normalized by hydroxyl radical scavengers.8 The levels of malondialdehyde (MDA) increases, and superoxide dismutase (SOD) and catalase (CAT) decrease in kidney tissue after MARF in rats.9 As a result, oxidative stress causes tubular injury.7,10 Damaged tubular epithelial cells pour into the lumen and cause distal tubular obstruction. Furthermore, excess myoglobin may form intraluminal plugs in the tubular lumen and contribute to tubular obstruction.10 Hypovolemia-induced activation of the renin–angiotensin system and sympathetic nervous
system facilitates intrarenal vasoconstriction. Hypovolemia, together with renal vasoconstriction concentrates myoglobin along the tubular lumen and increase the tubular obstruction.

Hyperbaric oxygen (HBO) therapy involves inhalation of 100% oxygen at higher atmospheric pressures. HBO therapy is used to reduce muscle damage after crush injury in earthquake victims. However, our knowledge regarding the effect of HBO therapy on renal functions in such patients is limited. A study by Sever et al reporting a 15.2% mortality rate among 639 earthquake victims with acute renal failure revealed that all 28 patients who received HBO therapy had survived. On the other hand, experimental studies conducted by our group and others have shown that HBO therapy alleviates renal injury induced by chemotherapy, ischemia/reperfusion (I/R) injury, and sepsis. Furthermore, Ayvaz et al. reported that HBO therapy (2.5 ATA 90 min, for 2 days) had a protective effect in an experimental MARF model in rats. Therefore, further studies to elucidate the potential protective role of HBO therapy in MARF are warranted.

Heme oxygenase-1 (HO-1), known to be protective against cellular stress, may have a key role in the pathogenesis of MARF. It was shown that increased HO-1 expression reduces kidney damage, whereas HO-1 inhibitors increase kidney damage at MARF models, while increased kidney damage was observed in HO-1 knockout mouse. HBO therapy was also shown to increase renal HO-1 levels in experimental models of renal I/R injury and sepsis. We hypothesized that HBO therapy may alleviate renal injury in MARF by inducing renal HO-1 expression. In this study, we investigated the effects of HBO therapy on renal functions, oxidant/antioxidant system and renal HO-1 expression in an experimental MARF model in rats.

Materials and methods

Animals

A total of 20 male 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. Rats were housed under standard laboratory conditions (22 ± 1C, 12 h light/dark cycle) and fed with standard rat chow and tap water ad libitum.

Experimental groups

Animal were randomly assigned into three groups: Control (n = 4), MARF (n = 8), and MARF + HBO (n = 8). In the control group 8 mL/kg saline was injected into the hind limb of rats and in the MARF and MARF + HBO groups 50% glycerol (8 mL/kg) was injected into hind limb of rats. Animals in the MARF + HBO group received two sessions of HBO therapy (two and 18 h after glycerol injection) in an experimental hyperbaric chamber. The chamber was compressed with 100% O2 at 2.4 ATA in 10 min. After 70 min at 2.4 ATA, the chamber was decompressed to normal atmospheric pressure in 10 min. Twenty-four hours after glycerol injection, animals were anesthetized with intramuscular ketamine and chlorpromazine. Anterior abdominal laparotomy was performed 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+, and creatine kinase (CK) in plasma were measured with a Cobas 6000 biochemistry analyzer (Roche, Germany) using commercial kits.

Half of the right kidney was put into formalin solution for histological and immunohistochemical examination. Rest of the kidney tissues 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).

Biochemical analysis

Preparation of tissue samples and determination of oxidative stress

Kidney tissue samples were weighed and homogenized in 2 mL buffer (Tris (10 mM), diethylenetriaminepentaacetic acid (1 mM), phenylmethanesulfonyl fluoride (1 Mm; pH 7.4)) at +4°C by a homogenizer (Thermo Scientific, Waltham, MA). The tissue samples were diluted to a final concentration of 100 mg/mL. The diluted samples were centrifuged at 3000 rpm for 20 min (Heraeus Biofuge A, Germany). SOD, CAT, GPx, 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, Sunnyvale, CA).

The protein content of kidney homogenates was measured by using Biuret’s method.
Histology and immunohistochemistry
Kidney tissues were fixed with formalin, embedded in paraffin, sectioned at 4 μm thickness and then stained with hematoxylin-eosin (H&E) and examined using a light microscope. Kidney tissue damage was assessed using a modified semi-quantitative scale as previously reported. 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. For each parameters 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 was carried out in formaldehyde fixed and, paraffin-embedded kidney 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 min for antigen retrieval. The tissue sections were then incubated for 30 min in 3% H₂O₂ to block the endogenous peroxidase activity. After subsequent treatment with normal goat serum, sections were incubated at 37°C for 1 h with the primary anti-HO-1 antibody (1:200; AB1284; Millipore, Boston, MA). Biotin-labeled goat antirabbit IgG secondary antibody (GGHL-15P; ICLLab, Newberg, OR) was used, and diaminobenzidine was used for the 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.

Statistical analysis
SPSS version 20.0 (SPSS Inc., Chicago, IL) 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’s correction. p Values less than .05 were considered as statistically significant.

Results
One animal in the MARF + HBO group died during the study and the results of seven animals were included in the final analysis.

Biochemistry
Urea, creatinine, AST, and ALT levels were significantly higher (p < .05) in MARF and MARF + HBO group compared to the control group. Mean CK level in MARF group was found to be significantly higher than the control group (p < .05). These results are shown in Table 1.

MARF + HBO and MARF groups had statistically higher MDA levels compared to control group. There was no statistical difference in tissue levels of SOD, CAT, and GPx among the groups. These results are shown in Table 2.

Histology and immunohistochemistry
Control group sections showed a normal structure of kidney tissue. Tubular necrosis, glomerular damage, tubular dilation, myoglobin accumulation were observed in MARF and MARF + HBO groups (Figure 1). Histological scores were statistically higher (p < .05) in MARF (3.0 ± 0.81) and MARF + HBO group (3.57 ± 0.53) compared with the control group. Histological scores were similar in MARF and MARF + HBO groups (p > .05). Cytoplasmic HO-1 immunostaining was seen in all groups (Figure 1). Distal tubules showed more intense HO-1 staining. HO-1 immunostaining score in the control group was 0.75 ± 0.5. Immunostaining scores were statistically higher (p < .05) in MARF (2.25 ± 0.46) and MARF + HBO (2.71 ± 0.48) groups compared to the control group. Figure 1 summarizes H&E staining and immunohistochemistry findings.

Discussion
We used a well-established experimental model, intramuscular glycerol injection-induced rhabdomyolysis, to induce MARF in rats. Renal failure was demonstrated by increased serum levels of urea and creatinine and histological findings; glomerular damage, tubular dilatation, tubular necrosis, and intraluminal plugs. However, HBO therapy did not prevent kidney injury in experimental MARF model in this study.

The effect of HBO therapy on renal injury may be related to duration and frequency of the treatment. It was shown that seven sessions of HBO therapy (at 2.5 ATA, for 60 min) reduced plasma urea and creatinine levels in addition to proximal tubular necrosis and apoptotic cell count in cisplatin-induced acute renal failure. HBO therapy also prevented histological damage after experimental renal I/R injury. It was reported that HBO therapy could reduce the urea and creatinine levels and improve histological findings after I/R injury.
In different studies, HBO therapy was administered more frequently and longer compared to our study. Studies indicating negative effects of HBO therapy are also available. In a study of nephrotoxicity model due to cyclosporine A, they found that there were more apoptotic cells on histopathologic examination in cyclosporine A + HBO group compared to only cyclosporine A group. They suggested that HBO therapy might potentiate the nephrotoxic effect of cyclosporine.

When studies demonstrating beneficial effects of HBO therapy in different ARF models were compared, it was seen that HBO administration frequency was different from each other. The therapy pressure changes 2 to 2.5 ATA, frequency 1–4 per days, duration 60 to 90 min and number of session 4 to 5. In our study, we performed two sessions of HBO therapy and our study has the least session compared with other studies. Future studies should have more HBO therapy sessions and longer follow-up after MARF.

Ayvaz et al. compared once a day and twice a day HBO therapy in cisplatin-induced nephrotoxicity model. They performed HBO therapy in the first group at 2.5 ATA, for 60 min, once a day, for 6 days and HBO therapy at 2.5 ATA, for 60 min, twice a day, for 6 days in the second group. Beneficial effects of HBO therapy was observed in once a day HBO session, whereas renal derangement was reported histologically in twice a day HBO session. Twice a day HBO session could potentiate nephrotoxicity induced by cisplatin. In our study, HBO therapy (at 2.4 ATA, for 90 min) was performed 2 h and 18 h after intramuscular injection. The absence of optimal HBO therapy frequency may be a reason that we could not reach expected results about HBO therapy.

Ayvaz et al. reported the effectiveness of HBO therapy in MARF model with intramuscular glycerol injection (both hind legs, totally 8 mL/kg). They showed that urea, creatinine, and CK levels were significantly decreased in MARF + HBO group compared to MARF group. They stated that HBO therapy could significantly improve MARF induced renal failure in histological examination. They found that SOD and CAT activities were significantly higher in MARF + HBO group compared to MARF group (p < .001) and significantly lower in MARF group compared to control group (p < .05). In contrast to our study, the rats received HBO therapy (2.5 ATA %100 oxygen for 90 min) once a day for two consecutive days. This may explain the differences between their results and ours. Twice daily HBO therapy did not prevent renal injury after MARF.

The most appropriate method for diagnosing rhabdomyolysis is the long-term increase in plasma levels of CK compared to myoglobin. In our study, we evaluated CK level instead of plasma myoglobin. Ayvaz et al showed that increase of CK was significantly lower in MARF + HBO group compared to MARF group. In our study, there was no significant difference between MARF + HBO group and MARF group in terms of CK levels. The absence of significant difference might be again related to duration and frequency of HBO therapy.

### Table 1. Serum urea, creatinine, CK, Na+, K+, AST, and ALT levels in each group (mean ± SD).

|                      | Control | MARF  | MARF + HBO |
|----------------------|---------|-------|------------|
| Urea (mg/dL)         | 59.0 ± 3.3 | 122 ± 62* | 185 ± 61*  |
| Creatinine (mg/dL)   | 0.46 ± 0.02 | 1.18 ± 0.68* | 1.70 ± 0.42* |
| CK (U/L)             | 599 ± 179 | 3600 ± 3347* | 1274 ± 652  |
| Na+ (mmol/L)         | 139 ± 1.08 | 141 ± 3.44 | 141 ± 2.44  |
| K+ (mmol/L)          | 5.61 ± 0.11 | 5.41 ± 0.21 | 5.10 ± 0.35  |
| AST (U/L)            | 126 ± 16 | 1144 ± 620* | 992 ± 245*  |
| ALT (U/L)            | 65.2 ± 10.3 | 305 ± 153* | 450 ± 667*  |

*p < .05 compared with Control.

### Table 2. Renal MDA, SOD, CAT, and GPx levels in each group (mean ± SD).

|                      | Control | MARF  | MARF + HBO |
|----------------------|---------|-------|------------|
| MDA (nmol/mg protein)| 0.019 ± 0.002 | 0.284 ± 0.121* | 0.321 ± 0.128* |
| SOD (U/g protein)    | 19.4 ± 5.5 | 21.2 ± 7.7 | 24.2 ± 13.4  |
| CAT (ng/mg protein)  | 7.1 ± 4.2 | 5.8 ± 2.6 | 8.6 ± 5.1   |
| GPx (U/g protein)    | 23.8 ± 4.2 | 28.9 ± 12.6 | 34.0 ± 21.7  |

*p < .05 compared with Control.

![Figure 1. Rhabdomyolysis promotes kidney damage and HO-1 expression in rat kidney tissues. Representative images showing hematoxylin and eosin (H&E) staining images shows tubular damage (double stars), tubular dilatation (star), glomerular damage (arrow), myoglobin accumulation (double arrows) are seen in MARF and MARF + HBO groups. Representative immunohistochemical expression of HO-1 images indicates weak (+), moderate (++), strong (+++) intensity of staining. MARF + HBO group shows more intense immunopositivity. Scale bar 200 μM.](image)
therapy. We found that serum Na+ and K+ levels unchanged in our study. Although this finding is contradictory to the study by Ayvaz et al, these may be due to shorter duration of our study.

Rhabdomyolysis-induced hypovolemia and vasoconstriction aggravate renal perfusion defect. In our study, rats did not receive water during HBO therapy for 90 min, which started 2 h after glycerol injection. This may have contributed to hypovolemia in HBO treated rats. Furthermore, HBO is known to induce vasoconstriction and reduce renal blood flow. Taken together, water abstinence during HBO therapy and HBO-induced renal vasoconstriction may be responsible for histological changes observed in HBO-treated rats. Experimental hyperbaric chambers should be modified to allow water during HBO therapy. It is also reasonable to monitor the weight of animals to evaluate dehydration due to MARF. Future studies should consider these points.

The HO-1 system is a cytoprotective mechanism activated during cellular stress and it is reported that high levels of HO-1 protein secretion could protect the renal tissue against injury mediated by free radicals. HBO therapy applied before lung damage increases the expression of HO-1 in rats. But there is no previous study whether HBO therapy had an effect on the HO-1 enzyme in MARF model. In this study, HO-1 expression was higher in MARF and MARF + HBO groups compared to the control group (p < .05), however, there was no significant difference between MARF (2.25 ± 0.46) and MARF + HBO (2.71 ± 0.48) groups. Our findings do not support the idea that HBO therapy is protective against MARF through the activation of renal HO-1 levels in rats.

Conclusions
We found that HBO therapy did not reduce renal injury in experimental MARF model. HBO therapy is used to reduce the muscle damage after crush injury, which may be accompanied by MARF. Therefore, more studies are needed to understand the effects of HBO treatment on renal functions after MARF.

Disclosure statement
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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