Hyperbaric oxygen treatment improves GFR in rats with ischaemia/reperfusion renal injury: a possible role for the antioxidant/oxidant balance in the ischaemic kidney

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Abstract

Background. Ischaemic kidney injury continues to play a dominant role in the pathogenesis of acute renal failure (ARF) in many surgical and medical settings. A major event in the induction of renal injury is related to the generation of oxygen-free radicals. Hyperbaric oxygen therapy (HBO) is indicated for treatment of many ischaemic events but not for ARF. Therefore, the present study examined the effects of HBO on kidney function and renal haemodynamics in rats with ischaemic ARF.

Methods. Renal ischaemia was induced by unilateral renal artery clamping (45 min) in rats. Within 24 h following ischaemia, rats were treated twice with HBO of 100% O2 at 2.5 absolute atmospheres for 90 min each (+HBO). Untreated rats (−HBO) served as a control. Forty-eight hours later, GFR, RBF and endothelial-dependent vasorelaxation were measured. In addition, the immunoreactive staining of 4-hydroxy-2-noneal (4-HNE), a major product of endogenous lipid peroxidation, and superoxide dismutase (SOD) were assessed.

Results. In the −HBO group, GFR was reduced by 94% compared with the untouched normal kidney (ischaemic: 0.06 ± 0.03 ml/min, normal: 1.02 ± 0.13 ml). In contrast, in the +HBO group, GFR of the ischaemic kidney (0.36 ± 0.07 ml/min) was reduced only by 68% compared with the contralateral normal kidney (1.12 ± 0.12 ml/min). In line with these findings, HBO improved the vasodilatory response to ACh as expressed in enhancement of both total and regional renal blood flow. In addition, HBO reduced the formation of 4-HNE by 33% and 76% and increased SOD by 30% and 70% in the cortex and outer stripe region of the medulla of the ischaemic kidney, respectively.

Conclusion. HBO attenuates the decline in GFR following renal ischaemia, and improves endothelial-dependent vasorelaxation, suggesting that treatment with HBO may be beneficial in the setting of ischaemic ARF.

Keywords: acute kidney injury; hyperbaric oxygen; ischaemia-reperfusion injury; rat

Introduction

Acute renal failure (ARF) continues to be a frequent threatening complication following trauma and complex surgical procedures, and in patients hospitalized in intensive care units [1,2]. Despite the increasing awareness of this syndrome, the advances in clinical care and extensive research of its pathophysiology, the mortality from ARF remains high and has not changed significantly during the last several decades [2].

Ischaemic kidney injury continues to play a dominant role in the pathogenesis of ARF in many surgical and medical settings. Recent investigations into the mechanisms of renal damage following ischaemia in animal models suggest an early contribution of endothelial dysfunction and a local inflammatory response as major components of the evolving ischaemic injury to the kidney [3–5]. As a consequence of these events, the recovery of blood supply to the ischaemic kidney may be significantly delayed and reduced leading to further impairment of tissue oxygenation.

A major event in the induction of tissue injury is related to the generation of oxygen-free radicals and the associated oxidative and nitrosative stress [4–6]. In particular, it has been demonstrated that the highly reactive hydroxyl radical and the nitric oxide (NO) metabolic product, peroxynitrite (ONOO−), are generated during the renal reperfusion phase and have diverse cytotoxic properties. These include damage to DNA, protein oxidation and nitrosylation, lipid peroxidation and induction of apoptosis [3,6,7].

Hyperbaric oxygen therapy (HBO) is defined as a treatment in which a patient is intermittently exposed to 100% oxygen while the treatment chamber is pressurized to a...
pressure above sea level (>1 ATA, 760 mmHg) [8]. HBO therapy has been used in a number of medical conditions with a proven efficacy in a limited number of disorders [8–10]. In ischaemic rat tissues, HBO was shown to inhibit neutrophil adherence to the wall of ischaemic vessels and to decrease post-ischaemic vasoconstriction in skin grafts [11]. Furthermore, we showed in our laboratory that HBO has striking beneficial anti-inflammatory effects on experimental colitis in rats [12]. In several experimental conditions, HBO has been claimed to exert its beneficial effects in part by increasing the activity of Cu/Zn superoxide dismutase (Cu/Zn-SOD) and other antioxidant cellular defence mechanisms, thereby altering the balance between generation and removal of oxygen-free radicals [13–15].

Several investigators reported that HBO treatment may modulate kidney function in rats with sepsis [16], rats with adriamycin-induced nephrotic syndrome [17] and experimental cyclosporine nephrotoxicity [18]. Recently, Solmazgul et al. [19] demonstrated that HBO attenuated the elevation in plasma creatinine and histological damage in Sprague-Dawley rats subjected to renal ischaemia/reperfusion injury. Moreover, HBO treatment has been reported to be beneficial in the management of the muscle compartment syndrome, a devastating complication been reported to be beneficial in the management of the muscle compartment syndrome, a devastating complication.

Here we report that HBO treatment following renal ischaemia/reperfusion attenuated the deterioration in glomerular filtration rate (GFR) in the ischaemic kidney, in parallel with an improvement in the antioxidant/oxidant balance.

**Materials and methods**

Studies were conducted on male Sprague-Dawley rats (Harlan Laboratories, Jerusalem, Israel) weighing 280–320 g. Animals were maintained on regular rodent chow (0.45% NaCl) and water ad libitum prior to experiments. The experiments were conducted according to the guidelines of the institutional Animal Use and Care Committee, Technion, Israel.

**In vivo protocols**

**Induction of ischaemic ARI.** Forty-eight hours prior to clearance and renal haemodynamic studies, rats were lightly anaesthetized by intraperitoneal injection of Nembutal (pentobarbitone sodium, 30 mg/kg BW) and placed on a heated surgical table to maintain body temperature. The left kidney was then exposed through a flank incision, and renal ischaemia was induced by complete occlusion of the left renal artery for 45 min by a surgical clamp. The contralateral, right kidney was left untouched and served as an internal control. Following ischaemia, the clamp was released, the incision was sutured and the rat was allowed to recover.

**Hyperbaric oxygen treatment.** Rats with left kidney ischaemia were randomly divided into two experimental groups. The first group (+HBO, n = 9) was placed in a hyperbaric chamber for two HBO treatments of 90 min each, 2 h and 22 h after renal ischaemia. This protocol was chosen based on previous experiments from our laboratory in rats with crush syndrome where HBO exerted beneficial effects on muscle function (unpublished observations). The chamber was pressurized with 100% oxygen to 2.5 absolute atmospheres (ATA). The second group (−HBO, n = 6) was left at room atmosphere for 48 h, and served as a control for group 1.

**Clearance studies.** On the second day, ~48 h after renal ischaemia, animals from both groups were anaesthetized by injection of Inactin (100 mg/kg BW, i.p.), and prepared for clearance experiments as previously described from this laboratory [22,23]. In short, after tracheotomy, the rat was placed on a heated surgical table and the left carotid artery and right jugular vein were cannulated by polyethylene tubing (Portex, Hythe, Kent, UK) for continuous monitoring of mean arterial pressure (MAP) and infusion of various solutions, respectively. A solution of 2% inulin in 0.9% saline was infused at a rate of 1.0–1.5% BW/h, throughout the experiment. The abdominal cavity was then exposed through a mid-abdominal incision, and the left ureter was identified, separated from the surrounding fat and cannulated by a PE-10 tube for urine collection. The urinary bladder was exposed, and a funnel-shaped PE-50 catheter was inserted for urine collection from the contralateral right kidney. Urine was collected separately from each kidney into pre-weighed tubes containing mineral oil, and the urine volume was measured gravimetrically. Following surgery, a 60-min equilibration period was allowed before the first clearance period was obtained. Four clearance periods of 20–30 min each were obtained, and blood samples (0.3 ml) were withdrawn every second clearance period. Blood samples were separated by centrifugation, and plasma and urine aliquots were kept at −20°C for determination of inulin concentration. For each experimental group, GFR was determined for the left ischaemic kidney, the right control kidney and the ratio of GFR in ischaemic/control kidneys were calculated.

**Renal haemodynamic measurements**

(A) Total renal blood flow: to evaluate the effects of HBO on renal haemodynamic parameters, studies were conducted in rats subjected to unilateral ischaemia 48 h earlier, treated (+HBO, n = 9) or untreated (−HBO, n = 6) with hyperbaric oxygen, as described in the previous protocol. An additional group of sham-operated control rats (n = 6) was used to test the effects of the operation itself on relevant haemodynamic parameters. In these rats, a flank incision was performed, but the left renal artery was not clamped. The rats were anaesthetized by Inactin as described in the previous protocol. The left renal artery was exposed through a mid-abdominal incision. Measurements of total renal blood flow (RBF) were performed by an ultrasonic flowmeter (model T206, Transonic Corp., Ithaca, NY, USA), using a flow-probe (1RB) placed around the left renal artery, as previously described [23,24]. MAP was continuously monitored by a pressure transducer, and RVR was calculated on-line according to the formula RVR = MAP/RBF using data acquisition.
software (Labtech Notebook®). After surgery and equilibration, baseline measurements were obtained for 20 min. The endothelial-dependent renal vasodilation in response to incremental doses of acetylcholine (ACh) infusion (1, 10 and 100 µg/kg/min, for 20 min each dose, followed by a recovery period) was determined as previously described [22].

(B) Intrarenal blood flow: to evaluate the effects of HBO on intrarenal haemodynamic parameters, studies were conducted in rats subjected to unilateral ischaemia 48 h earlier, treated (+HBO, n = 9) or untreated (−HBO, n = 6) with hyperbaric oxygen. For this purpose, the left kidney was exposed through a mid-abdominal incision. Cortical and medullary blood flow (CBF and MBF, respectively) were measured simultaneously by a dual channel laser-Doppler flowmeter (Master Perimed AB, model 4001) using two needle probes as previously described [23]. ACh was administered at a dose of 10 µg/kg/min, and CBF was measured at 5-min intervals for 30 min after administration of the drug. The location of the laser needle probes was verified at the end of each experiment.

**In vitro protocols**

To test the hypothesis that HBO exerted its beneficial effects on renal function in part by improving the antioxidant/oxidant balance, we evaluated the immunohistochemical localization of the activities of the antioxidant enzyme Cu/Zn-SOD and that of 4-hydroxy-2-noneal (4-HNE), a marker of lipid peroxidation.

The unclamped, normal kidney and the ischaemic kidney were removed 48 h following ischaemia/reperfusion in the −HBO and +HBO groups under pentobarbitone sodium anaesthesia. Kidneys were fixed in 10% neutral buffered formalin, dehydrated in ascending ethanols and embedded in paraffin wax. Five-micrometre thick microtome sections were mounted on SuperFrost® Plus slides (Menzel-Glaser, Braunschweig, Germany).

Immunostaining was performed with an antibody against 4-HNE (1:600, Alpha Diagnostic Inc., San Antonio, TX, USA) and anti-superoxide dismutase–CuZn enzyme (1:600, Oxis International Inc., Portland, OR, USA), using a histostain-SP kit (Zymed Laboratories, San Francisco, CA, USA).

The immunoreactive stained areas of the end-products were observed using an Olympus CH-40 brightfield microscope (Olympus, Tokyo, Japan) with an attached CCD camera. The areas observed and analysed in the sections were taken from the medulla and from throughout the cortex apart from that immediately under the capsule. Quantitative histomorphometric analysis was determined in randomly standardized fields of view by means of commercial image analysis software (analySIS® 3.0, Soft Imaging System, Münster, Germany).

**Chemical analysis**

Inulin concentration in plasma and urine samples was determined by the anthrone method [25]. GFR was equated with the clearance of inulin. Sodium concentrations were determined by flame photometry (model IL 943, Instrumentation Laboratories, Milano, Italy).

**Statistical analysis**

The paired student t-test (for comparison between the ischaemic and untouched, normal, kidney in the same animal) and either the unpaired t-test or analysis of variance (ANOVA), as appropriate, were used for statistical analysis of the data. A P-value of 0.05 or less was considered statistically significant. Data are expressed as means ± standard error of mean (S.E.M).

**Results**

**Clearance protocols**

Figure 1 summarizes the results of the clearance experiments in the −HBO and +HBO groups. In the −HBO
group, 48 h after renal ischaemia, GFR was reduced by 94% compared with the untouched, normal kidney (ischaemic: $0.06 \pm 0.03$ ml/min, normal: $1.02 \pm 0.13$ ml/min). In contrast, in the +HBO group, GFR in the ischaemic kidney ($0.36 \pm 0.07$ ml/min) was reduced only by 68% compared with the contralateral normal kidney ($1.12 \pm 0.12$ ml/min). The difference in GFR of the ischaemic kidneys in the groups treated or untreated with HBO was statistically significant ($P < 0.05$, Figure 1). Also, when expressed as GFR of ischaemic/normal kidney, this ratio was significantly higher in the +HBO group compared with the −HBO group ($0.39 \pm 0.1$ versus $0.08 \pm 0.05$, respectively, $P = \text{N.S.}$).

Renal and intrarenal haemodynamics measurements

Baseline values of MAP, RBF and calculated RVR in the sham-operated control group and in the ischaemic kidney of −HBO and +HBO groups are summarized in Figure 2. Baseline MAP did not differ significantly in the three experimental groups. Baseline RBF decreased by 26% in +HBO rats versus −HBO rats. However, both were significantly lower compared with normal control kidney. In line with these measurements, calculated baseline RVR was higher (by 52%) in the ischaemic kidney of the +HBO group compared with the −HBO group.

The results of the endothelial-dependent renal vasodilatation in response to ACh administration are depicted in Figure 3. As shown, infusion of incremental doses of ACh produced a similar decrease in MAP in the three experimental groups (sham, ischaemic −HBO and ischaemic +HBO), followed by a similar recovery response upon cessation of ACh infusion. In spite of the decrease in MAP, there was a dose-related rise in RBF in response to ACh in all groups, apparently as a result of an endothelial-dependent renal vasodilatation. Of interest, treatment with HBO (ischaemic kidney of +HBO) significantly improved the renal vasodilatory response to ACh as compared to ischaemic kidney of −HBO. Indeed, as shown in the lower panel of Figure 3, there was a dose-dependent decrease in RVR in all three experimental groups, especially in ischaemic kidney of +HBO rats. This notion is further supported by the measurements of intrarenal blood flow in response to the ACh as depicted in Figure 4. As shown, administration of ACh to control rats caused a gradual increase in CBF, which reached maximal magnitude of 35% ± 8% above baseline value at 30 min after the drug injection (Figure 4A), whereas MBF did not change significantly (Figure 4B). In contrast, in the ischaemic kidney of the −HBO group, ACh elicited an attenuated increase in CBF (18 ± 3% peak increase at 30 min after injection of the drug). The cortical vasodilatory response to ACh significantly improved following HBO treatment (CBF reached a peak increase of 28 ± 11% at 30 min after injection of the drug). These findings may explain the observed stimulatory effects of HBO on total RBF, and suggest that treatment with HBO may improve endothelial-dependent vaso-relaxation mediated by eNOS. It should be emphasized that basal CBF...
Effects of acetylcholine (ACh) administration (1, 10 and 100 µg/kg/min) on (A) mean arterial pressure (MAP), (B) renal blood flow (RBF) and (C) renal vascular resistance (RVR), in the sham-operated control group and in the ischaemic kidney of rats treated with hyperbaric oxygen than in untreated rats (+HBO: 12.7 ± 0.84%, −HBO: 53.1 ± 1.55, P < 0.001, Figure 5).

Figure 6 summarizes the immunohistological data on Cu/Zn-SOD in the cortex and outer stripe region of the medulla of control and ischaemic kidneys treated with or without HBO. In the control, untouched kidneys, immunostaining for Cu/Zn-SOD in the cortex and the medulla was negligible. There was a marked increase in Cu/Zn-SOD immunoreactive staining in the outer stripe of the medulla of the ischaemic kidneys in both −HBO and +HBO groups, compared with the control non-ischaemic kidneys. However, in the +HBO group, the percentage of staining for Cu/Zn-SOD was significantly higher than that in the −HBO group (+HBO: 17.3 ± 1.11%, −HBO: 10.2 ± 0.83%, P < 0.001, Figure 6).

Thus, HBO treatment was associated with an increase in the immunohistochemical staining of the antioxidant Cu/Zn-SOD and a parallel decrease in the immunohistochemical staining for the lipid peroxidation marker, 4-HNE, in the outer stripe of the medulla of the ischaemic kidneys.

Discussion

The findings of the present study provide novel information on the mechanisms underlying ARF and support the use of HBO for treatment of ischaemic renal injury. Our data clearly demonstrate that treatment with HBO substantially attenuated the reduction in GFR characterizing ARF. The HBO-induced renal vasodilatation was associated with a significant increase in total RBF in response to ACh, due to enhanced perfusion of the renal cortex in the ischaemic kidney. Despite the improvement in renal haemodynamics, HBO did not cause a concomitant increase in urine flow or sodium excretion. Moreover, our findings demonstrate that the improvement in renal function following HBO occurs in parallel with a similar increase in the antioxidant/oxidant ratio in the ischaemic kidney. This suggests that the latter mechanism may partially contribute to the improvement in renal function in kidneys recovering from ischaemic injury, and support the use of HBO for treatment of ARF.

Previously, Solmazgul et al. [19] demonstrated that HBO treatment produced marked protection against ischaemia of 30 min followed by 24-h reperfusion after the right nephrectomy. These authors applied HBO therapy for 60 min, starting at the initial 15th min of reperfusion. Animals treated with HBO showed regression of the elevated plasma creatinine and blood urea nitrogen levels to normal concentrations in association with reversal of tubular damage and neutrophil infiltration. However, the mechanisms underlying the beneficial effects of HBO therapy were not studied in this study. Our data clearly show that HBO treatment partially corrected the decline in GFR complicating ARF and provide new insights into the mechanisms underlying the therapeutic actions of HBO in the I/R...
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Fig. 4. Effects of acetylcholine (ACh) administration (10 µg/kg/min) on (A) cortical blood flow (CBF) and (B) medullary blood flow (MBF) in the sham-operated control group and in the ischaemic kidney of -HBO and +HBO groups 48 h after the induction of ischaemia. $n = 6–9$ in each group. It is evident that administration of ACh to control rats caused a gradual increase in CBF, whereas MBF did not change significantly. In contrast, in the ischaemic kidney of the -HBO group, ACh elicited an attenuated increase in CBF. The cortical vasodilatory response to ACh significantly improved following HBO treatment. These findings may explain the observed stimulatory effects of HBO on total RBF, and suggest that treatment with HBO may improve endothelial-dependent vasorelaxation mediated by eNOS. $P < 0.05$ vs. -HBO.

Fig. 5. Representative pictures of 4-HNE staining in the medulla of -HBO (A) and +HBO (B) ischaemic kidneys (objective $\times20$). (C) Percentage staining area of 4HNE in the renal outer strip of the outer of -HBO and +HBO of control and ischaemic kidneys. $n = 6–9$ in each group. This figure indicates that 4-HNE is localized primarily in the renal tubular cells of the outer stripe of the medulla of the ischaemic kidneys, and is almost undetectable in the cortex. The area of the outer stripe of the medulla stained for 4-HNE is significantly less than in the ischaemic kidneys of rats treated with hyperbaric oxygen than in untreated rats.

For instance, the beneficial effects of HBO on GFR in the +HBO group could be attributed to the observed concurrent improvement in endothelial function of the injured kidney following HBO. In that respect, rats with ARF that were treated with HBO displayed a more pronounced endothelial-dependent vasorelaxation in response to ACh compared with control animals, and the -HBO group. This improvement was accompanied by a concomitant decrease in the 4-HNE level in renal tissue. 4-HNE, a major product of endogenous lipid peroxidation, is considered as a reliable indicator of oxidative damage [26,27]. It is well established that ROS directly cause vasoconstriction in
Fig. 6. Representative pictures of Cu/Zn-SOD staining in the medulla of −HBO (A) and +HBO (B) ischaemic kidneys (objective × 20). (C) Percentage staining area of Cu/Zn-SOD in the renal medulla of −HBO and +HBO control and ischaemic kidney. n = 6–9 in each group. It is evident that immunostaining for Cu/Zn-SOD in the cortex and the outer stripe of the medulla of untouched kidneys is negligible. A marked increase in Cu/Zn-SOD immunoreactive staining in the outer stripe of the medulla of the ischaemic kidneys in both −HBO and +HBO groups, compared with the control non-ischaemic kidneys, is notable. However, in the +HBO group, the percentage of staining for Cu/Zn-SOD is significantly higher than that in the −HBO group. Thus, HBO treatment induces an increase in the immunohistochemical staining of the antioxidant Cu/Zn-SOD in the outer stripe of the medulla of the ischaemic kidneys.

kidney microcirculation [28]. Furthermore, they indirectly increase vascular tone by affecting the action or production of vasodilatory substances of endothelial origin such as NO [29]. Therefore, the observed increased oxidative stress in the ischaemic kidney probably composes additional mechanism that could be responsible for the renal dysfunction occurring during ARF. The latter is compatible with our findings that the beneficial effect of HBO treatment on GFR was accompanied by enhancement of SOD abundance and decline in 4-HNE in the renal tissue. Similar results were obtained in sepsis-induced ARF, where renal dysfunction was associated with the reduction of renal SOD and catalase (CAT) activities and the increase in malondialdehyde (MDA) levels [16,30]. Interestingly, GFR recovered to normal values along with the reduction in oxidative damage by either HBO treatment [16] or administration of an SOD mimetic [29]. Beneficial renal effects of antioxidant agents such as vitamin E, edaravone (radical scavenger) and melatonin were also reported in experimental models of cyclosporine nephrotoxicity [31], ischaemic ARF [32] and glycerol-induced ARF [33], respectively. Taken together, these studies indicate that oxygen-free radicals play a major role in renal injury of different aetiologies and that HBO treatment has a beneficial effect on renal dysfunction. The beneficial renal effect of HBO could be attributed to its antioxidative capacity. This notion is supported by our findings that HBO augmented the SOD immunoreactivity in the renal tissue to normal levels and abolished the increase in 4-HNE. Similarly, several studies have demonstrated that HBO treatment increased the SOD and/or CAT activities in rat extensor digitorum longus muscle [13], in pancreas and erythrocytes of rats with induced acute necrotizing pancreatitis [34], in the lungs of rats and guinea pigs [35] and in the erythrocytes of human patients with multiple sclerosis [36].

The stimulatory effects of HBO treatment on the endothelial-dependent vasorelaxation induced by ACh in the ischaemic kidney hint that HBO treatment improves the function of the NO system, which plays an important role in the regulation of renal haemodynamic and kidney function. In line with these findings, several studies have shown that HBO increases the NO levels in the perivascular part of the aorta [35], and the brain [37,38]. Similarly, Cabigas and coworkers [39] have recently demonstrated a cardioprotective effect of HBO in rats subjected to myocardial ischaemia/reperfusion. The authors suggested that the conferred cardioprotection was due to HBO-mediated increased association between heat-shock protein 90 (hsp90) and eNOS, which further augmented NO production [39]. Additional studies are required to elucidate if similar mechanisms contributed to the enhanced recovery of GFR in our rats with ischaemic renal injury.
Nevertheless, despite the restoration and even enhanced endothelial-dependent response to ACh 48 h after ischemic injury, baseline RBF was still lower in the –HBO and +HBO ischemic kidneys compared with the non-ischemic control kidney. This could reflect the predominant effects of various vasoconstrictor, NO-independent, systems that are still present at this post-ischemic stage and may control renal haemodynamics.

Since basal RBF was lower in the +HBO group as compared to the –HBO group, this further decline in basal RBF following HBO treatment is consistent with the vasoconstrictive action of hyperoxygenation [40]. The mechanism(s) by which HBO treatment caused the increase in GFR were not clarified in the present study. Thus, the finding that despite the reduction in basal RBF, HBO caused a concomitant increase in GFR of the ischemic kidneys might suggest that HBO induced a preferential vasoconstriction of the efferent arteriole. Alternatively, it might be speculated that the observed enhancement in GFR was not related to haemodynamic changes, but rather to an improvement in the tubular integrity and function of the injured nephrons. Further studies will be required to elucidate the exact mechanisms of GFR improvement and the contribution of the alterations to oxidant/antioxidant balance in that regard.

In summary, this study demonstrates that acute ischemic renal injury is associated with enhancement of renal oxidative stress and that HBO treatment has a potential in the recovery of GFR by improving the antioxidant/oxidant balance in the ischemic kidney.

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