Application of normobaric hyperoxia therapy for amelioration of haemorrhagic shock-induced acute renal failure*

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Abstract

Background. Hypoxia resultant from haemorrhagic shock is the primary cause of kidney damage. Application of normobaric hyperoxia therapy (NHT) is an acceptable treatment for acute haemorrhagic shock. We investigated the effect of NHT on amelioration of haemorrhagic shock-induced rat renal failure.

Methods. Twenty-four Sprague-Dawley rats were subjected to gradual blood withdrawal/reperfusion, followed by 12-h, 24-h or 48-h NHT. Verification/monitoring of intrarenal hypoxia was performed using Hypoxyprobe-TM-1. Subsequently, cystatin C, urea and creatinine were assessed in serum by a Hitachi autoanalyser, and NO, 3-nitro-tyrosine, STAT-8-isoprostane and NF-kB in renal medullae and cortices by specific ELISAs.

Results. In rats subjected to haemorrhagic shock, 12- to 48-h NHT significantly reduced intrarenal Hypoxyprobe-TM-1 stained areas and attenuated augmentation of urea, creatinine and cystatin C. Haemorrhagic shock resulted in a 10-fold drop of intrarenal NO availability. 12-h and 24-h, but not 48-h, NHT significantly increased cortical/medullar NO synthesis, the latter, however, not approaching the pre-shock values. Significant shock-induced accumulation of STAT-8-isoprostane and 3-nitro-tyrosine was further exacerbated by NHT. Haemorrhagic shock activated NF-kB in ischaemic tissues, which was not attenuated by NHT.

Conclusions. (1) 12- to 48-h NHT decreased intrarenal hypoxia signs and ameliorated deterioration of renal functions in a rat model of haemorrhagic shock-induced renal failure. (2) 12- to 24 h NHT improved bioavailability of NO in cortices/medullae of kidneys recuperating from haemorrhagic shock. (3) If any anti-inflammatory activities were stimulated by NHT, they would not be mediated via the NF-kB pathway. (4) Despite NHT-associated elevation of reactive oxygen species (ROS), early oxygen supply proved mandatory for effective recuperation of ischaemic kidney from detrimental consequences of haemorrhagic shock.

Keywords: acute renal failure; haemorrhagic shock; ischaemia-reperfusion; normobaric hyperoxia; oxidative stress

Introduction

Shock is a pathophysiologic condition characterized by a significant systemic and local reduction in tissue perfusion. Multiple organ failure is a common outcome of a shock condition, the kidney being one of the primary target organs. Pre-renal azotaemia, ischaemia-induced apoptotic cell death and acute tubular necrosis (ATN) occur on a continuum of the same hypoxic process and, taken together, account for 66–75% of the cases of acute renal failure [1,2].

Hypoxia due to hypoperfusion is the primary cause of kidney damage. However, the damage might progress further during the reperfusion phase, when the blood flow to the ischaemic tissues is restituted. Three main factors are responsible for further progression of renal damage following the reperfusion period: intrarenal inflammation initiated by inflammatory cells infiltrating the kidney, augmented generation of reactive oxygen species (ROS) and persistent tissue hypoxia [3,4].

Normobaric hyperoxia therapy (NHT) is currently accepted as a treatment for acute phase of a number of diseases [5]. In several clinical and experimental conditions NHT, in addition to serving as a tool for enhancement of oxygen delivery, was shown to exert anti-inflammatory effects [6]. The anti-inflammatory outcome of hyperoxia following ischaemia/reperfusion could be related to the direct inhibitory action of oxygen on activation, infiltration and adhesion of inflammatory cells to the ischaemic tissue. In addition, NHT may indirectly inhibit inflammation by ameliorating renal hypoxia, the main trigger of the latter [7].
On the other side, the commonly accepted paradigm of ischaemia/reperfusion injury emphasizes the role of oxygen-derived free radical formation in intrarenal toxicity as well as activation of the inflammatory cascade [7,8]. This would evoke the initial concern that hyperoxia, by adding extra oxygen to the system and thus increasing free radical formation, might further exacerbate the injury [7,8].

Application of 100% oxygen at 1 atm (NHT) is a standard treatment for a patient suffering from haemorrhagic shock. No sufficient information concerning NHT application at the reperfusion stage is available at present. Furthermore, the role of NHT in haemorrhagic shock-induced acute renal failure has not been systematically investigated. Therefore, estimation of the effect of NHT on progression of haemorrhagic shock-induced acute renal failure at different time points of intervention would bear a significant clinical importance.

The aim of the present study was to evaluate the effects of NHT on renal functioning, intrarenal hypoxia markers, inflammatory parameters and oxidative stress in a rat model of haemorrhagic shock-induced acute renal failure.

Methods

Animals

Sixty 3-month-old Sprague-Dawley male rats weighing 300–350 g were included in the experiment. All the animals were born and bred in significant pathogen free conditions (s.p.f.) in the animal facilities of Assaf Harofeh Medical Center. The maintenance was according to the guidelines of the Local Ethics Committee for Animal Experimentation, and the experimental protocol received the approval of the latter.

The rats were fed regular chow routinely purchased by our animal facilities via Harlan Laboratories and had free access to water. The experiments were conducted according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

In vivo experimentations

Induction of haemorrhagic shock

Prior to the induction of haemorrhagic shock, all the rats were anaesthetised intraperitoneally by a 0.3 ml bolus (0.1 ml/100 g rat weight) injection of ketamine–xylazin mixture, prepared as follows: 0.15 ml of a 20 mg/ml xylazin mixture with 0.85 ml of a 100 mg/ml ketamine. Subsequently, the rats were maintained on isoflurane mixture containing 2% isoflurane and 98% oxygen, at a rate of 200 ml/min. PE-50 catheters were introduced into the left carotid artery for blood pressure recording and blood withdrawal and into the left external jugular vein for blood reinfusion.

In order to maximally approach simulation of haemorrhagic shock following massive blood loss, fractional withdrawal of blood via a heparinized syringe through the carotid catheter was performed, so as to decline the animals’ mean arterial pressure to 30 ± 5 mmHg. Blood withdrawal, accompanied by continuous invasive blood pressure monitoring, was carried out according to the following protocol:

- First withdrawal: 5 ml of blood.
- After 10 min: 2 ml of blood.
- After additional 5 min: 2 ml of blood.
- Subsequently, 1 ml of blood was withdrawn every 5 min, until the constantly dropping mean arterial pressure was stabilized at 30 ± 5 mmHg. The time of the last blood withdrawal was considered the starting time point of the experiment.

Three rats did not survive the haemorrhagic shock procedure and were replaced by three similarly treated animals. Subsequently, during the follow-up period, all the remaining rats participating in the study survived the experimental protocol.

The blood samples were kept in lithium heparin pre-coated test tubes (Beckton-Dickinson, UK) at room temperature in a rotating shaker until re-infused to the animal. Following stabilisation of the mean arterial pressure at 30 ± 5 mmHg, 1.5 h later the blood reperfusion process was started according to a protocol constituting a mirror reflection of the blood withdrawal protocol. At the end of the reperfusion procedure, the systolic blood pressure of all animals was >100 mmHg. At this point, the PE-50 catheters were withdrawn, anaesthetic procedure was terminated and during 24 h following the reperfusion period all the experimental rats were subjected to subcutaneous injections of 2 ml 0.9% saline every 6 h, to ensure preservation of body hydration.

In our preliminary experiments, application of this protocol resulted in induction of acute renal failure in 100% of experimental animals, with a 2.5- to 3-fold increase in serum cystatin C at 12 h time point and a 2.5- to 3-fold increase in serum creatinie at 24 h time point.

The animals were divided into the following experimental groups (n = 15 in each):

- Group 1: rats subjected to surgical intervention of a similar quality and duration, without undergoing the blood withdrawal procedure (sham operation).
- Group 2: rats subjected to the sham operation procedure followed by NHT (sham operation + O2).
- Group 3: rats subjected to haemorrhagic shock.
- Group 4: rats subjected to haemorrhagic shock and subsequently receiving NHT.

Each group was subjected, or not, to NHT for 12 h, 24 h or 48 h.

At each stage, the animals were killed, their blood samples withdrawn for biochemical studies and the internal organs allocated for ex vivo procedures.

Normobaric hyperoxia therapy (NHT)

Animals destined for NHT were placed in a custom-made pressure chamber, 100 cm long, with inner diameter 50 cm. They were subjected to 1 atm/100% oxygen pressure for 12 h, 24 h or 48 h. Control animals were exposed to standard pressure/oxygen concentrations (1 atm/20% oxygen) in the same chamber and for the same time period.
No additional anaesthetics or analgesics were necessary for the animals at the period of post-haemorrhagic shock observation, be the latter combined or not with NHT application. All the animals moved freely within the cage or the press-chamber space and had free access to food and water.

**Blood pressure measurement**

At the end of each follow-up period, prior to killing, tail systolic blood pressure measurements were performed in two animals out of each experimental group, using a Narcosystem Blood Measurement Device (ND, USA). Each animal was individually caged before blood pressure determination, and body temperature was adjusted to 37°C in an incubator for at least 15 min. Thereafter, the animal was transferred to a fixed measurement cage pre-heated to 37°C. The rat was then restrained relative to its body length and weight. Three to five sequential tail cuff measurements were then performed, and the mean values were calculated from the last three measurements.

**The in vivo marking of intrarenal hypoxia**

Intrarenal hypoxia was marked in vivo, using a Hypoxyprobe-TM-1 Plus Kit for the Detection of Tissue Hypoxia (Chemicon International, USA), according to the manufacturer’s protocol. In brief, equal doses of 60 mg/kg b.w. of Hypoxyprobe-TM-1 in a 0.5 ml bolus were injected into two animals from each experimental group via caudal vein 1 h prior to killing. After the animals being killed, their kidneys were immediately removed and placed in 4% formalin, to be used for immunohistochemical staining as described later.

**Ex vivo experimentations**

**Preparation of cell extracts for evaluation of intrarenal oxidative stress and inflammatory responses**

Four rats from each experimental group were destined for in vitro investigations. They were killed by halothane overdose following exposure to NHT for 12, 24 or 48 h. Their kidneys were removed under sterile conditions in a laminar flow. The kidneys were de-capsulated, and the cortices were separated from the medullae by a procedure described elsewhere [9]. Cortical and medullar cell extracts were prepared using a Chemicon Nuclear Extraction kit (Chemicon International, USA) as previously described [10]. The prepared extracts were stored at −80°C until used for the NO, STAT-8-isoprostane and NF-kB assays.

**Biochemical studies**

**Blood creatinine and cystatin C measurements** were performed on a Roche/Hitachi 717 autoanalyser (Roche Diagnostics, Mannheim, Germany) as previously reported [11]. In brief, creatinine was assessed using a highly sensitive Roche modification of the Jaffe method (the lower detection limit 18 µmol/L; CV, the coefficient of variability, <2.3%). Urea was determined on a spectrophotometer (Carmira, USA), using a modification of Talke and Schubert’s enzymatic procedure. In brief, urea in the sera samples was hydrolyzed by urease to ammonium and carbon dioxide, and ammonium, in turn, reacted with 2-oxoglutarate and the reduced cofactor in the presence of glutamate dehydrogenase (GLDH). The decrease in the concentration of the reduced cofactor was then measured at 340 nm versus appropriate standards and translated into urea concentrations, yielding the lowest detection limit <1.4 mmol/L and CV <2.9%. Cystatin C was measured by a particle-enhanced immunoturbidimetry method, with a commercially available Dako Cystatin C PET Reagent Set (DAKO, Hamburg, Germany). A polyclonal rabbit antibody against cystatin C was used for the immunoturbidimetric reaction, providing the lower detection limit <0.1 mg/L and CV <2.5%.

**Total NO synthesis** was evaluated by a specific two-step photocolourimetric assay (R&D Systems, MN, USA), enabling a separate measurement and calculation of available nitrite (NO2) prior to conversion of nitrite to nitrate (NO3) and the final assessment of total nitrate using the Griess reagent.

**STAT-8 isoprostane PGE2 alpha content** was estimated by a specific ELISA (Cayman Chemical Company, Ann Arbor, MI, USA) based on the competition between 8-isoprostane and 8-isoprostane-alkaline phosphatase conjugate for a limited number of the specific STAT-8 isoprostane PGE2 alpha antibody binding sites and subsequent reaction of the immobilized complex with para-nitrophenyl-phosphate. The intensity of the developing yellow colour was then measured in the ELISA reader according to the manufacturer’s instructions.

3-Nitro-tyrosine was assessed by a specific competitive ELISA (Chemicon International, USA) based on quantitation of tyrosine nitration.

**NF-kB p50/p65 transcription factor assay** was performed on renal cortical and medullar nuclear extracts obtained using a Chemicon Nuclear Extraction kit (Chemicon International, USA) according to the manufacturer’s protocol. The NF-kB p50/p65 transcription factor assay (Chemicon International, USA) was performed as described elsewhere [10], using a kit combining the principle of the electrophoretic mobility shift assay (EMSA) with the 96-well-based ELISA, which enables significantly greater sensitivity compared to conventional EMSA assays. Whole cell extract from the HeLa cell sample was provided by the manufacturer, to serve as a positive control probe. In brief, the prepared nuclear extracts were mixed with biotinylated oligonucleotide containing the flanked DNA binding sequence for NF-kB (5′-GGGACTTTCC-3′). Following 2-h incubation, allowing the active (i.e. not bound to Ik-B chain) form of NF-kB to bind to the consensus sequence, the sample mix was transferred to the streptavidin-coated 96-well plates. Rabbit anti-NF-kB p50/p65 antibodies were used as specific primary antibodies. Goat anti-rabbit horseradish peroxidase-conjugated antibody was used as a secondary antibody. The TMB substrate was added to the wells for the final colour development. The intensity of the colour was
Pathologic evaluation

At the end of the study protocol, three randomly selected rats from each group were killed by halothane overdose. Their kidneys were immediately removed, preserved in 4% formalin and subsequently embedded in paraffin. Large sections (1 µm) were cut perpendicularly to the renal capsule, in order to ensure that both cortex and medulla are presented in each section. The samples were stained with haematoxylin-eosin dye, and 20 microscopic fields (magnification ×400) were randomly selected for light microscope evaluation. Stratification of the kidney damage was evaluated by differential counting of the percentage of necrotizing cells in damaged tubuli per total count of the tubuli, and tubular lumen obstruction was established by calculating the percentage of granular casts or cellular fragments per total tubuli count.

Detection of intrarenal tissue hypoxia

The kidneys removed from animals injected with Hypoxyprobe-TM-1 were embedded in paraffin and cut as described earlier. The prepared slides were deparaffinized in xylene and cleared in 100% ethanol. Following subsequent gradual hydration procedure in serial ethanol dilutions and quenching of tissue peroxidase with 3% hydrogen peroxide, the antigen retrieval was performed in a 10 mM citrate buffer in a steamer device. The slides were then incubated for 30 min with the primary antibody (FITC-conjugated Hypoxyprobe-1 MAb, diluted 1:50), repeatedly rinsed and maintained for additional 30 min with the secondary antibody, the Anti-FITS Mab conjugated with horseradish peroxidase (1:50). ImmPACT DAB chromogen was used for primary staining, while counterstaining was performed in Mayer’s haematoxylin solution.

Statistical analysis

Statistical analysis was performed using SPSS-version 13 software. Parametric data were expressed as means ± standard deviations. Statistical differences between the groups were evaluated by one-way ANOVA with subsequent application of the Bonferroni test for post hoc analysis. Differences yielding P values <0.05 were considered statistically significant.

Results

Blood creatinine, urea and cystatin C measurements

All the rats had normal basal creatinine, urea and cystatin C prior to starting the experiment. Irrespective of NHT application, all the rats participating in the 48-h follow-up part of the study survived the experimental protocol. All those animals subjected to haemorrhagic shock demonstrated elevated creatinine, urea and cystatin C values (Figure 1a–c). In ischaemic rats subjected to 12-h through 48-h NHT, augmentation of urea, creatinine and cystatin C was significantly attenuated compared to the respective NHT untreated animal groups (Figure 1a–c).

Blood pressure measurement

Prior to killing, tail systolic blood pressure was assessed in two animals out of each experimental group. The mean results were 125 ± 11 mmHg in all animals and were found not statistically different between the experimental groups (P = NS in each comparison, data not shown).

Pathologic evaluation

Light microscopy evaluation of kidney tissue samples from control animals not subjected to haemorrhagic shock revealed no ATN or any other signs of tubular obstruction. Microscopic examination of renal tissues from kidneys of rats subjected to haemorrhagic shock revealed visible tubular damage, represented by extensive protein deposition and sloughed epithelial cells. Haemorrhagic shock brought about modest infiltration of inflammatory cells from blood circulation, which was not appreciably altered by NHT application. The damage was found attenuated in animals subjected to NHT following blood reperfusion. After 48 h, tubular lumen obstruction by broad granular casts and cell fragments was present in 36.2 ± 10.8% of counted tubules in the haemorrhagic shock group, compared to 16.4 ± 4.2% in haemorrhagic shock treated with HBOT animals (P = 0.004, Figure 2a–c). Similarly, the percentage of necrotizing tubular cells was significantly lower in haemorrhagic shock treated with the NHT group: only 22.1 ± 6.7% of tubular cells were found damaged, compared to 54.8 ± 13.1 in the non-NHT treated P = 0.003.

Evaluation of intrarenal tissue hypoxia status

Histopathologic evaluation of ischaemic tissue revealed a significant expression of pimonidazole protein (Hypoxyprobe-1), the marker of tissue ischaemia, 12–48 h after reperfusion. No significant expression of pimonidazole protein was evident in the post-shock NHT group. Figure 2d–f demonstrate Hypoxyprobe-1 staining of renal tissues after the 48-h follow-up in normal controls (Figure 2d) and in post-shock groups without (Figure 2e) or with NHT (Figure 2f).

Total NO synthesis

Haemorrhagic shock brought about a 10-fold drop of NO availability in renal tissues from all experimental groups (Figure 3, P < 0.001). Application of NHT following haemorrhagic shock resulted in a significant 2-fold increase of NO synthesis both in medullae and in cortices. It was evident after 12 h and 24 h of NHT (P < 0.007 in each comparison), but not after 48 h (P = 0.82 for cortex, P = 0.85 for medulla). However, the NHT-associated increased NO concentrations did not reach the pre-shock values in any experimental variation (Figure 3).
Hyperoxia attenuates ischaemia-induced renal failure

**STAT-8 isoprostane PGE$_2$ alpha accumulation**

Haemorrhagic shock brought about a significant increase of STAT-8 isoprostane PGE$_2$ alpha content in cortices after 24 h and 48 h ($P < 0.001$ for each comparison). In medullae, the increase was already evident following 12 h and remained elevated after 24 h and 48 h ($P < 0.001$ for each comparison). NHT application resulted in a significant, about 10-fold, outburst of Isoprostane production both in cortices and medullae of non-haemorrhagic shock rats. The NHT-induced elevation of Isoprostane synthesis in animals subjected to haemorrhagic shock was even more pronounced (Figure 4).

**NF-kB p50/p65 transcription factor assay**

NF-kB p50/p65 transcription factor activity in medullae and cortices of non-haemorrhagic shock groups exposed to oxygen was extremely low NHT (Figure 5).

By contrast, in medullae and cortices from kidneys of animals subjected to haemorrhagic shock, NF-kB activity was significantly augmented ($P < 0.001$ for medulla and cortices). Similar to non-haemorrhagic shock groups, NHT did not bring about any significant alterations in NF-kB activity at any time point of the experiment (Figure 5).
3-Nitrotyrosine content

As demonstrated in Figure 6, 3-nitrotyrosine concentrations in renal tissue extracts from animals subjected to haemorrhagic shock were moderately albeit significantly elevated compared to control animals. In contrast, in all NHT-exposed animals, irrespective of shock, a profound increase of intrarenal 3-nitrotyrosine formation was evident.

Discussion

The results of the present investigation demonstrated that NHT at 1 atm/100% O₂ regimen attenuated deterioration of renal function, as estimated by serum cystatin C, creatinine and urea, in rats subjected to haemorrhagic shock. In addition, histopathologic evaluation revealed profoundly decreased intrarenal expression of pimonidazole protein (Hypoxyprobe-1), the marker of tissue hypoxia, and attenuated signs of ATN in kidneys of NHT-treated animals. Furthermore, 12- to 24-h NHT application improved intrarenal NO availability. It is noteworthy that NHT-induced improvement in renal function parameters and NO availability was evident despite the expectant augmentation of intrarenal oxidative stress, as represented by the outburst of isoprostane and 3-nitrotyrosine formation. Concomitantly with elevation of oxidative stress, haemorrhagic shock resulted in a profound activation of NF-kB, which was not ameliorated by NHT application.

Renal ischaemia-induced injury is one of the most common causes of acute renal failure [3,4,9]. In turn, the following reperfusion phase is essential for survival of at least a part of the ischaemia-damaged renal tissue. However, the reperfusion process is, in turn, accompanied by additional cell damage (reperfusion injury), which has been attributed to the generation of ROS, exhilarated inflammation and hypoxia of remaining renal tissue, still persistent at the cellular level [3,4,9].

Hypoxic injury of renal tissue plays a key role in deterioration of renal functional parameters and exacerbation of renal disease [9–13]. Renal hypoxia is characterized by tubular obstruction, mainly due to ATN, and adjacent tissue oedema. Both are contributory to the increase in intrarenal pressure and, accordingly, to the decrease in perfusion pressure. The demand of oxygen is further expanded in the tissue undergoing the post-injury repair processes. The decrease in perfusion pressure and elevation of oxygen demand during the reperfusion phase are responsible for continuation of hypoxia at the cellular level. In the present study, post-reperfusion ischaemia, demonstrable by intrarenal expression of pimonidazole protein, a marker of tissue hypoxia, was evident as long as 48 h following the acute insult. NHT application almost completely abolished the intrarenal signs of pimonidazole staining 48 h following reperfusion. This would tend to conclude that NHT, by providing enhanced oxygen supply to the oxygen-deprived tissue, markedly decreased intrarenal hypoxia-induced damage during the reperfusion phase.

On the other side, augmented oxygen supply might be associated with the formation of ROS [13]. Consequently, oxygen supply via NHT during the reperfusion phase might further aggravate the already augmented intrarenal ROS accumulation. Certainly, ROS are not solely responsible for ischaemia/reperfusion-induced renal injury. However, in several animal models treatment with free radical scavengers, such as superoxide dismutase (SOD), was shown to ameliorate the ischaemia-induced deterioration of renal function [14,15]. This would indicate that excessive ROS generation is, indeed, contributory to the development of post-ischaemic renal tissue damage [4,14,15]. In the present study, NHT application resulted in an expectant significant accumulation of ROS in ischaemic as well as non-ischaemic kidneys, as estimated by the outburst of isoprostane.
Hyperoxia attenuates ischaemia-induced renal failure

Fig. 2. Pathologic evaluation. (a) Normal renal tissue from the sham group. (b) Renal tissue from the haemorrhagic shock exposed group, with no NHT treatment. (c) Renal tissue from the haemorrhagic shock group, subjected to NHT at 1 atm of 100% oxygen. (d) Renal tissue from the sham control group stained for Hypoxiprobe-1. (e) Renal tissue from the haemorrhagic shock exposed group, with no NHT treatment, stained for Hypoxiprobe-1. (f) Renal tissue from the haemorrhagic shock group, subjected to NHT at 1 atm of 100% oxygen, stained for Hypoxiprobe-1. The pathologic evaluation was performed in all groups after 48 h, irrespective of NHT application. The tissues from the sham group (a) demonstrate normal renal histology. In the haemorrhagic shock groups (b and c), tubular damage is represented by extensive protein deposition and sloughed epithelial cells. The percentage of tubular lumen obstruction and necrotizing tubular cells is more prominent in the group subjected to haemorrhagic shock group without NHT application. Similarly, the Hypoxiprobe staining, not evident in sham group (d), is prominent in the haemorrhagic shock tissue (e), but almost completely absent in tissue from kidneys of NHT exposed animals (f).
Fig. 3. Nitric oxide (NO) content in renal cortical and medullar cell extracts. Shock: rats exposed to gradual blood withdrawal/reperfusion; Sham: rats not exposed to gradual blood withdrawal/reperfusion; NHT: normobaric hyperoxia treatment; + Oxygen: rats exposed to NHT; 12 h, 24 h, 48 h: the time periods of exposure to NHT. *P < 0.05 compared to the respective sham group. #P < 0.05 compared to the shock exposed, NHT non-treated group.

Fig. 4. STAT-8 Isoprostane PGE2 alpha content in renal cortical and medullar cell extracts. Shock: rats exposed to gradual blood withdrawal/reperfusion; Sham: rats not exposed to gradual blood withdrawal/reperfusion; NHT: normobaric hyperoxia treatment; + Oxygen: rats exposed to NHT; 12 h, 24 h, 48 h: the time periods of exposure to NHT. *P < 0.05 compared to the respective sham group. #P < 0.05 compared to the shock exposed, NHT non-treated group.

production and 3-nitrotyrosine formation. Nevertheless, NHT significantly improved renal functioning parameters in kidneys recovering from haemorrhagic shock, despite augmentation of intrarenal oxidative stress.

The ischaemia-injured renal cells have been demonstrated to produce bioactive proteins serving as chemotactants recruiting inflammatory mononuclear and polymorphonuclear cells from blood circulation to the damaged kidney. In turn, the infiltrating inflammatory cells locally secrete chemokines initiating renal cell activation, cytokine production and extracellular matrix deposition, thus exacerbating the inflammation and compromising renal microvascular blood flow [16,17]. The impact of the latter is sensed more in the outer medulla than in the cortex, as indicated by marked vascular congestion typically seen in this region of the kidney. In a number of studies performed on non-renal tissues, NHT application at the reperfusion stage has been demonstrated to affect the inflammatory cascades induced by ischaemia/reperfusion injury [18]. The observed effects included altered secretory and adhesive
Hyperoxia attenuates ischaemia-induced renal failure

Fig. 5. NF-κB activation in renal cortical and medullar cell extracts. Shock: rats exposed to gradual blood withdrawal/reperfusion; Sham: rats not exposed to gradual blood withdrawal/reperfusion; NHT: normobaric hyperoxia treatment; + Oxygen: rats exposed to NHT; 12 h, 24 h, 48 h, the time periods of exposure to NHT. ∗P < 0.05 compared to the respective sham group. #P < 0.05 compared to the shock exposed, NHT non-treated group.

Fig. 6. 3-Nitrotyrosine content in renal cortical and medullar cell extracts. Shock: rats exposed to gradual blood withdrawal/reperfusion; Sham: rats not exposed to gradual blood withdrawal/reperfusion; NHT: normobaric hyperoxia treatment; + Oxygen: rats exposed to NHT; 12 h, 24 h, 48 h, the time periods of exposure to NHT. ∗P < 0.05 compared to the respective sham group. #P < 0.05 compared to the shock exposed, NHT non-treated group.

Properties of circulating and residual mononuclear cells, as well as augmentation of NO production [18]. At the cellular level, activation of nuclear factor NF-κB is considered to be one of the main events triggering and controlling the progression of inflammation. The results of the present study, similar to some other investigations [18,19], demonstrated a profound activation of the NF-κB-regulated inflammatory pathways in the ischaemia/reperfusion-injured tissue. However, within the present experimental protocol, NF-κB activation could be neither prevented nor attenuated by NHT application. Therefore, even if NHT therapy did exert any positive effects on haemorrhagic shock-induced renal inflammation, they were not mediated via the NF-κB pathway.

In the present investigation, clinical and histopathologic manifestations of haemorrhagic shock-induced renal failure were ameliorated in animals subjected to NHT. Concomitantly, within the first 24 h of NHT, a significant 2-fold increase in intrarenal NO availability was evident. NO is famous for its dual role in induction and progression of renal disease. The consensus is that augmentation of intrarenal NO synthesis beyond the physiologic levels is, indeed, damaging for the kidney [20,21]. However, sufficient availability of constitutive intrarenal NO is mandatory for regulating intracellular signalling and counteracting the vasoconstrictor activities of vasopressin, noradrenaline and angiotensin II, thereby maintaining intravascular tone and sustaining blood perfusion in most capillary beds [22–25].
The inhibition of constitutive NO synthesis was shown to decrease blood perfusion both in renal cortices and medul- lae [22–25]. NO is a strong competitive inhibitor of oxygen consumption [26,27], and the magnitude of NO-mediated inhibition of the latter is inversely associated with the magnitude of partial oxygen pressure (pO$_2$) [28]. Therefore, a steep, about 10-fold, drop of intrarenal NO availability observed in ischaemic renal tissues appears to be re- sultant from the ischaemia-induced reduction in pO$_2$. On the other side, 3-nitrotyrosine formation was found augmented in renal tissues subjected to haemorrhagic shock. NHT application resulted in a further profound increase in 3-nitrotyrosine formation, indicating augmented peroxynitrite generation in the oxygen-exposed kidneys. ROS have been previously shown by some authors to react with NO, forming peroxynitrates [21,27], and thus decrease bioavail- ability of the NO molecule [27]. It is plausible that in the present experimental setting NHT application also resulted in an initially sharp increase of intrarenal NO. However, the intense concomitant peroxynitrite formation compromised the availability of the latter. Still, it appears that even a mod- erate elevation of intrarenal NO appreciably contributed to the recuperation processes, despite concomitant accumula- tion of intrarenal ROS.

**Conclusion**

1. 12- to 48-h NHT ameliorated deterioration of renal func- tion and expression of histopathologic signs of intrarenal hypoxia in a rat model of haemorrhagic shock-induced renal failure.

2. Concomitantly, 12- to 24-h NHT application increased bioavailability of NO in renal cortices and medullae of kidneys recuperating from haemorrhagic shock.

3. As far as the NF-kB pro-inflammatory pathway is con- cerned, NHT had no effects on inflammatory processes ongoing within the ischaemic kidneys.

4. Despite the NHT-associated accumulation of ROS, early oxygen supply appears essential for effective recuperation of the ischaemic kidney from detrimental conse- quences of haemorrhagic shock.

**Conflict of interest statement:** None declared.

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