The effect of combined hyperbaric oxygen and iloprost treatment on the prevention of spinal cord ischaemia–reperfusion injury: an experimental study

Gokhan Ilhan[a,*], Murat Aksun[b], Berkan Ozpak[b], Tevfik Gunes[b], Sahin Bozok[a], Murtaza Emre Durakoglugil[c], Hasan Gucer[d] and Ali Gurbuz[b]

[a] Faculty of Medicine, Department of Cardiovascular Surgery, Recep Tayyip Erdogan University, Rize, Turkey
[b] Department of Cardiovascular Surgery, Izmir Katip Celebi University, Ataturk Education and Research Hospital, Izmir, Turkey
[c] Faculty of Medicine, Department of Cardiology, Recep Tayyip Erdogan University, Rize, Turkey
[d] Faculty of Medicine, Department of Pathology, Recep Tayyip Erdogan University, Rize, Turkey

* Corresponding author. Faculty of Medicine, Department of Cardiovascular Surgery, Recep Tayyip Erdogan University, Islampasa Mah, Rize 53100, Turkey.
  Tel: +90-4642123009; fax: +90-4642123015; e-mail: drgknilhan@yahoo.com (G. Ilhan).

Abstract

OBJECTIVES: Hyperbaric oxygen (HBO) has been shown to be effective in preventing neurological injuries in animal models of ischaemia, whereas iloprost (IL) prevents ischaemia-related mitochondrial dysfunction and reduces infarction size after focal cerebral ischaemia in animal models. The aim of the present study was to investigate the effect of combined HBO and IL treatment on spinal cord ischaemia–reperfusion (IR) injury by neurological, histopathological and biochemical methods in an experimental study.

METHODS: Eighty New Zealand white male rabbits were randomly allocated into one of five study groups. The HBO group received a single session of HBO treatment and the IL group received an infusion of 25 ng/kg/min IL; the HBO + IL group received both HBO and IL and the control group received only 0.9% saline; the fifth group was the sham group. Levels of S100β protein, neuron-specific enolase (NSE) and nitric oxide (NO) were measured at onset, at the end of ischaemia period and at the 24th and 48th hour of reperfusion. Physical activity was assessed using Tarlov criteria 24, and the spinal cords of the sacrificed rabbits were evaluated histopathologically. Additionally, tissue malondialdehyde (MDA) and antioxidant enzyme activities [total superoxide dismutase (SOD); catalase (CAT) and glutathione peroxidase (GSH-Px) were assessed.

RESULTS: Neurological scores in the HBO, IL and HBO + IL groups were statistically significantly better compared with the control group at the 24th ($P = 0.001$ for all) and 48th hour ($P = 0.001$ for all). Histopathological scores in the HBO, IL and HBO + IL groups were also significantly better compared with the control group ($P = 0.003$, 0.001 and 0.001, respectively). Whereas MDA, NSE, S100β protein and NO concentrations were significantly lower, CAT and GSH-PX levels were significantly higher in either sham or treatment groups compared with the control group.

CONCLUSIONS: Since we demonstrated beneficial effects on spinal cord IR injury, we think that both HBO and IL, either alone or in combination, may be reasonable in the treatment of IR injury. Furthermore, there did not appear to be synergistic effects with combined treatment. More research is needed for practical application in humans, following thoracoabdominal aortic surgery.

Keywords: Spinal cord • Ischaemia–reperfusion injury • Hyperbaric oxygen • Iloprost

INTRODUCTION

Paraplegia secondary to spinal cord injury (SCI) remains a disastrous and unpredictable complication of descending thoracic and thoracoabdominal aorta surgery. Even though several anaesthetic, surgical and perfusion techniques have been introduced, the incidence of paraplegia still remains high, reported between 4 and 33% [1]. Therefore, there is considerable effort on preventing paraplegia. Several procedures have been suggested to minimize this problem, including temporary shunts or partial bypass, hypothermia, drainage of cerebrospinal fluid and pharmacological agents. However, a reliable preventive method has not been reported until now.

Paraplegia is usually due to temporary or permanent ischaemia of the spinal cord caused by disruption of blood flow during aortic cross-clamping. Inflammatory processes, as well as free oxygen radical formation, and activation of phospholipase-A₂ have been blamed in etiopathogenesis of ischaemia–reperfusion (IR) injury. Reactive oxygen species (ROS), produced initially during reperfusion, aggravate this damage [2]. Eventually, neuronal necrosis ensues due to free radical generation, lipid peroxidation and accumulation of intracellular calcium.

Hyperbaric oxygen (HBO) is occasionally used as a neuroprotective adjuvant therapy for the treatment of cerebral ischaemia. The beneficial effects of HBO in IR stem from lower endothelial

adhesion molecule expression and decreased neutrophil activation and migration. Moreover, HBO stabilizes lysosomal enzymes and promotes nitric oxide (NO) production [3]. Prostacyclin (PGI_2) may reduce SCI during aortic occlusion due to cytoprotective, antioxidant and vasodilator properties [4]. Iloprost (IL), a stable analogue of PGI_2, has a similar profile of action. In addition to inhibition of ROS production and release of lysosomal enzymes, IL decreases neutrophil activation and aggregation. The overall effect of HBO results in increased tissue oxygen and improved collagen synthesis, angiogenesis and epithelization [3]. In addition, IL infusion, started before aortic occlusion and continued throughout ischaemia, was reported to have a possible effect on improving spinal cord protection [4]. However, the underlying mechanisms are poorly understood. That is why different studies still reach different results, without a final conclusion.

The purpose of this experimental study was to investigate the effectiveness of HBO and IL treatment, either alone or in combination, on SCI due to ischaemia and reperfusion by neurological, histopathological and biochemical methods in an animal model.

MATERIALS AND METHODS

Animal model

The experimental procedure was in accordance with the Position of the American Heart Association on Research Animal Use. Animal care complied with the Principles of Laboratory Animal Care as formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (National Institutes for Health publication No. 5377-3, 1996). This study was approved by the Animal Research Ethics Committee of Recep Tayyip Erdogan University (29 July 2011, no: 2011/19). All animals were given 5 days of adaptation to their environment prior to experiments. The room temperature was kept between 26 and 28°C.

Study groups

Eighty New Zealand white male rabbits, weighing 2.3–3.2 (mean 2.8) kg, were randomly allocated into one of the five study groups. The HBO group ($n = 16$) received a single session of HBO treatment, the IL group ($n = 16$) received an infusion of 25 ng/kg/min IL; the HBO + IL group ($n = 16$) received both HBO and IL; the control (IR) group ($n = 16$) received only 0.9% saline and the fifth group was the sham group ($n = 16$). HBO treatment and IL infusion were administrated during 60 min of ischaemia and 60 min of reperfusion in the treatment groups.

Three random rabbits from every group were sacrificed in order to compare with the sham group and to perform biochemical measurements prior to any surgical procedure or medical treatment. The remaining rabbits ($n = 13$ for each group) were subjected to a spinal cord ischaemia for 60 min. During the reperfusion period, animals were reanesthetized and killed at 24 ($n = 5$ for each group) and 48 h after reperfusion ($n = 8$ for each group). Sham-operated animals underwent the same operative conditions, without aortic occlusion.

Anaesthesia and monitoring

Initial anaesthesia was achieved using intramuscular ketamine (50 mg/kg) and xylazine (5 mg/kg) without endotracheal intubation.

Body temperature was maintained close to 38°C using a thermostatically controlled heated operation table. All animals received a similar volume of maintenance fluids (0.9% sodium chloride, 20 ml/h) during the whole procedure. An arterial catheter (20-gauge) was placed in an ear artery to monitor blood pressure (Petas KMA 800, Ankara, Turkey), and arterial blood was sampled for gas analysis. Blood pressure tracings were drawn following the induction of anaesthesia (pre-ischaemia) and 2 h after release of aortic cross-clamping (post-ischaemia) via the ear artery catheter. Arterial and venous samples were gathered at the same periods.

Surgical technique

Animals were placed in supine position. After sterile preparation, the infrarenal abdominal aorta was exposed through a midline incision and a transperitoneal approach with the abdominal contents reflected to the right. The aorta was isolated from the left renal artery down to the aortic bifurcation. Each animal was given 150 U/kg heparin intravenously 5 min prior to aortic occlusion. Heparin effect was not reversed at the end of the procedure. The aorta was cross-clamped just below the left renal artery and proximal to the aortic bifurcation using arterial bulldog clamps (Vascu-statt, Scanlan International, St. Paul, MN, USA). The inferior mesenteric artery was also clamped at origin, and loss of aortic pulse was confirmed by palpation. The clamp was removed after 60 min and restoration of blood flow was verified visually. Sham-operated animals underwent the same operative conditions, without aortic occlusion. The abdomen was closed and the catheters were removed.

Local bupivacaine hydrochloride was applied around the wound for postoperative analgesia. Gentamicin (40 000 IU) was administered intramuscularly immediately after the operation. Afterwards, the animals were restored to their home cage and survived for 2 days. Bladder content was compressed manually if necessary. Animals were allowed to recover in their cages, with free access to food and water in the postoperative period.

Hyperbaric oxygen treatment procedure

Animals received HBO therapy in an animal monoplace chamber during 60 min of ischaemia and 60 min of reperfusion at a pressure of 2.5 atm. Before pressurization, 100% medical oxygen was flushed through the chamber for 10 min to displace ambient air. The oxygen pressure was then increased slowly and reached 2.5 atm. in 5 min. The chamber was ventilated during HBO therapy to avoid carbon dioxide (CO_2) accumulation. The concentration of CO_2 was not allowed to rise above 0.1%. An environmental control system maintained inner temperature and relative humidity at 25 ± 1°C and 50 ± 20%, respectively. After 120 min at 2.5 atm., the chamber was decompressed to normal atmospheric pressure in 5 min.

Evaluation of neurological function

The rabbits were neurologically assessed by an observer, unaware of the treatment group, using the modified Tarlov criteria at 24 and 48 h after reperfusion. Modified Tarlov criteria are as follows: 0: paraplegic with no lower extremity function; 1: poor lower

extremity function with only perceptible movement of joints; 2: active movement but no ability to stand; 3: ability to stand but no ability to walk; 4: completely normal hind limb motor function.

Sacrifice and tissue preparation

All animals were humanely sacrificed by a lethal cardiac injection of pentobarbital (100 mg/kg), at the predetermined time. Spinal cords were harvested immediately and the L3–6 segments (6–8 cm in length) were fixed in neutral buffered 10% formalin solution and stored for 24 h prior to embedment in paraffin blocks for sectioning and histological examination.

Histopathological examination

Sections 4–7 μm in thickness, obtained from distal caudal portions of the harvested spines corresponding to the L3–6 segments, were affixed to glass slides. Afterwards, slides were stained with haematoxylin–eosin (H–E) and examined by light microscopy. The extent of ischaemic damage and leucocyte infiltration in motor neurons in ventral horns of the spinal cord were assessed.

Cells that had eosinophilic cytoplasm and lost their nucleus were considered injured. Neurons having prominent nucleolus with fine chromatins and cytoplasmic nissle bodies were considered viable. The neurons from each spinal cord section were examined and categorized as injured or viable by a blinded observer. A viability index was calculated via dividing the number of viable cells by the total number of neurons counted within the entire microscopic section for each animal (viability index = the number of viable cells/total number of neurons). In addition, inflammatory response was also graded semi-quantitatively by counting the number of infiltrated leucocytes in randomly selected fields and scored according to the number of leucocytes as follows: '0' for none, '1' for <20, '2' for 20–50 and '3' for >50 leucocytes.

Serum biochemical evaluation

Blood pH, pO_2, pCO_2 and HCO_3 values were determined using a blood-gas analyser (Ciba-Corning Blood Gas Analyzer Model 860, Ciba Corning Diagnostics Corp., Irvine, CA, USA). Blood samples were drawn via the ear artery catheter following the induction of anaesthesia (pre-ischaemia), and 24 and 48 h after release of aortic cross-clamping (post-ischaemia). Blood was allowed to clot for 20–30 min at room temperature, centrifuged (5000 rpm, 10 min at +4°C) and kept frozen at −85°C until studied.

We also analysed neuron-specific enolase (NSE), a presumed quantitative marker of ischaemic injury, levels of which increase in the first week of ischaemic stroke; S100β protein, as a marker of cerebral injury and NO (nitrite + nitrate), as a marker of oxidative stress.

Determination of neuron-specific enolase and S100β

NSE and S100β were measured by a commercial kit (Roche, Cat.) via a Moduler E170 hormone autoanalyzer. This method is based

on chemiluminescent enzyme immunoassay and results were expressed as mg/l. There was no significant haemolysis in any of the samples.

Determination of nitric oxide

As NO measurement is very difficult in biological specimens, tissue nitrite (NO_2^-) and nitrate (NO_3^-) were estimated as an index of NO production. The method for spinal cord, nitrite and nitrate levels was based on the Griess reaction. Samples were initially deproteinized with Somogyi reagent. Total nitrite (nitrite + nitrate) was measured after conversion of nitrate into nitrite by copporized cadmium granules using a spectrophotometer at 545 nm (Ultraspec Plus, Pharmacia LKB Biochrom Ltd., UK). A standard curve was established with a set of serial dilutions (10^{-8}–10^{-3} mol/l) of sodium nitrite, and an absorbance–concentration graphic was prepared. Linear regression was performed by using the peak area from nitrite standard. The resulting equation (slope) was used to calculate the unknown sample concentrations. Results were expressed as micromole per gram protein for spinal cord tissue, and micromole per litre for serum measurements.

Tissue biochemical evaluation

Spinal cord tissues of L3–6 levels were homogenized in cold saline with a weight-to-volume ratio of 1:10. Spinal cord tissues were kept at −30°C until analysis (about 3 days). Tissues were weighed, homogenized in Tris–HCl buffer (pH 7.4, 50 mM) containing 0.50 ml/l Triton X-100 with a homogenisator (Tempest Virtishear, Model 278069, The Virtis Company, Inc., Gardiner, NY, USA) and then centrifuged at 5000 *g* for 30 min to remove debris. For further extraction, the supernatant was extracted in an alcohol/chloroform mixture. Following a second centrifugation at 5000 *g* for 60 min, the clear upper layer was taken and used in the enzymatic assays. All procedures were performed at +4°C. Malondialdehyde (MDA) levels were determined in the homogenate, catalase (CAT) in supernatant and total superoxide dismutase (SOD) in the extracted samples. Protein measurements were made in all stages. Protein concentrations were determined according to Lowry's method [5].

SOD activity was measured using reduction of nitrobluetetrazolium by the xanthine–xanthine oxidase system, which is a superoxide generator. Enzyme activity leading to 50% inhibition was accepted as one unit. Results were expressed as U/mg protein. CAT activity was determined according to Aebi [6]. The principle of the CAT activity was based on the determination of the rate constant (k, s^{-1}) or the hydrogen peroxide decomposition rate at 240 nm. Results were expressed as kg/g protein. Tissue MDA levels were determined by the method described by Wasowicz *et al.* [7]. Briefly, MDA was reacted with thiobarbituric acid by incubating for 1 h at 95–100°C. Afterwards, fluorescence intensity was measured in the *n*-butanol phase with a fluorescence spectrophotometry (Hitachi, Model F-4010; excitation at 525 nm, emission at 547 nm) by comparing with a standard solution of 1,1,3,3 tetramethoxypropane. Results were expressed in terms of nmol/g wet tissue. Glutathione peroxidase (GSH-Px) activity was measured by the method of Paglia *et al*. The enzymatic reaction in the tube, which contains the following items: NADPH, reduced glutathione (GSH), sodium azide and glutathione reductase (GR), was initiated by addition of H_2O_2 and the change in absorbance at 340 nm was monitored by a spectrophotometer [8]. Activity was given as units

per gram protein in spinal cord tissue. All samples were assayed in duplicate.

Statistical analysis

Data were analysed using the Statistical Package for Social Sciences (SPSS) software (version 10.0 for Windows). All differences associated with a chance probability of <0.05 were considered statistically significant. All data are presented as mean ± SD. Statistical analysis was performed using the analysis of variance (ANOVA) test. The one-way ANOVA test was followed by *post hoc* Dunnett's test.

RESULTS

Physiological and haemodynamic parameters

Physiological and haemodynamic parameters are summarized in Table 1. Physiological parameters were not statistically significant between groups in pre- and post-ischaemic periods, except controls. Blood pH, PO_2 and HCO_3 levels of the control group were significantly lower in the post-ischaemic phase compared with both the pre-ischaemic period and the treatment groups.

Neurological outcome

Tarlov scores in both assessments were summarized in Table 2. All animals survived until the final neurological behaviour assessment

at the 24th and 48th hour following reperfusion. Rabbits in the sham-operated group did not have any neurological deficit. Conversely, the most severe neurological deficit was noted in the control group in both assessments. The neurological examination at the 24th hour revealed that pretreated rabbits were significantly more impaired than the sham group and less impaired than controls. Importantly, final neurological assessment (48 h) showed that rabbits in the HBO, IL and HBO + IL groups had a significantly better neurological score compared with 24th hour, which were not statistically different from the sham group (Table 3).

Histopathological changes

Sham-operated animals had spinal cords with high viability indices. Extensive ischaemic damage was observed in ventral horns of spinal cords harvested from controls, which was consistent with a low viability index (Fig. 1). All animals in the HBO, IL and HBO + IL groups had viability indices that were not statistically different from the sham group. However, viability indices in the HBO, IL and HBO + IL groups were statistically higher than the control group (P < 0.001) (Table 3, Figs 1 and 2), indicating minor or no neuronal damage. The mean score of inflammatory response grade was significantly higher in the control group.

Biochemical findings

Results of NSE, S-100B and NO are summarized in Table 4.

Table 1: Physiological and haemodynamic variables in all groups at pre- (after induction of anaesthesia) and post-ischaemia (2 h after removal of cross-clamp)

	Group HBO	Group IL	Group HBO + IL	Group Control	Group Sham
Mean arterial pressure (mmHg)					
Pre-ischaemia (*n* = 16)	87 ± 17	90.5 ± 5.5	89 ± 14	91 ± 6	82 ± 16
Post-ischaemia (*n* = 13)	74 ± 10	83.8 ± 6.2	73 ± 9	78 ± 9	77 ± 12
Heart rate (beat/min)					
Pre-ischaemia (*n* = 16)	232 ± 26	234 ± 20	238 ± 22	240 ± 18	239 ± 10
Post-ischaemia (*n* = 13)	227 ± 16	228 ± 14	234 ± 13	236 ± 14	231 ± 8
Rectal temperature (°C)					
Pre-ischaemia (*n* = 16)	38.4 ± 0.5	38.5 ± 0.2	38.3 ± 0.4	38.2 ± 0.3	38.5 ± 0.4
Post-ischaemia (*n* = 13)	38.0 ± 0.3	37.9 ± 0.3	38.1 ± 0.3	37.8 ± 0.5	37.9 ± 0.2
pH					
Pre-ischaemia (*n* = 16)	7.35 ± 0.02	7.34 ± 0.04	7.34 ± 0.03	7.36 ± 0.03	7.35 ± 0.04
Post-ischaemia (*n* = 13)	7.40 ± 0.06	7.33 ± 0.12	7.32 ± 0.02	7.18 ± 0.02*	7.34 ± 0.05
pO_2 (mmHg)					
Pre-ischaemia (*n* = 16)	110.7 ± 3.3	119 ± 3.7	112.6 ± 4.7	107.1 ± 9.8	104.6 ± 12.7
Post-ischaemia (*n* = 13)	234 ± 27.7#	96.6 ± 3.2	212 ± 23.8#	78.6 ± 4.7*	98.2 ± 5.2
pCO_2 (mmHg)					
Pre-ischaemia (*n* = 16)	35.6 ± 5.7	38.1 ± 4.2	38.6 ± 3.5	36.4 ± 3.8	35 ± 6
Post-ischaemia (*n* = 13)	32.7 ± 6.3	35.3 ± 3.7	34.5 ± 2.8	39 ± 5	31.6 ± 4.4
HCO_3 (mmol/l)					
Pre-ischaemia (*n* = 16)	14.0 ± 1.7	16 ± 1.4	15 ± 1.3	14 ± 1.1	15 ± 2.7
Post-ischaemia (*n* = 13)	13.5 ± 2.5	14 ± 1.5	14.6 ± 1.2	7.2 ± 1.3*	14.8 ± 1.6
Glucose (mmol/l)					
Pre-ischaemia (*n* = 16)	168 ± 34	180 ± 43	201 ± 46	192 ± 30	159 ± 25
Post-ischaemia (*n* = 13)	203 ± 37	212 ± 52	226 ± 35	208 ± 36	189 ± 20

*Significantly lower than the treatment groups, sham and pre-ischaemia (P < 0.001).
**Significantly higher than pre-ischaemia, the control, sham and IL groups (P < 0.001).

Table 2: Neurological outcome of rabbits according to the Tarlov score

Tarlov score	Group HBO 24 h (n = 13)	48 h (n = 8)	Group IL 24 h (n = 13)	48 h (n = 8)	Group HBO + IL 24 h (n = 13)	48 h (n = 8)	Group control 24 h (n = 13)	48 h (n = 8)	Group sham 24 h (n = 13)	48 h (n = 8)
0							8	5		
1	3	1	2		1		5	2		
2	7	5	7	3	7	2		1		
3	3	1	4	4	5	4				
4		1		1		2			13	8

Table 3: Comparison between neurological outcome and histopathological findings

	Group HBO	Group IL	Group HBO + IL	Group control	Group sham	
24th hour neurological score (n = 13)	2.00 ± 0.52	2.15 ± 0.42	2.30 ± 0.55	0.38 ± 0.52	4 ± 0	HBO vs control, P = 0.001* IL vs control, P = 0.001* HBO + IL vs control, P = 0.001*
48th hour neurological score (n = 8)	2.25 ± 0.46	2.75 ± 0.74	3.00 ± 0.83	0.5 ± 0.76	4 ± 0	HBO vs control, P = 0.001* IL vs control, P = 0.001* HBO + IL vs control, P = 0.001*
48th hour histopathological score (n = 8)	1.53 ± 0.82	1.14 ± 0.90	0.92 ± 0.73	2.28 ± 0.74	0.58 ± 0.42	HBO vs control, P = 0.003* IL vs control, P = 0.001* HBO + IL vs control, P = 0.001*
48th hour viability index (n = 8)	0.73 ± 0.09	0.76 ± 0.03	0.80 ± 0.07	0.30 ± 0.05	0.85 ± 0.08	HBO vs control, P = 0.001* IL vs control, P = 0.001* HBO + IL vs control, P = 0.001*

*$P < 0.05$.
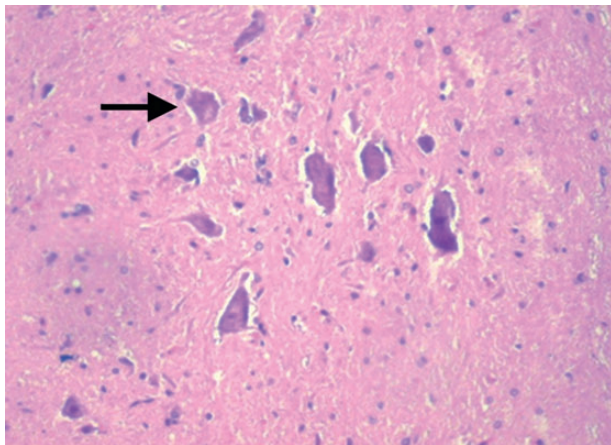
Figure 1: Neuronal damage (arrow) in some of the anterior horn cells of the spinal cord (haematoxylin–eosin ×100).
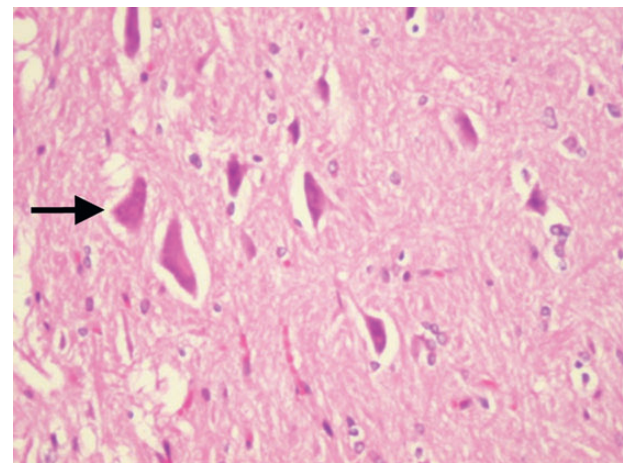
Figure 2: Severe neuronal damage characterized by caryolysis, hyperchoromasia and wrinkling in all of the anterior horn cells of the spinal cord (arrow) (hamatoxylin–eosin ×100).

Malondialdehyde content in spinal cord tissue

MDA content increased significantly in the control group at both 24 and 48 h after reperfusion compared with pre-ischaemic state (vs pre-ischaemic period: 24 h, $P = 0.001$; 48 h, $P = 0.001$). MDA content in spinal cord tissue of treatment groups was significantly lower at 24 and 48 h after reperfusion compared with controls (24th hour HBO vs control, $P = 0.001$; IL vs control, $P = 0.002$; HBO + IL vs control, $P = 0.001$; 48th hour HBO vs control, $P = 0.001$; IL vs control, $P = 0.001$; HBO + IL vs control, $P = 0.001$). On the other hand, MDA levels were significantly higher at 24 h compared with the pre-ischaemic state and sham group ($P < 0.001$). Moreover, MDA levels in the HBO + IL group were

significantly lower at the 48th hour compared with that of the 24th hour and other treatment groups ($P = 0.005$; Fig. 3 and Table 4).

Antioxidant enzyme activities in spinal cord

Superoxide dismutase. The most prominent increase in SOD levels at the 24th and 48th hour was in the control group, which was significantly higher than that in the pre-ischaemic period and other groups (control vs pre-ischaemic period, $P = 0.001$; 24th hour: control vs HBO, $P = 0.002$; control vs IL, $P = 0.001$; control vs HBO + IL, $P = 0.001$ and control vs sham, $P = 0.001$; 48th hour control vs HBO, $P = 0.001$; control vs IL, $P = 0.002$; control vs HBO + IL, $P = 0.001$ and control vs sham, $P = 0.001$) (Fig. 3).

Catalase. CAT activity was significantly lower in the control group compared with the pre-ischaemic period and remaining groups (vs pre-ischaemic period, $P = 0.003$; vs HBO, $P = 0.004$; vs IL, $P = 0.002$; vs HBO + IL, $P = 0.001$ and vs sham, $P = 0.001$) (Fig. 3).

Glutathione peroxidise. The lowest levels of GSH-Px were documented in the control group (vs pre-ischaemic period, $P = 0.001$; vs HBO, $P = 0.001$; vs IL, $P = 0.002$; vs HBO + IL, $P = 0.001$ and vs sham, $P = 0.001$). GSH-Px activity decreased in the control group at the 24th and 48th hour after reperfusion in comparison with pre-ischaemic state (24th hour, $P = 0.001$ and 48th hour, $P = 0.002$) (Fig. 3).

Serum neuron-specific enolase and S100β levels

Post-ischaemic serum NSE and S100β protein concentrations in the control group were significantly higher compared with both pre-ischaemic state and other groups (vs pre-ischaemic period, $P = 0.001$; vs HBO, $P = 0.005$; vs IL, $P = 0.002$; vs HBO + IL, $P = 0.001$ and vs sham, $P = 0.001$). NSE and S100β concentrations decreased among treatment groups at the 48th hour of reperfusion, the most prominent change being at the HBO + IL group. This decrement in NSE and S100β values is meaningful and correlates with histopathological and neurological assessments (Table 4).

Serum nitric oxide (nitrite + nitrate)

Serum NO levels were significantly higher in the control group in comparison with the pre-ischaemic period and remaining groups (vs pre-ischaemic period, $P = 0.001$; vs HBO, $P = 0.002$; vs IL, $P = 0.001$; vs HBO + IL, $P = 0.001$ and vs sham, $P = 0.001$) (Table 4).

DISCUSSION

Ischaemic SCI is a major complication of thoracic and thoracoabdominal aneurysm surgery and remains a persistent clinical problem. Numerous spinal cord protection methods including temporary shunts or partial bypass, hypothermia, drainage of cerebrospinal fluid and pharmacological measures have been
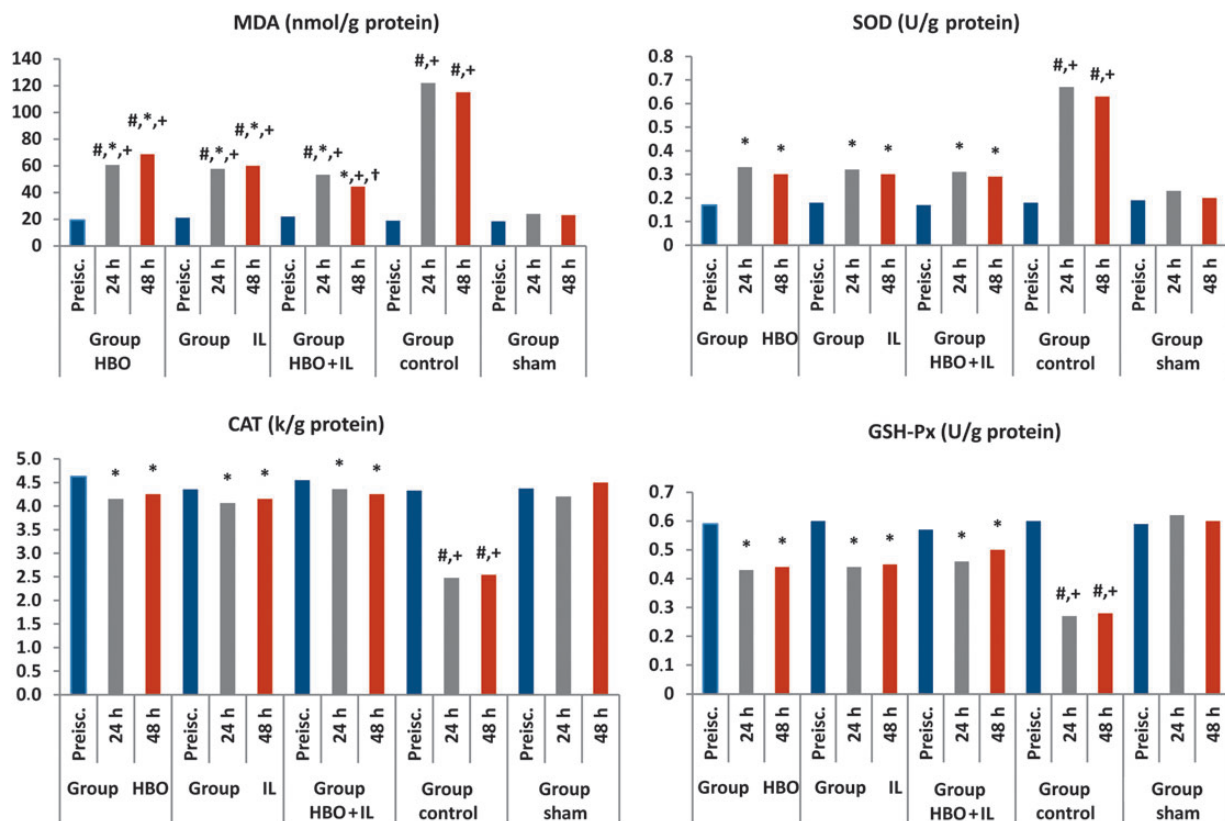
Figure 3: Spinal cord oxidant and antioxidant status in the groups in rabbits. HBO: hiperbaric oxygen; IL: iloprost; MDA: malondialdehyde (nmol/g protein); SOD: superoxide dismutase (U/g protein); CAT: catalase (kg/g protein); GSH-Px: glutathione peroxidase (U/g protein); Preisc: pre-ischaemia; #Significantly different from values of the sham group ($P < 0.001$); *significantly different from untreated control values ($P < 0.001$); +significantly different from the pre-ischaemic period ($P < 0.005$); †significantly different from 24 h ($P < 0.02$).

Table 4: Comparison of changes in biochemical markers

		NSE (μg/l)	S-100 β (μg/l)	NO (μM/l)
Group HBO	Preisc. (n = 16)	6.78 ± 0.32	1.72 ± 0.25	21.5 ± 2.5
	24 h (n = 13)	11.1 ± 0.55**	3.75 ± 0.36**	27.5 ± 3.6**
	48 h (n = 8)	9.5 ± 0.44**	3.12 ± 0.23**	24.7 ± 2.8**
Group IL	Preisc. (n = 16)	6.25 ± 0.45	1.75 ± 0.27	22.7 ± 3.09
	24 h (n = 13)	10.72 ± 0.5**	3.70 ± 0.33**	28 ± 2.15**
	48 h (n = 8)	9.1 ± 0.58**	3.10 ± 0.35**	25 ± 3.4**
Group HBO + IL	Preisc. (n = 16)	6.43 ± 0.38	1.72 ± 0.38	19.5 ± 2.10
	24 h (n = 13)	9.65 ± 0.35**	3.15 ± 0.26**	29.7 ± 4.10**
	48 h (n = 8)	8.6 ± 0.47**	2.87 ± 0.37**	21.5 ± 2.5**
Group control	Preisc. (n = 16)	6.32 ± 0.42	1.77 ± 0.35	22.5 ± 2.7
	24 h (n = 13)	26.2 ± 1.25*.***	6.25 ± 0.40*.***	43.5 ± 3.25*.***
	48 h (n = 8)	31.6 ± 1.00*.***	5.57 ± 0.45*.***	38.5 ± 2.80*.***
Group sham	Preisc. (n = 16)	6.57 ± 0.73	1.68 ± 0.28	22.7 ± 4.15
	24 h (n = 13)	6.9 ± 0.29	1.97 ± 0.25	25.6 ± 2.40
	48 h (n = 8)	7.0 ± 0.25	2.0 ± 0.32	24. ± 2.60

HBO: hyperbaric oxygen; IL: iloprost; NSE: neuron-specific enolase (μg/l); S-100 beta protein (μg/l); NO: nitric oxide metabolites nitrite and nitrate (μM/l).
*Significantly different from values of the sham group (P < 0.001).
**Significantly* different from untreated control values (P < 0.001).
***Significantly different from the pre-ischaemic period (P < 0.002).

suggested to prevent ischaemic SCI. Several studies demonstrated the effect of HBO on spinal cord ischaemia in experimental models. The overall effect results in increased tissue oxygen and improved collagen synthesis, angiogenesis and epithelization; however, the underlying mechanism is poorly understood. That is why different studies still reach different results, without a final conclusion.

Experimental studies with HBO revealed protection against cerebrovascular diseases and improved outcomes in rats and other animals. Preliminary studies evaluating the effect of HBO in 1976 and 1977 by Yeo *et al.* [9, 10] demonstrated that HBO applied 2 h after SCI produces significant recovery of motor function in paraplegic sheep, compared with untreated controls. The 1977 study documented a better histological outcome in the HBO group, with less central cystic degeneration [10]. Murakami *et al.* [11], investigating the influence of HBO on delayed neuronal cell death in spinal motor neurons, demonstrated that one session of HBO given 30 min after SCI has protective effects against ischaemic spinal cord damage. The animals had less neurological deficit and less degeneration of spinal motor neurons in ventral grey matter. These protective effects were not observed in the group that received HBO 6 h after SCI. Yu *et al.* [12] investigated the effects of HBO treatment on the progress of secondary damage following SCI induced by a weight-drop device in rats. They compared three groups: a single HBO administration, repeated applications of HBO once daily in the following 4 days and control group. Their results showed that early onset of HBO significantly diminished the number of apoptotic cells, a day after injury. Another study by Huang *et al.* [13] revealed a benefit of multiple sessions of HBO that extended the therapeutic window to 6 h after acute SCI with a rat model.

The results of our study clearly demonstrated that aortic occlusion causes severe injury of the spinal cord without treatment. We created extensive SCI resulting in pronounced functional neurological deficit. We initiated both IL and HBO treatments at the onset of ischaemia and continued until the first hour of reperfusion. Neurological evaluation 24 and 48 h after injury revealed that treatment groups (HBO, IL and HBO + IL) had preserved

motor function compared with controls. Among treatment groups, neurological scores in the HBO, IL and HBO + IL groups were statistically significantly better compared with the control group at 24th (P = 0.001, 0.001 and 0.001, respectively) and 48th hour (P = 0.001, 0.001 and 0.001, respectively). Neurological scores in the HBO, IL and HBO + IL groups were similar at the 24th and 48th hour.

Histopathological assessment of the spinal cord showed that perineural oedema was significantly higher in the saline-treated (control) group. Moreover, higher viable cell counts were noted in the IL and HBO groups. These results clearly demonstrated the protective effect of HBO, IL and HBO + IL on SCI. Histopathological scores in the HBO, IL and HBO + IL groups were statistically significantly better compared with the control group (P = 0.003, 0.001 and 0.001, respectively). Histopathological scores in the HBO, IL and HBO + IL groups were similar.

SCI significantly increased spinal cord tissue MDA and decreased SOD, GSH-Px and CAT enzyme activities in controls. Kahraman *et al.* [14] compared the effect of HBO, given immediately after SCI, to methylprednisolone after experimental SCI in rats. Five days after SCI, oxidative status of spinal cord was evaluated by thiobarbituric acid reactive substances, SOD and GSH-Px concentrations. Interestingly, only HBO treatment reduced the level of oxidative damage after SCI. Moreover, Aydinoz *et al.* reported that the beneficial effect of HBO on cisplatin-induced nephrotoxicity seems to be partially mediated by a significant reduction in lipid peroxidation and by increased SOD and GSH-Px activities in the kidneys. Another study by Topuz *et al.* [15] revealed that HBO decreased MDA concentrations and increased SOD, GSH-Px and CAT levels.

NSE and S100β protein were analysed in order to monitor neuronal necrosis. NSE, neuronal form of the intracytoplasmic glycolytic enolase, is found in neuronal cell bodies, axons, neuroendocrine cells and in neuroendocrine tumours. Blood NSE concentrations significantly increase in the early period following neuronal injury.

NO, a free radical, has been implicated as one of the mediators of neuronal damage following ischaemia. Neuronal damage

Table 5: Comparison of changes in biochemical markers

		MDA (nmol/g)	SOD (U/mg)	CAT (kg/g)	GSH-Px (U/g protein)
Group HBO	Preisc. (*n* = 3)	19.2 ± 1.66	0.17 ± 0.02	4.62 ± 0.27	0.59 ± 0.02
	24 h (*n* = 5)	60.5 ± 6.43*,**,***	0.33 ± 0.05**	4.15 ± 0.21**	0.43 ± 0.03**
	48 h (*n* = 8)	68.7 ± 4.82*,**,***	0.30 ± 0.08**	4.25 ± 0.27**	0.44 ± 0.03**
Group IL	Preisc. (*n* = 3)	21 ± 2.26	0.18 ± 0.01	4.35 ± 0.30	0.6 ± 0.02
	24 h (*n* = 5)	57.7 ± 4.23*,**,***	0.32 ± 0.02**	4.06 ± 0.23**	0.44 ± 0.02**
	48 h (*n* = 8)	60 ± 4.20*,**,***	0.3 ± 0.02**	4.15 ± 0.22**	0.45 ± 0.03**
Group HBO + IL	Preisc. (*n* = 3)	22 ± 2.40	0.17 ± 0.03	4.55 ± 0.30	0.57 ± 0.02
	24 h (*n* = 5)	53.2 ± 3.45*,**,***	0.31 ± 0.02**	4.36 ± 0.31**	0.46 ± 0.02**
	48 h (*n* = 8)	44.3 ± 3.5*,**,***,****	0.29 ± 0.01**	4.25 ± 0.23**	0.5 ± 0.02**
Group control	Preisc. (*n* = 3)	18.7 ± 2.4	0.18 ± 0.01	4.33 ± 0.28	0.60 ± 0.03
	24 h (*n* = 5)	122 ± 9.7*,***	0.67 ± 0.02*,***	2.48 ± 0.32*,***	0.27 ± 0.04*,***
	48 h (*n* = 8)	115 ± 7.33*,***	0.63 ± 0.02*,***	2.54 ± 0.20*,***	0.28 ± 0.03*,***
Group sham	Preisc. (*n* = 3)	18.3 ± 3.02	0.19 ± 0.02	4.37 ± 0.25	0.59 ± 0.03
	24 h (*n* = 5)	24 ± 3.2	0.23 ± 0.01	4.20 ± 0.32	0.62 ± 0.02
	48 h (*n* = 8)	23 ± 2.6	0.2 ± 0.02	4.5 ± 0.25	0.6 ± 0.02

HBO: hyperbaric oxygen; IL: iloprost; MDA: malondialdehyde (nmol/g); SOD: superoxide dismutase (U/mg); CAT: catalase (kg/g); GSH-Px: glutathione peroxidase (U/g protein); Preisc: pre-ischaemia.
*Significantly different from values of the sham group (*P* < 0.001).
**Significantly different from untreated control values (*P* < 0.001).
***Significantly different from the pre-ischaemic period (*P* < 0.005).
****Significantly different from 24 h (*P* = 0.02).

following vascular stroke has been shown to be markedly diminished in animals with NO synthase inhibitors [16]. HBO reduced iNOS (inducible NO synthase) mRNA synthesis in mouse peritoneal macrophages. Accordingly, inhibition of NO generation in hypoxic spinal cells might result in decreased production of peroxynitrite or other free oxygen radicals. Severe spinal cord ischaemia generated in the present model resulted in significantly elevated NO levels. As long as spinal cord was protected using HBO and IL, NO concentrations remained low. Conversely, a recent double-blind trial mentioned the possibility that HBO may actually worsen neurological outcomes [17]. There have been concerns regarding the use of HBO for IR, based on the hypothesis that providing extra oxygen would increase free radical production and tissue damage.

Pablos *et al.* [18] observed increased lipid peroxidation products, total and oxidized glutathione levels; reduced GR and GPH activity after HBO therapy at a pressure of 4 atm. for 90 min, in rat lung and brain tissues. On the other hand, Boadi *et al.* [19] reported elevated GSH, GR, GSH-PX and SOD concentrations, which increased more with dietary antioxidants (Vitamin E, riboflavin and selenium). Investigating a different aspect, Puglia and Loeb [20] reported serious central nervous system toxicity using HBO with pressures >2.8 atm., which may occur more rapidly given the inhibition of SOD.

In contrast, HBO has also been shown to decrease lipid peroxidation in a number of ischaemia–reperfusion studies. Gurer *et al.* [21] showed decreased production of free oxygen radicals, namely, MDA, among animals pretreated with HBO. The protective effect of HBO treatment was proposed to be due to suppression of specific enzymes that catalyze lipid peroxidation and through oxygen-mediated termination reactions. Despite the fact that HBO may increase lipid peroxidation and ROS, especially superoxide and hydrogen peroxide, Jamieson [22] did not discover significant changes in MDA concentrations with HBO at several pressures up to 5.9 atm. Similarly, even though MDA and SOD concentrations increased at the 24th hour only in the HBO group compared with sham and pre-ischaemic period, their concentrations were significantly lower than the control group. Importantly, MDA levels in combined therapy were significantly lower at the 48th hour compared with both control group and remaining treatment groups.

Clinical feasibility of both treatment modalities, especially HBO, is controversial. Although several recent studies reported that repeated HBO preconditioning prior to spinal cord ischaemia provides neuroprotection, information about the mechanism of how this neuroprotection works is still very limited [23]. In both animal and human models, HBO therapy has sporadically demonstrated a decrease in the size of infarcts [24]. A Cochrane review concluded that there is little evidence to support the use of HBO for IRI patients [25]. IL, a PGI2 analogue, has anti-inflammatory properties such as inhibition of thrombocyte aggregation and neutrophil functions, vasodilatation and cytoprotection. Several studies reported that IL infusion, started before aortic occlusion and continued throughout ischaemia, might significantly improve spinal cord protection.

Several limitations of this work should be noted. Neurological outcome and histopathology were measured at only two time points. It is possible that clinical deficits and neuronal losses may only be delayed in treatment groups and that injury may mature with time. Further studies should extend these preliminary investigations to at least 72 h to fully define neuronal injury and protection. It is also important to note that our estimates of neuronal injury are based on simple histopathology. Further studies should explore this question immunohistochemically, examining specific markers for neuronal death.

In conclusion, our results suggest that HBO and IL may reduce IR injury following transient spinal ischaemia and provide better neurological outcome. Although the present study confirms the efficacy of HBO and IL in SCI regarding neurological, histopathological and biochemical outcomes, the optimal number of applications needs to be investigated additionally, so that the effect of the treatment may remain constant (Table 5).

Conflict of interest: none declared.

REFERENCES

[1] Chen S, Xiong L, Wang Q, Sang H, Zhu Z, Dong H *et al*. Tetramethylpyrazine attenuates spinal cord ischemic injury due to aortic cross-clemping in rabbits. BMC Neurol 2002;2:1.

[2] Dong HL, Zhang Y, Su BX, Zhu ZH, Gu QH, Sang HF *et al*. Limb remote ischemic preconditioning protects the spinal cord from ischemia-reperfusion injury: a newly identified nonneuronal but reactive oxygen species-dependent pathway. Anesthesiology 2010;112:881–91.

[3] Dayan K, Keser A, Konyalioglu S, Erturk M, Aydin F, Sengul G *et al*. The effect of hyperbaric oxygen on neuroregeneration following acute thoracic spinal cord injury. Life Sci 2012;90:360–4.

[4] Carbajal D, Arruzazabala ML, Noa M, Molima V, Mas R, Arango E *et al*. Protective effect of D-003 on experimental spinal cord ischemia in rabbits. Prostaglandins Leukot Essent Fatty Acids 2004;70:1–6.

[5] Lowry O, Rosenbraugh N, Farr L, Rondall R. Protein measurement with the theofilin-phenol reagent. J Biol Chem 1951;183:265–75.

[6] Aebi H. Catalase. In: Bergmeyer HU (ed). Methods of Enzymatic Analysis. New York: Academic Press, 1974, 673–7.

[7] Wasowicz W, Neve S, Peretz A. Optimized steps in fluorometric determination of thiobarbituric acid-reactive substances in serum: importance of extraction pH and influence of sample preservation and storage. Clin Chem 1993;39:252–6.

[8] Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967; 70:158–70.

[9] Yeo JD, McKenzie B, Hindwood B, Kidman A. Treatment of paraplegic sheep with hyperbaric oxygen. Med J Aust 1976;1:538–40.

[10] Yeo JD, Stabback S, McKenzie B. A study of the effects of hyperbaric oxygen on the experimental spinal cord injury. Med J Aust 1977;2: 145–7.

[11] Murakami N, Horinouchi T, Sakurai M, Ejima Y, Matsukawa S, Kato M *et al*. Hyperbaric oxygen therapy given 30 min after spinal cord ischemia attenuates selective motor neuron death in rabbits. Crit Care Med 2001;29:814–8.

[12] Yu Y, Matsuyama Y, Yanase M, Ito S, Adachi K, Satake K *et al*. Effects of hyperbaric oxygen on GDNF expression and apoptosis in spinal cord injury. Neuroreport 2004;15:2369–73.

[13] Huang L, Mehta MP, Nanda A, Zhang JH. The role of multiple hyperbaric oxygenation in expanding therapeutic windows after acute spinal cord injury in rats. J Neurosurg 2003;99(2 Suppl):198–205.

[14] Kahraman S, Düz B, Kayali H, Korkmaz A, Oter S, Aydin A *et al*. Effects of methylprednisolone and hyperbaric oxygen on oxidative status after experimental spinal cord injury: a comparative study in rats. Neurochem Res 2007;32:1547–51.

[15] Topuz K, Colak A, Cemil B, Kutlay M, Demircan MN, Simsek H *et al*. Combined hyperbaric oxygen and hypothermia treatment on oxidative stress parameters after spinal cord injury: an experimental study. Arch Med Res 2010;41:506–12.

[16] Dawson TM, Dawson VL. In: Batjer HH (ed). Protection of the Brain from Ischemia in Cerebrovascular Disease. Philadelphia, PA: Lippincott-Raven, 1997, 319–25.

[17] Anderson DC, Bottini AG, Jagiella WM. A pilot study of hyperbaric oxygen in the treatment of human stroke. Stroke 1991;22:1137–42.

[18] Pablos MI, Reiter RJ, Chuang JI, Ortiz GG, Guerrero JM, Sewerynek E *et al*. Acutely administered melatonin reduces oxidative damage in lung and brain induced by hyperbaric oxygen. J Appl Physiol 1997;83:354–8.

[19] Boadi WY, Thaire L, Kerem D, Yannai S. Effects of dietary factors on antioxidant enzymes in rats exposed to hyperbaric oxygen. Vet Hum Toxicol 1991;33:105–9.

[20] Puglia CD, Loeb GA. Influence of rat brain superoxide dismutase inhibition by diethyldithiocarbamate upon the rate of development of central nervous system oxygen toxicity. Toxicol Appl Pharmacol 1984;75:258–64.

[21] Gurer A, Ozdogan M, Gomceli I, Demirag A, Gulbahar O, Arikok T *et al*. Hyperbaric oxygenation attenuates renal ischemia reperfusion injury in ratstransplantation proceedings. 2006;38:3337–40.

[22] Jamieson DD. Lipid peroxidation in brain and lungs from mice exposed to hyperoxia. Biochem Pharmacol 1991;41:749–56.

[23] Li J, Liu W, Ding S, Xu W, Guan Y, Zhang JH *et al*. Hyperbaric oxygen preconditioning induces tolerance against brain ischemia–reperfusion injury by up-regulation of antioxidant enzymes in rats. Brain Res 2008;1210:223–9.

[24] Helms AK, Whelan HT, Torbey MT. Hyperbaric oxygen therapy of cerebral ischemia. Cerebrovasc Dis 2005;20:417–26.

[25] Bennett MH, Wasiak J, Schnabel A, Kranke P, French C. Hyperbaric oxygen therapy for acute ischaemic stroke. Cochrane Database Syst Rev 2005; CD004954.