

*Original Research*

# Screening Serum Biomarkers for Rats Preconditioned with Hyperbaric Oxygen: Potential of Predicting Prognosis for Stroke

Beibei Dong<sup>1,†</sup>, Yuanyuan Bai<sup>2,†</sup>, Qi Jia<sup>3,†</sup>, Lin Su<sup>1</sup>, Xinyue Zhang<sup>1</sup>, Shiquan Wang<sup>3,\*</sup>, Zongping Fang<sup>3,4\*</sup><sup>1</sup>Department of Anesthesiology, Tianjin Medical University General Hospital, 300052 Tianjin, China<sup>2</sup>Department of Anesthesiology, Tianjin Baodi Hospital, Baodi Clinical College of Tianjin Medical University, 301800 Tianjin, China<sup>3</sup>Department of Anesthesiology and Perioperative Medicine and Department of Critical Care Medicine, Xijing Hospital, Fourth Military Medical University, 710032 Xi'an, Shanxi, China<sup>4</sup>Translational Research Institute of Brain and Brain-Like Intelligence and Department of Anesthesiology and Perioperative Medicine, Shanghai Fourth People's Hospital Affiliated to Tongji University School of Medicine, 200434 Shanghai, China\*Correspondence: [Wangshiquan-301@163.com](mailto:Wangshiquan-301@163.com) (Shiquan Wang); [Zongping03@163.com](mailto:Zongping03@163.com) (Zongping Fang)

†These authors contributed equally.

Academic Editor: Gernot Riedel

Submitted: 24 August 2022 Revised: 19 November 2022 Accepted: 29 November 2022 Published: 20 July 2023

## Abstract

**Background:** Stroke is a major health concern and a leading cause of mortality and morbidity. We and other groups have documented that hyperbaric oxygen preconditioning could significantly alleviate neuronal damage in ischemia–reperfusion models through various mechanisms. However, we found that some of the subjects did not benefit from preconditioning with hyperbaric oxygen. The preconditioning phenomenon is similar to vaccination, in which the endogenous survival system is activated to fight against further injuries. However, with vaccine inoculations, we could test for specific antibodies against the pathogens to determine if the vaccination was successful. Likewise, this experiment was carried out to explore a biomarker that can reveal the effectiveness of the preconditioning before neuronal injury occurs. **Methods:** Middle cerebral artery occlusion (MCAO) was used to induce focal cerebral ischemia-reperfusion injury. 2D-DIGE-MALDI-TOF-MS/MS proteomic technique was employed to screen the differentially expressed proteins in the serum of rats among the control (Con) group (MCAO model without hyperbaric oxygen (HBO) preconditioning), hyperbaric oxygen protective (HBOP) group (in which the infarct volume decreased after HBO preconditioning vs. Con), and hyperbaric oxygen nonprotective (HBOU) group (in which the infarct volume remained the same or even larger after HBO preconditioning vs. Con). Candidate biomarkers were confirmed by western blot and enzyme linked immunosorbent assay (ELISA), and the relationship between the biomarkers and the prognosis of cerebral injury was further validated. **Results:** Among the 15 differentially expressed protein spots detected in the HBOP group by Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE), 3 spots corresponding to 3 different proteins (haptoglobin, serum albumin, and haemopexin) products were identified by MALDI-TOF-MS/MS. Serum albumin and haemopexin were upregulated, and haptoglobin was downregulated in the HBOP group ( $p < 0.05$  vs. Con and HBOU groups). After the western blot study, only the changes in haemopexin were validated and exhibited similar changes in subjects from the HBOP group in accordance with MALDI-TOF-MS/MS proteomic analysis and enzyme linked immunosorbent assay (ELISA) analysis. The serum level of the hemopexin (HPX) at 2 h after HBO preconditioning was correlated with the infarct volume ratio after MCAO. **Conclusions:** Haemopexin may be developed as a predictive biomarker that indicated the effectiveness of a preconditioning strategy against cerebral ischaemic injury.

**Keywords:** HBO preconditioning; stroke; serum biomarker; 2D-DIGE-MALDI-TOF-MS/MS; haemopexin

## 1. Introduction

As a leading cause of morbidity and mortality, stroke is a major concern in health care [1]. It is widely accepted that the risk factors for stroke involve old age, hypertension, diabetes, smoking, atrial fibrillation, etc. [2]. In addition to the common risk factors, surgical stimulus is also an important risk factor for stroke. In complicated cardiac and vascular surgeries, the incidence of perioperative stroke increases from 0.8% in general surgery up to 10% [3]. Moreover, perioperative stroke often results in even worse outcomes, as these strokes involve higher morbidity and mortality rates [4]. As a complication of surgery, perioperative stroke usu-

ally occurs during or after operation [5]. In this view, the time frame in which a perioperative stroke occurs is designated so that a pretreatment or preconditioning strategy can be conducted against the stroke, and this strategy is especially important for organs sensitive to ischaemic damage, such as the brain.

We and other groups have demonstrated that repeated hyperbaric oxygen (HBO) preconditioning could induce neuroprotection in a rat model of cerebral ischemia and reperfusion, which could be attributed to inhibition of ischemia/hypoxia-induced mitochondrial apoptosis and energy metabolism disturbance, and promote autophagic flux [6,7]. The protective effects of HBO preconditioning have



been conformed in various animal models, such as myocardial ischemia, spinal cord ischemia, hepatic ischemia, neonatal hypoxia-ischemia, intestinal ischemia, surgical brain injury, intracerebral hemorrhage, traumatic brain injury and renal ischemia, in which the underlying mechanism included antioxidant and anti-inflammation. Our previous clinical evidence has demonstrated the repeated preconditioning by HBO reduced the release of the neurobiochemical markers of S100B, neuron-specific enolase (NSE), and cTnI and induced neuroprotection in patients undergoing on-pump coronary artery bypass grafting (CABG) surgery [8]. However, after carefully analysing the prospective, randomized, controlled clinical trial, we concluded that although preconditioning could be beneficial for most of the patients and animals, there were still some individuals that did not benefit. How can we tailor the therapeutic protocol to patients' individual needs instead of using a universal strategy for all, in which not all individuals benefit? We also considered this question for some time.

It has been well clarified that preconditioning can activate endogenous survival signals and pathways, thus inducing ischaemic tolerance against ischaemic insults. Electroacupuncture pre-treatment increased extracellular glutamate levels in the ischemic penumbra, then reduced brain damage produced during ischemia-reperfusion and contributes to protracted protection which was evaluated by the infarct volumes and a 28-point neurological deficit assessment of mice [9]. Preconditioning with isoflurane could reduce the neurological deficit scores and the brain infarct volumes in rats, in which process the adenosine A1 might mediate the neuroprotective effect of isoflurane preconditioning. However, whether the endogenous survival signals in each subject can be effectively activated by the same preconditioning protocol remains unclear. To address this issue, we identified some easy-to-test biomarkers, which can be used to predict that endogenous survival signals are effectively activated by HBO preconditioning through a proteomic approach. Identifying and verifying the biomarkers would facilitate the clinical use of preconditioning strategies, and the biomarkers may also serve as new therapeutic targets if they are proven to be functionally related to the protective effect.

Three criteria for biomarkers must be considered. The first is the sensitivity and accuracy, which can only be revealed through verification. The second is the accessibility of the material, such as body fluids or sample biopsies, that is used for screening. The last is the availability of rapid detection technology. Considering that blood is the most common clinical test material and HBO has a systematic effect on the body, serum samples were chosen as the research subject to screen the predictive biomarkers for the effectiveness of HBO preconditioning against cerebral ischaemic injury in the current study.

Recently, Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) has been suggested to be

among the most powerful proteomics methodologies in the search for potential biomarkers in different diseases due to its high sensitivity and good reproducibility [10]. In this study, we performed a 2D-DIGE technique with rat serum to screen and determine predictive biomarkers that indicate the protective effect of HBO preconditioning against brain ischaemic reperfusion injuries. We screened proteins in the serum from the subjects in the HBO preconditioning group who benefitted from the treatment, and these proteins were differentially expressed from both the control group and the subjects in the HBO group that had no benefits; these proteins were candidates for biomarkers that predicted the protective effect of HBO preconditioning. To differentiate the benefit subjects, we used a retrospective subgroup method to discriminate the subjects that benefitted from the treatment and those with no benefit in the HBO preconditioning group by brain infarct volume ratio. To differentiate the benefit subjects, we used a retrospective subgroup method to discriminate the subjects that benefitted from those that did not in the HBO preconditioning group by brain infarct volume ratio. To the best of our knowledge, this is the first study that investigated proteomic changes to assess predictive biomarkers for the effectiveness of a preconditioning strategy against cerebral ischaemic injuries.

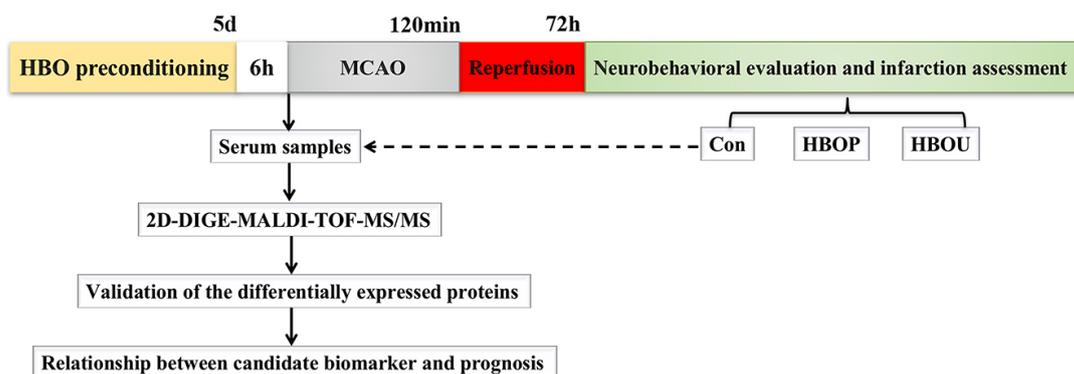
## 2. Experimental Procedures

### 2.1 Animals and Experimental Design

Male Sprague–Dawley (SD) rats weighing 280 to 300 g were provided by the Experimental Animal Center of the Fourth Military Medical University. The animals were housed in a controlled environment ( $25 \pm 2$  °C, 50% humidity, and 12 h light-dark cycle from 8 to 8) and were given free access to food and water. All animal experiments were carried out in compliance with the Guidelines for the Care and Use of Laboratory Animals of the Fourth Military Medical University. A flowchart of the experimental design is shown in Fig. 1. The animals were randomly assigned to the control (Con, MCAO model without HBO preconditioning) group or the HBO group. HBO preconditioning combined with the ischaemia/reperfusion model was employed to screen predictive biomarkers for the protective effect of HBO preconditioning. The infarct volumes between the Con and HBO groups were calculated and analysed by independent sample *T* test analysis. Based on the mean and standard deviation of the infarct volume of the Con group, the rats in the HBO group in which the infarct volumes were less than the means minus the standard deviation ( $< \text{Mean (Con)} - \text{SD}$ ) were defined as the protective group (HBOP, hyperbaric oxygen protective), and the other rats in the HBO group were defined as the nonprotective group (HBOU, hyperbaric oxygen nonprotective).

### 2.2 HBO Preconditioning and Sample Collection

Rats in the HBO group accepted oxygen (2.5 ATA, 100% O<sub>2</sub>, 1 h/d) for five days, while rats in the control



**Fig. 1. Experimental design of the proteomic screening and subsequent validation of biomarkers for the neuroprotective effect of HBO preconditioning against cerebral ischaemia–reperfusion injury.** HBO, hyperbaric oxygen; MCAO, middle cerebral artery occlusion; Con, control; HBOP, hyperbaric oxygen protective; HBOU, hyperbaric oxygen nonprotective.

group did not receive HBO preconditioning but were placed into a hyperbaric chamber breathing normal baric air under the same conditions. Rat blood was got from the femoral artery at 6 h after the last time of HBO preconditioning. The samples were left to clot for 2 h at 4 °C followed by 3000 g centrifugation force for 15 min at 4 °C. The serum was then aliquoted and stored at –80 °C before processing.

### 2.3 Transient Focal Cerebral Ischaemia and Cerebral Blood Flow Measurement

As was previously described, the rat transient focal cerebral ischaemia was induced by middle cerebral artery occlusion (MCAO) [11,12]. Briefly, anesthesia was induced by an intraperitoneal injection of sodium pentobarbital (50 mg/kg in normal saline). The right common carotid artery (CCA) and right external carotid artery (ECA) were exposed through a ventral midline incision of the neck and were ligated proximally. The 4/0 single silk nylon suture (Sunbio Biotechnology Co. Ltd., Beijing, China) was inserted into the common carotid artery below the carotid bifurcation through a small incision, followed by inserting into the internal carotid artery (ICA) about 18–20 mm distal to the carotid bifurcation until slight resistance was felt. Two hours after ischemia, the animals were anesthetized again and reperfusion was accomplished by retracting the sutures.

### 2.4 Regional Cerebral Blood Flow Monitoring

Regional cerebral blood flow (rCBF) of all animals subjected to ischaemia/reperfusion injury was monitored using a PF 5000 Laser Doppler Perfusion Monitoring Unit (PeriFlux 5000, Perimed AB, Stockholm, Sweden). Stroke models were considered successful if rCBF decreased to 30% of the baseline level immediately after MCAO and increased to 70% of the baseline within 10 min after reperfusion.

### 2.5 Neurobehavioural Evaluation and Infarction Assessment

At 72 h after reperfusion, neurologic behaviours of rats were assessed according to the Garcia scoring system (an 18-point scoring system) by an observer who was blinded to the grouping information [13]. Six aspects of neurological deficit were measured, including (1) spontaneous activity, (2) symmetry of four limb movements, (3) forepaw outward extension, (4) climbing ability, (5) body proprioception, and (6) response to whisker touch. Each aspect was scored from 0 to 3 points, and the total score was calculated. All behavioral tests were performed in a quiet room. The higher the score was lead to the better the neurobehavioral function. The animals were then sacrificed under deep anaesthesia with pentobarbital sodium and decapitated, and their brains were evacuated and sliced into six 2-mm-thick coronal sections with a matrix and stained with 2% TTC (Cat.#17779, Sigma, St. Louis, MO, USA) in normal saline at 37 °C for 10 min to evaluate the infarct volume ratio. The stained slices were then transferred to 4% formalin for postfixation before being photographed with a digital camera (Canon, Tokyo, Japan). White or pale pink areas were defined as infarction and were measured using image analysis software (Adobe Photoshop CS5, Adobe Systems, CA, USA). To exclude the effect of cerebral oedema that is induced by ischaemia on the infarct size, the percentage of the infarct volume was calculated using a corrected algorithm: (total contralateral hemispheric volume – total ipsilateral hemispheric stained volume)/total contralateral hemispheric volume × 100%.

### 2.6 Immunoaffinity Depletion of High-Abundance Proteins

Immunoaffinity Depletion of High-Abundance Proteins Collected serum was pooled into three groups, each containing serum from 6 rats. Serum pools were depleted of the three most abundant proteins (albumin, IgG, and transferrin) using Multiple Affinity Removal Column (M-3) fol-

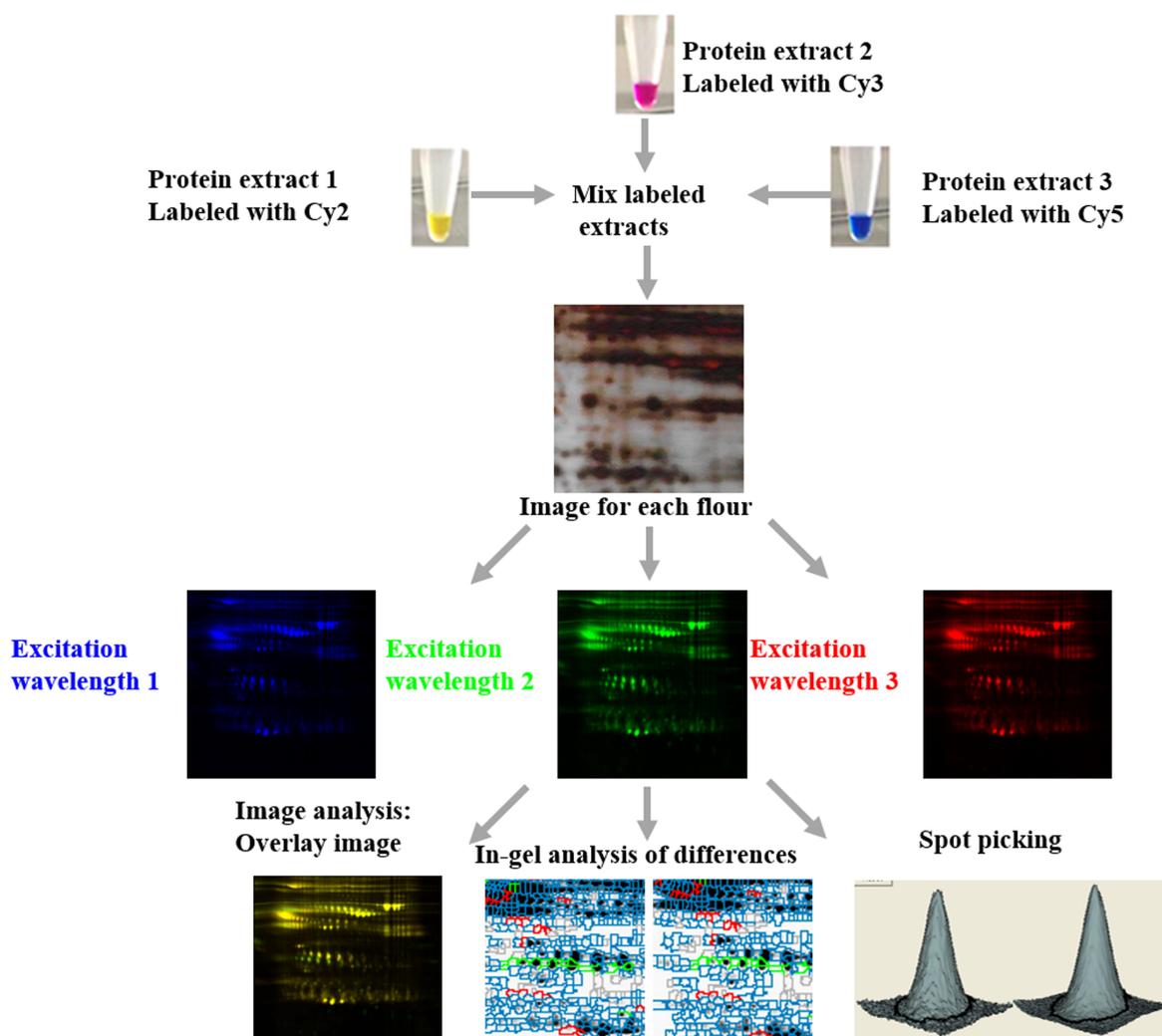


Fig. 2. Screening process for the differential proteins by 2D-DIGE-MALDI-TOF-MS.

lowing the manufacturer's protocol (Agilent Technologies Co., Ltd., Santa Clara, CA, USA). Briefly, the serum samples were diluted 5-fold with Agilent buffer A. The 100  $\mu$ L diluted sample was loaded into a 0.22  $\mu$ m spin cartridge and centrifuged at 16000 g for 2 min. Then, the bound proteins were eluted with buffer B at 1 mL/min for 3.5 min. Desalination was performed in 5K ultrafiltration tubes. The depleted serum was concentrated using the EZQ<sup>®</sup> protein quantification kit (Bio-Rad, Hercules, CA, USA). The collected samples were exchanged with a buffer of sodium bicarbonate and 0.5 M sodium carbonate solution.

### 2.7 Protein Digestion and Labelling

A 2-D clean-up kit (GE Healthcare, Bucks, United Kingdom) was used to remove the substances that interfered with plasma two-way electrophoresis. Serums from 6 rats in each group were pooled. The serums of the three groups were labelled respectively with different fluorescent dyes later and then mixed before analysis. The protein concentration in the serum samples was determined with the

Ettan TM 2-D Quant Kit (GE Healthcare Life Sciences, Piscataway, NJ, USA). protein quantification kit. Fifty micrograms of six serum samples from each group were randomly selected. Labelling reactions were performed with either Cy3 or Cy5 fluorescent dyes. The internal standard was prepared by mixing 25  $\mu$ g of each sample protein and performing the labelling reaction with Cy2 dye. Fluorescent dye-labelled plasma proteins top-sample to solid-phase nonlinear dry adhesive strips for hydration. The Ettan<sup>™</sup> IPGPhor (GE Healthcare) isoelectric focusing apparatus was applied, and the Ettan<sup>™</sup> DALT (GE Life Sciences) Six vertical electrophoresis system was used to perform first- and second-direction electrophoresis.

### 2.8 Gel Image Analysis

Cy2, Cy3 and Cy5 were scanned with excitation light at 488 nm, 532 nm and 633 nm. The glue images were collected and analysed with the Typhoon multifunction scanning imaging system (GE Healthcare). The images of samples labelled with Cy2, Cy3, and Cy5 fluorescent dyes are

coloured blue, green, and red, respectively. The DeCyder V6.5 analysis software (GE Healthcare) detected and matched the analysed collagen points. 3D images and manual proofreading were used to select the differential protein points and then match the corresponding protein points on the preparation glue (Fig. 2). The differential protein points were dug using the Ettan™ Spot picker automatic spot cutting system (GE Healthcare).

### 2.9 In-Gel Digestion

Digestion and picking were done by preparing the gels. Two-dimensional electrophoresis was performed. immobilized pH gradient (IPG) bands were loaded with 500 to 1000 µg of protein and gels were stained with Coomassie blue. Protein points of interest were excised and counter-stained with 25 mM ammonium bicarbonate and 50% acetonitrile. The gel was freeze-dried by centrifugation. It was performed with 0.01 µg/µL trypsin in 25 mM ammonium bicarbonate 10 for 15 hours at room temperature. The supernatant was collected and tryptic peptides were extracted with 5% trifluoroacetic acid at 40 °C for 1 h, then with 2.5% trifluoroacetic acid (TFA) and 50% Acetonitrile (ACN) for 1 h at 30 °C for 1 h.

### 2.10 Protein Identification by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

The peptide mixture was dissolved in 0.5% TFA, the 1 µL peptide solution was mixed with 1 µL matrix (4-hydroxy-cyanogenic acid in 30% ACN, 0.1% TFA) and then found on the target plate. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry and tandem TOF/TOF mass spectroscopy were performed on a 4800 Plus MALDI TOF/TOF instrument (Applied Biosystems, Foster City, CA, USA). Peptide mass maps were obtained in positive reflection mode, with an average of 5000 laser per MALDI-TOF spectrum and 5500 laser per TOF/TOF spectrum (20000 resolution). The 4800 calibration mixtures (Applied Biosystems, Foster City, CA, USA) were used to calibrate the spectrum to a mass tolerance of 0.1 Da. The parent mass peak with a minimum signal to noise ratio of 15 was selected for tandem TOF/TOF analysis. The Mascot database search algorithm (version 2.0, <https://www.matrixscience.com>) was used to interrogate the rat sequences in the International Protein Index Database (IPI\_Rat V3.52, <https://www.ebi.ac.uk/IPI>). All automated data analysis and database searches were performed using GPS Explorer™ software (Biosystems Applied Systems version 3.6, Applied Biosystems, Foster City, CA, USA). Confidence identification was statistically significant ( $p < 0.05$ ) for a protein score (based on the mass/mass spectrometry combination) and for an optimal ion score (based on mass/mass spectrometry). Any redundant proteins appearing with different names and accession numbers in the database were eliminated. If multiple proteins are identified at one point, the protein with the highest

protein score (the highest ranking) is removed. The molecular weight and isoelectric points of most proteins agree with the gel region where the spots were excised.

### 2.11 Western Blot Analysis for Rat Serum

The serum potential biomarkers were verified by western blot technique, which was carried out as previously described [14]. Another set of animals was used in this study and was randomly assigned into the control ( $n = 8$ ) and HBO groups ( $n = 16$ ). Blood serum was collected at 6 h after the last session of HBO, and the animals were subjected to 120 min MCAO at 24 h after HBO preconditioning. The brain infarct volume ratio was assessed at 72 h after reperfusion. Serum from the HBO group was regrouped as described above into HBOP ( $n = 10$ ) and HBOU ( $n = 6$ ) groups (infarct volume ratio for each group: control group  $0.39 \pm 0.032$ ; HBOP group  $0.16 \pm 0.036$ ; HBOU group  $0.36 \pm 0.014$ .  $p < 0.05$ , HBOP vs. Con and HBOP vs. HBOU). The serum samples in each group were randomly picked for a subsequent western blot analysis. An equal amount of protein (5 µL) was loaded onto each lane of 12% polyacrylamide-S ml DS gels. The gels were electrophoresed and transferred onto PVDF membranes. The membrane was then blocked at room temperature for 1 h and incubated with primary antibody at 4 °C overnight. Rabbit polyclonal anti-haemopexin (1:500 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and anti-rabbit  $\beta$ -actin antibodies (1:1000 dilution, CW-BIO, Peking, China) were used. The membranes were then incubated for 1 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:1000; Sigma Aldrich, St. Louis, MO, USA). Immunoreactive bands were visualized using enhanced chemiluminescence reagents (Applygen Technologies Inc., Beijing, China). The optical density was determined using Gel-pro32 software (Media Cybernetics, Marlow, UK).

### 2.12 Enzyme-Linked Immunosorbent Assay

Serum haemopexin levels were measured by a quantitative competitive enzyme linked immunosorbent assay (ELISA) kit (Westang, Shanghai, China). The same serum samples used in the western blot study above were applied here. All standards and samples were tested in duplicate. Absorbance was measured at 450 nm using a monochromator microplate reader (Denley Dragon Wellscan MK 3, Thermo, Finland).

### 2.13 Statistical Analysis

SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used to conduct statistical analysis. Measurement data were obtained, and tests of normality were performed using the Kolmogorov–Smirnov normality test. All the data except the neurologic score are presented as the Mean  $\pm$  SD and were analysed using a two-tailed Student's  $t$  test or one-way ANOVA followed by LSD post-hoc test. The neu-

**Table 1. Decyder image analysis results (AV.Ratio >1.2 or <-1.2;  $p < 0.05$ ).**

Protein	Con/HBOP		HBOU/HBOP	
	<i>p</i> value	AV.Ratio	<i>p</i> value	AV.Ratio
Pzp Alpha-1-macroglobulin	0.0075	-1.38	0.038	-1.44
LOC500180 Ig kappa chain C region	0.0017	2.28	0.0055	1.62
Gc Vitamin D-binding protein	0.0068	2.55	0.013	1.81
ApoE, Apolipoprotein E	0.0072	1.43	0.0066	1.5
Orm 1, Alpha-1-acid glycoprotein	0.0023	2.41	0.0063	1.72
Hp Isoform 2 of Haptoglobin	0.028	1.69	0.01	1.44
Sprb	0.0082	2.31	0.0041	1.78
Cfb complement factor B	0.0022	2.61	0.0028	1.82
ApoA1 Apolipoprotein A-I	0.011	-1.33	0.014	-1.4
Alb serum albumin	0.014	-1.47	0.028	-1.56
RGD 1564318, Ig lambda-2 chain C region	0.016	1.93	0.024	1.45
Haemopexin (HPX)	0.021	-1.21	0.0055	-1.46
Fetub Fetuin-B	0.016	1.32	0.045	1.27
B allele	0.018	-1.47	0.00032	-1.32
Complement C3	0.25	-1.06	0.0017	-1.39

HBOP, hyperbaric oxygen protective; HBOU, hyperbaric oxygen nonprotective; LOC, locus; RGD, Arg-Gly-Asp.

rologic scores were expressed as the median (range) and were analysed with the Kruskal–Wallis test followed by the Mann–Whitney *U* test using Bonferroni correction. Pearson correlation analysis was used to analyse the correlation between infarct size and hemopexin (HPX) serum concentration. Receiver operating characteristic (ROC) curve analysis for serum HPX concentration at 2 h after HBO preconditioning was used to calculate the area under the ROC curve (AUC), sensitivity, and specificity.  $p < 0.05$  was considered significantly different.

### 3. Results

#### 3.1 Only Some of the Rats Can Benefit from HBO Preconditioning Against Cerebral Ischaemia Reperfusion Injury

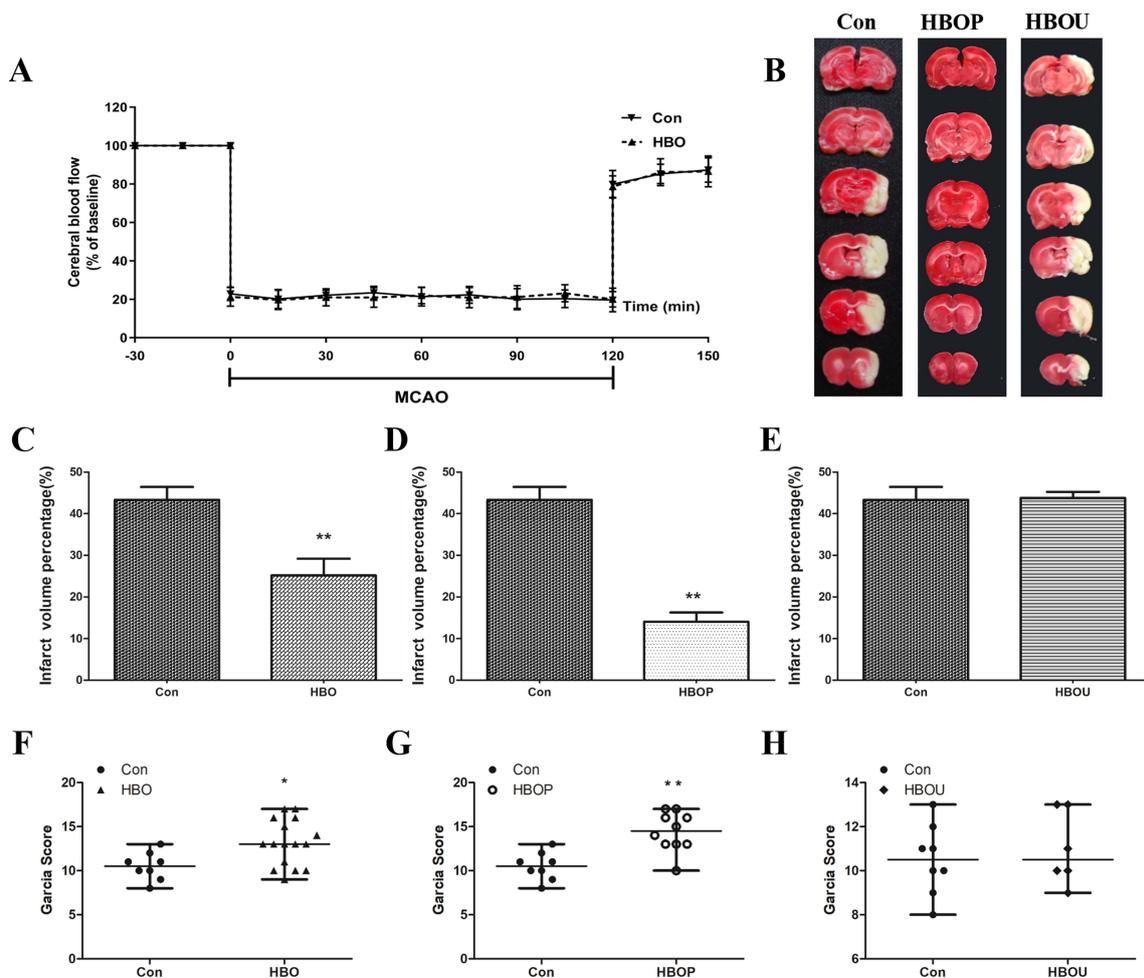
During occlusion, the cerebral blood flow (CBF) value decreased to 30% of the baseline in all rats. After reperfusion, CBF increased back to 70% of the baseline immediately and gradually grew back to 100% of the baseline (Fig. 3A).

To examine the long-term neuroprotection of HBO preconditioning, 72 h long-lasting neurological function and neuroprotective effects were assessed by infarction volume evaluation and behavioral assessment. The neurobehavioral evaluation was done through an 18-point scoring system Garcia score method and the neurobehavioral scores (NBS) were got in each group [13]. As shown in Fig. 3F, HBO preconditioning improved neurological function effectively at 3 d after transient cerebral ischaemia and reperfusion ( $p = 0.024$  Con vs. HBO). In addition, TTC staining revealed that HBO preconditioning also reduced the infarct volume ratio at 3 d after MCAO ( $p = 0.007$  Con vs. HBO, Fig. 3B–C). As expected, we found that not every

subject in the HBO group exhibited a smaller infarct volume ratio than that of the control group. The outcome in some of the subjects in the HBO preconditioning group was even worse than that in the Con group. Therefore, rats in the HBO group were subgrouped into HBOP (subjects that benefited) and HBOU (subjects that did not benefit) groups based on the infarct volume ratio. Briefly, we regrouped the serum according to different infarct volume ratios at 72 h after reperfusion within the HBO preconditioning group as the HBOP group ( $n = 10$ , with an infarct volume ratio <(Mean-SD) of the control group) and HBOU ( $n = 6$ , with an infarct volume ratio  $\geq$ (Mean-SD) of the Con group). After regrouping, the HBOP group showed significant improvement in both neurological function ( $p < 0.01$  Con vs. HBOP, Fig. 3G) and a reduced brain infarct volume ratio ( $p < 0.01$  Con vs. HBOP, Fig. 3D), whereas worse outcomes were observed for the animals in the HBOU group compared to the HBOP group, which were similar to those in the Con group ( $p > 0.05$  Con vs. HBOU, Fig. 3B,E,H).

#### 3.2 Three Proteins were Found to be Differentially Expressed in the Serum of Rats that Benefited from HBO Preconditioning Against Cerebral Ischaemic Injury, As Determined by 2D-DIGE-MALDI-TOF-MS/MS Proteomic Analysis

After regrouping, serum collected before MCAO was analysed by 2D-DIGE-MALDI-TOF-MS/MS. After 2-D DIGE, the Cy2, Cy3, and Cy5 fluorescence intensities of each gel were individually imaged and analysed with DeCyder 6.5 software (GE Healthcare, Little Chalfont, UK). To reveal the specific protein changes in the subjects that benefited (protective effect) from HBO preconditioning, serum proteomic comparisons were designed as follows: we separately investigated the differences in AV. ratios for the con-



**Fig. 3. Regional cerebral blood flows, infarction volume, and neurological outcome at 3 d after reperfusion.** (A) Regional cerebral blood flows in the ischaemic hemisphere of rats during the surgery procedure. Con: control group, in which the animals underwent MCAO without HBO preconditioning; HBO: hyperbaric oxygen preconditioning (2.5 ATA, 100% O<sub>2</sub>, 1 h/day, 5 d) group. (B) Representative TTC-stained brain slices of rats after regrouping at 3 d after reperfusion. HBOP: subjects that experienced a protective effect of HBO preconditioning with an infarct volume ratio <Mean-SD of Con group. HBOU: subjects in the HBO group that showed no benefit from HBO preconditioning with an infarct volume ratio ≥Mean-SD of the Con group at 3 d after reperfusion. (C–E) Infarct volume ratio after regrouping at 3 d after reperfusion. (F–H) Neurological functioning scores after regrouping at 3 d after reperfusion. N = 8 for Con, n = 10 for HBOP, and n = 6 for HBOU. \*  $p < 0.05$  compared to Con group; \*\*  $p < 0.01$  compared to Con group.

tol versus HBOP group and HBOU versus HBOP group. Proteins that showed significant differences ( $p < 0.05$ ) in both comparisons were selected as candidates for further verification. Among the 1926 matched protein spots, 15 spots were differentially expressed in the HBOP group compared to the other groups (AV.Ratio  $>1.2$  or  $<-1.2$ ,  $p < 0.05$ ; Table 1). Pzp (Alpha-1-macroglobulin), Apoal (Apolipoprotein A-I), Alb (serum albumin), Haemopexin, B allele, and Complement C3 expression levels were increased in HBOP samples, whereas LOC500180 Ig kappa chain C region, Gc Vitamin D-binding protein, Apoe (Apolipoprotein E), Orm 1 (Alpha-1-acid lycoprotein), Hp (Isoform 2 of Haptoglobin), Srprb, Cfb complement factor B, RGD 1564318 (Ig lambda-2 chain C region), and Fetub Fetuin-B showed lower expression levels in the HBOP

group (AV.Ratio  $>1.2$  or  $<-1.2$ ,  $p < 0.05$ ; Table 1) compared to the other groups.

Among the 15 differentially expressed spots in the HBOP group, 3 spots corresponding to 3 different genes (haptoglobin, serum albumin, and haemopexin) products were clearly identified by MALDI-TOF-MS/MS (Fig. 4). Serum albumin and haemopexin were upregulated, and haptoglobin was downregulated in the HBOP group ( $p < 0.05$  compared to other groups).

### 3.3 Verification of the Differential Expression of Serum Haemopexin in the HBO Group

We first excluded albumin as a biomarker candidate that can predict the outcome of stroke after preconditioning because it is the most abundant protein in plasma.

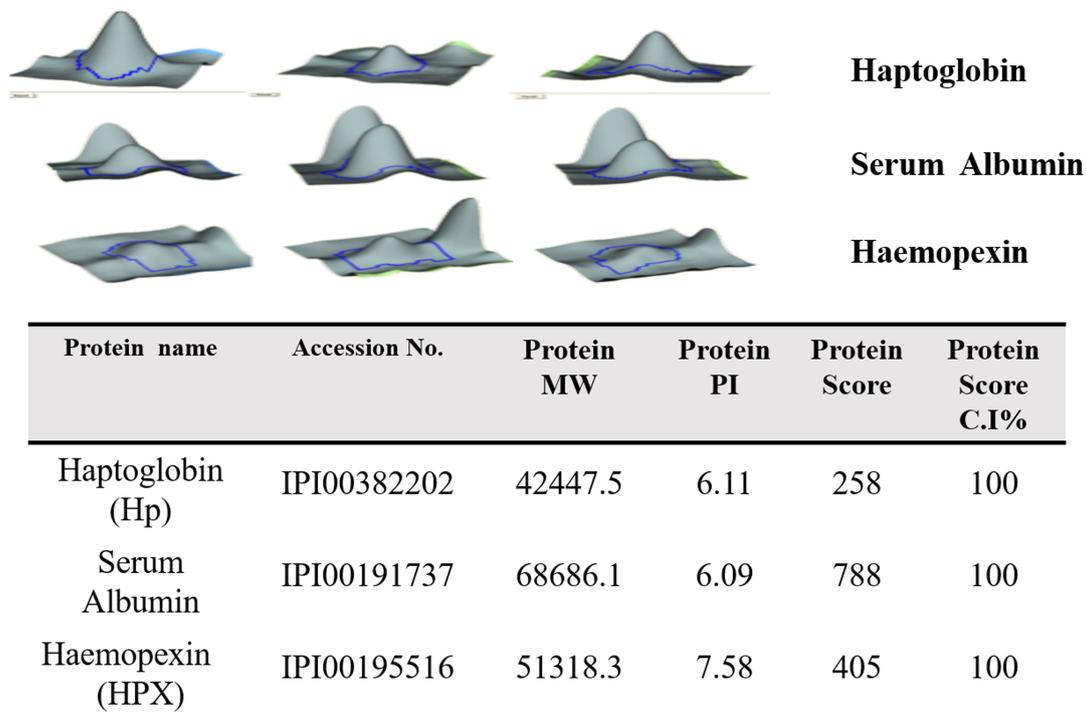


Fig. 4. Protein identification by matrix assisted laser desorption/ionization time-of-flight mass spectrometry. MW, molecular weight; PI, protein isoelectric point.

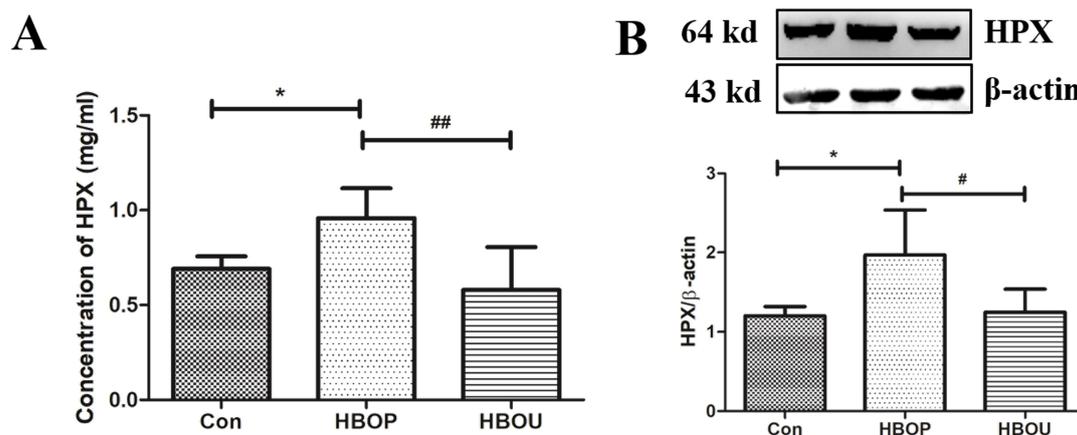


Fig. 5. Enzyme-linked immunosorbent assay (ELISA) and Western blot (WB) analysis of HPX levels in the serum of rats at 6 h after HBO preconditioning. (A) Enzyme-linked immunosorbent assay; (B) Western blot analysis. (n = 8 in the control group, n = 6 in the HBOU group, n = 10 in the HBOP group). The statistical results of WB after regrouping are shown under the representative bands. \*  $p < 0.05$  compared to the Con group; #  $p < 0.05$  compared to the HBOP group; ##  $p < 0.01$  compared to the HBOP group.

Haemopexin (HPX) and haptoglobin proteins, which stand out differently in both comparisons with the HBOP group, were selected for further verification using enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 5A, the changes in the HPX protein concentration in the HBOP groups were upregulated compared with that in the HBOU group and Con group, which was consistent with the data obtained from the MALDI-TOF-MS/MS proteomic analysis ( $p < 0.05$  HBOP vs. Con;  $p < 0.05$  HBOP vs. HBOU). To further verify the results, we validated the expression

of HPX in individual samples using western blot analysis (n = 6 for each group). After Bradford assay quantification, equal amounts of protein (5  $\mu$ g) from each sample were loaded onto each lane of polyacrylamide-SDS gels. After the western blot study, the changes in haemopexin were validated and exhibited similar changes in subjects from the HBOP group in accordance with MALDI-TOF-MS/MS proteomic analysis and ELISA analysis. As shown in Fig. 5B, the overall level of HPX in the HBOP group was higher than that in the control group and HBOU group

( $p < 0.05$  HBOP vs. Con;  $p < 0.05$  HBOP vs. HBOU). We also verified the expression of the haptoglobin protein among the different groups using the ELISA method and western blot, and there was no significant difference among the three groups (data not shown).

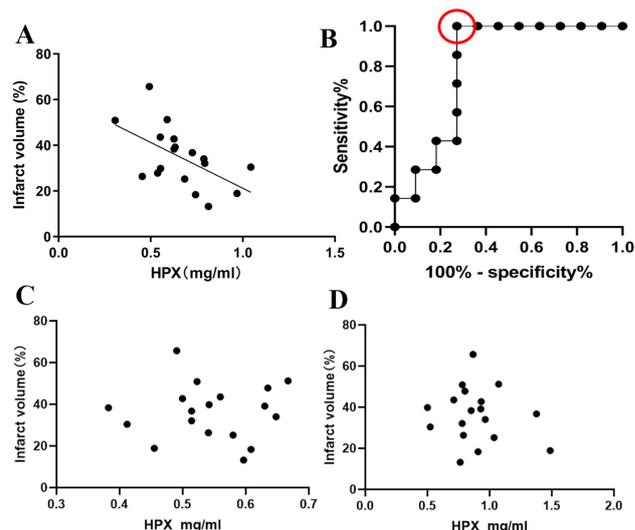
### 3.4 Serum HPX Levels at 2 h after HBO Preconditioning were Significantly Associated with the Prognosis of Cerebral Ischaemia Injury

To verify the feasibility of HPX as a biomarker for the neuroprotective effect of HBO pretreatment, the correlation between serum HPX content at different time points after HBO pretreatment and the infarct volume at 72 h following the MCAO procedure was evaluated. The serum HPX content was determined by ELISA. The results of the correlation analysis showed that the haemopexin concentration in the serum of rats at 2 h after HBO preconditioning was negatively correlated with the infarct volume ratio (correlation coefficient =  $-0.6199$ , 95% confidence interval (CI) ( $-0.8428, -0.2153$ ),  $p < 0.05$ , Fig. 6A). The ROC curve that discriminates the HBOP group from the HBOU group indicated that the area under the ROC curve was 0.9 (95% CI:  $0.7141-1$ ;  $p < 0.01$ ); Youden's index = 0.9 at HPX concentration =  $0.6265$  mg/mL ( $n = 18$ , Fig. 6B). We also determined the baseline HPX concentration in the serum of the rats, and the HPX concentration at 24 h after HBO preconditioning was not correlated with the infarct volume ratio at 72 h following MCAO ( $p > 0.05$ , Fig. 6C,D).

## 4. Discussion

In this study, we first reconfirmed the protective effect of HBO preconditioning on transient focal cerebral ischaemic injury. Consistent with previous studies, HBO preconditioning significantly improved neurological deficits and reduced the infarct volume ratio in rats that suffered from cerebral ischaemia/reperfusion injury [6]. However, some of the animals that were preconditioned with HBO did not experience favourable outcomes. To discriminate the fundamental protein differences in the subjects that benefit from those that did not benefit and to further test its possibility to be predictive biomarkers for the effectiveness of HBO preconditioning against cerebral ischaemia, we further divided the rats from the HBO group into HBOP (those that benefited) and HBOU (those that did not benefit) groups according to the infarct volume ratio of the control group (Mean-SD). Through comparing the serum protein expression between the control and HBOP groups, as well as the HBOU and HBOP groups by 2D-DIGE-MALDI-TOF-MS/MS, we identified 15 proteins in the HBOP group that were differentially expressed from both other groups, of which HPX and haptoglobin were further verified by western blot and ELISA.

Compared to cerebrospinal fluid (CSF) blood circulates all over the body and can be acquired more easily [15].



**Fig. 6. Analysis of HPX serum concentration at different time points after HBO pretreatment and prognosis of the rats in different groups.** Quantification of HPX by standard ELISA tests. (A) The HPX level at 2 h after HBO preconditioning was negatively correlated with the infarct volume at 3 d after reperfusion ( $r = -0.62$ ,  $p < 0.05$ ). (B) The receiver operating characteristic (ROC) curves of prognostic factors for distinguishing HBOP with HBOU. The area under the ROC curve was 0.9; 95% CI:  $0.7141-1$ ;  $p < 0.01$ ; Youden's index = 0.9 at HPX concentration =  $0.6265$  mg/mL. (C) No correlation was detected between the baseline HPX before HBO preconditioning and the infarct volume at 3 d after reperfusion ( $r = 0.01$ ,  $p > 0.05$ ). (D) No correlation was detected between HPX levels at 24 h after HBO preconditioning and the infarct volume at 3 d after reperfusion ( $r = -0.15$ ,  $p > 0.05$ ).  $n = 18$ . ROC, receiver operating characteristic; ELISA, enzyme linked immunosorbent assay.

Furthermore, HBO preconditioning can cause many pathophysiological changes in the brain, heart, spinal cord, liver, etc. [16–18]. All these changes in organs may cause variations in serum protein contents. Furthermore, each individual reacted to the HBO stimulus differently, resulting in different effects of tolerance against brain ischaemia, as shown in our study. Serum protein expression could change in accordance with the systemic reaction to HBO stimulation. These varying proteins in the serum may serve as potential biomarkers that can be used to predict the neuroprotective effect of HBO preconditioning against cerebral ischaemia. To identify sensitive and specific biomarkers, we performed 2D-DIGE-MALDI-TOF-MS/MS proteomic analysis to assess the differentially expressed serum proteins. To discriminate and define the “protective effect” and find proteins that are attributed to the valid protection provided by HBO and reduced infarct volume, we subgrouped the serum acquired from the HBO group into HBOP and HBOU groups according to their brain infarct volume.

In total, 1926 proteins were identified by 2D-DIGE-MALDI-TOF-MS/MS. Decyder image analysis revealed 15 proteins with over a 1.2-fold change in expression in the HBOP group. HPX and haptoglobin were further verified by western blot or ELISA to determine whether the protein changes corroborated the proteomic data discovered by 2D-DIGE-MALDI-TOF-MS/MS from pooled samples. The HPX level was found to be significantly higher in the HBO preconditioning groups, especially in the HBOP group. These data from both pooled and individual samples using three independent methods suggest that subjects in the HBOP group exhibit higher HPX levels than those in the HBOU and Con groups. Can the content of serum HPX reflect the protective effect of HBO preconditioning against cerebral ischaemia? We subsequently investigated the relationship between the content of serum HPX at different time points after HBO pretreatment and cerebral ischaemia outcomes. The serum HPX concentration at 2 h after HBO preconditioning was negatively correlated with the infarct volume ratio. The ROC curve that discriminates the HBOP group from the HBOU group also confirmed that the sensitivity and specificity of the serum HPX level are favourable for determining the prognosis of cerebral ischaemic injury with hyperbaric oxygen pretreatment. The above evidence corroborates that serum HPX may be a sensitive biomarker that indicates the protective effect of HBO preconditioning. We also tested the baseline HPX concentration before and after HBO preconditioning. The baseline HPX concentration before HBO preconditioning in the serum of the rats was about 1–1.5 g/L. HPX concentrations before and after HBO preconditioning in the serum were not correlated with the infarct volume ratio at 72 h following MCAO.

Although we pinned 15 proteins that were expressed differently among the three groups by proteomic study, only HPX was demonstrated to be consistent with the screening result by western blot (WB) and ELISA. It is evident that among western blot, ELISA and the proteomic method employed in the current study, there are differences in the sensitivity of quantifying protein abundances. The relative quantification of abundance determined by LC-MS/MS was calculated based on the differences in isoelectric focusing fractions. Multiple fractions of the proteins were detected without magnification. However, western blot and ELISA antibodies recognize only a single epitope and are detected through multiple magnifications. In the proteomic study, we aimed for a 1.2-fold change in expression in the pooled sample as a cut-off standard, but in WB and ELISA, each individual sample was tested, and the statistical results were used to describe the differences. Thus, these three methods might have different sensitivities for revealing differences in protein abundance.

We are most interested in HPX since it was demonstrated to be a potent endogenously protective molecule against cerebral ischaemia injury in our previous studies [19–21]. It has been reported that in haemolytic and haem-

orrhagic disease, a large amount of toxic free haem is produced in the blood, which can be quickly bound by HPX with high affinity ( $KD\ 0.32 \pm 0.04\ nM$ ) and transported into the cells, followed by enzymatic degradation to generate metabolites with antioxidant properties, thus alleviating subsequent oxidative stress and excitotoxic damage [22,23]. Interestingly, even in ischaemic disease, the reperfusion process also produces large amounts of harmful free heme [24]. HBO pretreatment induced HPX protein expression, and the upregulated HPX achieved the neuroprotective effects of HBO preconditioning through the above mechanisms, which provided important theoretical evidence that HPX is a biomarker for the neuroprotective effect of hyperbaric oxygen pretreatment against cerebral ischaemia injury. The serum concentration of HPX at 2 h rather than the basal HPX levels or the serum HPX levels at 24 h after HBO pretreatment was significantly associated with the prognosis of cerebral ischaemic injury. This may be related to the consistency between the blood metabolic chronergy of HPX that is associated with HBO preconditioning and the pathological process of cerebral ischaemia–reperfusion injury.

The following mechanisms may be involved in the neuroprotective effect of HPX: (1) Antioxidant and detoxification effects by binding to free heme [25,26]. The importance of HPX in combination with free haem, in which its enzymatic degradation into antioxidant metabolites occurs, has long been appreciated, in which process HPX also acts as a key regulator of intracellular iron homeostasis [27,28]. The antioxidant properties of HPX play an important role in the neuroprotective mechanism that functions in the early stage of cerebral ischaemic injury [19,21]. (2) HPX might help recovery after ischaemia through the induction of angiogenesis and neurogenesis. Our previous *in vitro* and *in vivo* studies verified that the angiogenic effect of HPX occurs by promoting the proliferation and differentiation of endothelial progenitors [20,29]. HPX can also contribute to adult neurogenesis in the subventricular zone, and deleting haemopexin leads to neurogenic abnormalities in the SVZ/olfactory bulb (OB) pathway [30]. (3) In the past years, heme-dependent anti-inflammatory effects of HPX have emerged [31]. In severe critically inflammatory diseases, such as acute respiratory distress syndrome (ARDS), burn patients and premature infants, some researchers have detected decreased HPX levels along with impaired haem clearance capacity [32]. HPX has been reported to be a novel biomarker for distinguishing chronic obstructive pulmonary disease (COPD) from asthma, which may be related to the anti-inflammatory mechanism of HPX [33].

In the current study, we identified multiple proteins that were differentially expressed in HBO preconditioning. Samples used for mass spectrum (MS) were designed to screen for the differentially expressed candidate proteins, in which most abundant proteins were eliminated to improve the sensitivity of mass spectrometry detection for the

low abundance proteins. To be more confidence about the screening results, we did additional MCAO models (with or without HBO preconditioning). Thereby, ELISA and WB were used to verify the different expression of hemopexin among the three groups. Using western blot and ELISA analyses, we confirmed that the expression of HPX was significantly changed in the HBOP group compared with the Con and HBOU groups. The serum HPX concentration at 2 h after HBO pretreatment exhibits good sensitivity and specificity as a predictor for evaluating the prognosis of cerebral ischaemia injury. Although further validation and evaluation in clinical settings are needed, our findings provide important evidence that may facilitate the translational use of preconditioning strategies for preventing stroke.

## 5. Conclusions

HPX could serve as a sensitive biomarker to distinguish whether patients benefit from HBO preconditioning against a possible stroke insult. Furthermore, HPX may even be a therapeutic target since its expression in the brain changed accordingly with serum after HBO preconditioning.

## Abbreviations

HBO, Hyperbaric oxygen; CABG, coronary artery bypass graft; 2D-DIGE, bidimensional electrophoresis difference gel electrophoresis; MCAO, middle cerebral artery occlusion; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; HPX, haemopexin; CBF, cerebral blood flow.

## Availability of Data and Materials

The data used to support the findings of this study are available from the corresponding author upon request.

## Author Contributions

SW conceived and coordinated the study. ZF designed experiments. BD, YB and LS carried out experiments. QJ participated in the data collection process. XZ analyzed experimental results. BD and QJ drafted the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

The experimental procedures in this study were approved by the Guidelines for the Care and Use of Laboratory Animals of the Fourth Military Medical University (approval number: 202010215).

## Acknowledgment

Not applicable.

## Funding

This work was supported by the National Natural Science Foundation of China (no. 82171322), the Natural Science Foundation of Shaanxi Province of China (no. 2022KJXX-102), and the Natural Science Foundation of Tianjin (no. 21JCQNJC01100).

## Conflict of Interest

The authors declare no conflict of interest.

## References

- [1] Saini V, Guada L, Yavagal DR. Global Epidemiology of Stroke and Access to Acute Ischemic Stroke Interventions. *Neurology*. 2021; 97: S6–S16.
- [2] Feigin VL, Brainin M, Norrving B, Martins S, Sacco RL, Hacke W, *et al.* World Stroke Organization (WSO): Global Stroke Fact Sheet 2022. *International Journal of Stroke*. 2022; 17: 18–29.
- [3] Reinert NJ, Patel BM, AlShaer QN, Wu L, Wisniewski S, Hager ES, *et al.* Cause-Specific Mortality as a Sequelae of Perioperative Stroke Following Cardiac and Vascular Surgery. *the Neurologist*. 2022; 27: 21–26.
- [4] Banco D, Dodson JA, Berger JS, Smilowitz NR. Perioperative cardiovascular outcomes among older adults undergoing in-hospital noncardiac surgery. *Journal of the American Geriatrics Society*. 2021; 69: 2821–2830.
- [5] Benesch C, Glance LG, Derdeyn CP, Fleisher LA, Holloway RG, Messé SR, *et al.* Perioperative Neurological Evaluation and Management to Lower the Risk of Acute Stroke in Patients Undergoing Noncardiac, Nonneurological Surgery: A Scientific Statement From the American Heart Association/American Stroke Association. *Circulation*. 2021; 143: e923–e946.
- [6] Wang S, Fu Y, Han X, Yong Z, Li Q, Hu Z, *et al.* Hyperbaric Oxygen Preconditioning Protects against Cerebral Ischemia/Reperfusion Injury by Inhibiting Mitochondrial Apoptosis and Energy Metabolism Disturbance. *Neurochemical Research*. 2021; 46: 866–877.
- [7] Fang Z, Feng Y, Li Y, Deng J, Nie H, Yang Q, *et al.* Neuroprotective Autophagic Flux Induced by Hyperbaric Oxygen Preconditioning is Mediated by Cystatin C. *Neuroscience Bulletin*. 2019; 35: 336–346.
- [8] Li Y, Dong H, Chen M, Liu J, Yang L, Chen S, *et al.* Preconditioning with Repeated Hyperbaric Oxygen Induces Myocardial and Cerebral Protection in Patients Undergoing Coronary Artery Bypass Graft Surgery: a Prospective, Randomized, Controlled Clinical Trial. *Journal of Cardiothoracic and Vascular Anesthesia*. 2011; 25: 908–916.
- [9] Yang C, Liu J, Wang J, Yin A, Jiang Z, Ye S, *et al.* Activation of astroglial CB1R mediates cerebral ischemic tolerance induced by electroacupuncture. *Journal of Cerebral Blood Flow and Metabolism*. 2021; 41: 2295–2310.
- [10] Dietrich MA, Irnazarow I, Adamek M, Jurecka P, Teich L, Rakus K, *et al.* 2D-DIGE proteomic analysis of blood plasma reveals changes in immune- and stress-associated proteins following hormonal stimulation of carp males. *Fish and Shellfish Immunology*. 2021; 118: 354–368.
- [11] Su Y, Sun B, Gao X, Dong X, Fu L, Zhang Y, *et al.* Intranasal Delivery of Targeted Nanoparticles Loaded With miR-132 to Brain for the Treatment of Neurodegenerative Diseases. *Frontiers in Pharmacology*. 2020; 11: 1165.
- [12] Fang Z, Wu D, Deng J, Yang Q, Zhang X, Chen J, *et al.* An MD2-perturbing peptide has therapeutic effects in rodent and rhesus monkey models of stroke. *Science Translational Medicine*. 2021; 13: eabb6716.

- [13] Garcia JH, Wagner S, Liu K, Hu X. Neurological Deficit and Extent of Neuronal Necrosis Attributable to Middle Cerebral Artery Occlusion in Rats. *Stroke*. 1995; 26: 627–635.
- [14] Fang Z, Deng J, Wu Z, Dong B, Wang S, Chen X, *et al.* Cystatin C is a Crucial Endogenous Protective Determinant against Stroke. *Stroke*. 2017; 48: 436–444.
- [15] Shahan B, Choi EY, Nieves G. Cerebrospinal Fluid Analysis. *American Family Physician*. 2021; 103: 422–428.
- [16] Kovacevic S, Ivanov M, Zivotic M, Brkic P, Miloradovic Z, Jeremic R, *et al.* Immunohistochemical Analysis of 4-HNE, NGAL, and HO-1 Tissue Expression after Apocynin Treatment and HBO Preconditioning in Postischemic Acute Kidney Injury Induced in Spontaneously Hypertensive Rats. *Antioxidants (Basel)*. 2021; 10: 1163.
- [17] Hu S, Feng H, Xi G. Hyperbaric oxygen therapy and preconditioning for ischemic and hemorrhagic stroke. *Medical Gas Research*. 2016; 6: 232.
- [18] Margarido MR, Silveira MRGD, Vanni JC, Feres O, Castro-e-Silva O. Hyperoxic preconditioning in partial liver ischemia. *Acta Cirurgica Brasileira*. 2014; 29: 19–23.
- [19] Dong B, Yang Y, Zhang Z, Xie K, Su L, Yu Y. Hemopexin alleviates cognitive dysfunction after focal cerebral ischemia-reperfusion injury in rats. *BMC Anesthesiology*. 2019; 19: 13.
- [20] Dong B, Zhang Z, Xie K, Yang Y, Shi Y, Wang C, *et al.* Hemopexin promotes angiogenesis via up-regulating HO-1 in rats after cerebral ischemia-reperfusion injury. *BMC Anesthesiology*. 2018; 18: 2.
- [21] Dong B, Cai M, Fang Z, Wei H, Zhu F, Li G, *et al.* Hemopexin induces neuroprotection in the rat subjected to focal cerebral ischemia. *BMC Neuroscience*. 2013; 14: 58.
- [22] Detzel MS, Schmalohr BF, Steinbock F, Hopp M, Ramoji A, Paul George AA, *et al.* Revisiting the interaction of heme with hemopexin. *Biological Chemistry*. 2021; 402: 675–691.
- [23] Garland P, Durnford AJ, Okemefuna AI, Dunbar J, Nicoll JAR, Galea J, *et al.* Heme–Hemopexin Scavenging is Active in the Brain and Associates with Outcome after Subarachnoid Hemorrhage. *Stroke*. 2016; 47: 872–876.
- [24] Cheng Y, Rong J. Therapeutic Potential of Heme Oxygenase-1/carbon Monoxide System against Ischemia-Reperfusion Injury. *Current Pharmaceutical Design*. 2017; 23: 3884–3898.
- [25] Ellis-Guardiola K, Soule J, Clubb R. Methods for the Extraction of Heme Prosthetic Groups from Hemoproteins. *BIO-PROTOCOL*. 2021; 11: e4156.
- [26] Rochette L, Lorin J, Zeller M, Guillard J, Lorgis L, Cottin Y, *et al.* Nitric oxide synthase inhibition and oxidative stress in cardiovascular diseases: Possible therapeutic targets? *Pharmacology and Therapeutics*. 2013; 140: 239–257.
- [27] Chen-Roetling J, Li Y, Cao Y, Yan Z, Lu X, Regan RF. Effect of hemopexin treatment on outcome after intracerebral hemorrhage in mice. *Brain Research*. 2021; 1765: 147507.
- [28] Leclerc JL, Santiago-Moreno J, Dang A, Lampert AS, Cruz PE, Rosario AM, *et al.* Increased brain hemopexin levels improve outcomes after intracerebral hemorrhage. *Journal of Cerebral Blood Flow and Metabolism*. 2018; 38: 1032–1046.
- [29] Yang Y, Dong B, Lu J, Wang G, Yu Y. Hemopexin reduces blood–brain barrier injury and protects synaptic plasticity in cerebral ischemic rats by promoting EPCs through the HO-1 pathway. *Brain Research*. 2018; 1699: 177–185.
- [30] Zhu Y, Qiu Y, Chen M, Zhang Y, Cao L, Su Z, *et al.* Hemopexin is required for adult neurogenesis in the subventricular zone/olfactory bulb pathway. *Cell Death and Disease*. 2018; 9: 268.
- [31] Ross AC. Impact of chronic and acute inflammation on extra- and intracellular iron homeostasis. *The American Journal of Clinical Nutrition*. 2017; 106: 1581S–1587S.
- [32] Lin T, Maita D, Thundivalappil SR, Riley FE, Hamsch J, Van Marter LJ, *et al.* Hemopexin in severe inflammation and infection: mouse models and human diseases. *Critical Care*. 2015; 19: 166.
- [33] Winter NA, Gibson PG, Fricker M, Simpson JL, Wark PA, McDonald VM. Hemopexin: A Novel Anti-inflammatory Marker for Distinguishing COPD From Asthma. *Allergy, Asthma & Immunology Research*. 2021; 13: 450–467.