1400W ameliorates acute hypobaric hypoxia/reoxygenation-induced cognitive deficits by suppressing the induction of inducible nitric oxide synthase in rat cerebral cortex microglia

Running title: 1400W ameliorates hypoxia/reoxygenation-induced cognitive deficits

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Abstract

Nitric oxide (NO) is involved in neuronal modifications, and overproduction of NO contributes to memory deficits after acute hypobaric hypoxia-reoxygenation. This study investigated the ability of the iNOS inhibitor 1400W to counteract spatial memory deficits following acute hypobaric hypoxia-reoxygenation, and to affect expression of NOS, NO, 3-NT and MDA production, and apoptosis in rat cerebral cortex. We also used primary rat microglia to investigate the effect of 1400W on expression of NOS, NO, 3-NT and MDA production, and apoptosis. Acute hypobaric hypoxia-reoxygenation impaired spatial memory, and was accompanied by activated microglia, increased iNOS expression, NO, 3-NT and MDA production, and neuronal cell apoptosis in rat cerebral cortex one day post-reoxygenation. 1400W treatment inhibited iNOS expression without affecting nNOS or eNOS. 1400W also reduced NO, 3-NT and MDA production, and prevented neuronal cell apoptosis in cerebral cortex, in addition to reversing spatial memory impairment after acute hypobaric hypoxia-reoxygenation. Hypoxia-reoxygenation activated primary microglia, and increased iNOS and nNOS expression, NO, 3-NT, and MDA production, and apoptosis. Treatment with 1400W inhibited iNOS expression without affecting nNOS, reduced NO, 3-NT and MDA production, and prevented apoptosis in primary microglia. Based on the above findings, we concluded that the highly selective iNOS inhibitor 1400W inhibited iNOS induction in microglial cells, and reduced generation of NO, thereby mitigating oxidative stress and neuronal cell apoptosis in the rat cerebral cortex, and improving the spatial memory dysfunction caused by acute hypobaric hypoxia-reoxygenation.

Keywords: hypobaric, hypoxia, reoxygenation, inducible nitric oxide synthase, nitric oxide, spatial memory

1. Introduction

A main feature of the natural environment of the plateau is low air pressure, with accompanying low air oxygen content. Most people entering the plateau elevation of 4000 meters above sea level will show different degrees of acute high altitude reactions ^[1,2], and have differing degrees of cognitive and neuropsychological dysfunctions, including slow reflection, memory deficits, and attention deficit disorder ^[3-5]. Cognitive dysfunction caused by the high altitude plateau is also related to hypoxia-reoxygenation, which happens during mountaineering, plateau rescue and emergency, and oxygen inhalation therapy. In addition, hypoxia-reoxygenation occurs after return to normal elevations after a period on the plateau; however, the mechanism of cognitive dysfunction due to plateau-related hypoxia-reoxygenation is not well understood. Previous studies have confirmed that hypobaric hypoxia-reoxygenation can reduce the learning and memory capacity of the brain, which is related to the increase of free radical production and apoptosis or necrosis ^[6-9]. At the same time, there is evidence indicating that endogenous nitric oxide (NO) is directly correlated with cognitive dysfunction caused by hypobaric hypoxia-reoxygenation ^[10-14].

NO is a bioactive molecule and is an important messenger molecule in the brain ^[15]. NO plays a role in cognition, which may be related to synaptic plasticity of the hippocampus ^[16,17]. NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS) ^[18]. NOS has three subtypes; neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) are expressed under normal conditions, and inducible nitric oxide synthase (iNOS) is expressed after injury. Under anoxic conditions, because of the decrease of oxygen supply, the formation of NO is reduced because NOS requires oxygen ^[19]. Hypoxia-reoxygenation triggers a number of events, including release of glutamate and activation of free radical, Ca^{2+} influx, upregulated NOS expression, and increased NO generation. During hypoxia-reoxygenation, the activated microglial cell (especially the M1-type) is the main source of NO ^[20-23]. Activated microglia upregulate expression of iNOS through the p38/MAPK pathway ^[24] and the PI3-kinase/AKT/mTOR pathway ^[25]. With the

increase in oxygen supply during reoxygenation, the level of NO increases via enhanced iNOS activity. This excessive NO is toxic to neurons through the combination of NO and oxygen free radical to produce peroxynitrite anion (ONOO⁻). Because ONOO⁻ is involved in cellular damage, energy exhaustion, and cell death [26-29], reoxygenation results in brain dysfunction including cognitive deficits.

Because excessive NO generated by hypoxia-reoxygenation is caused by iNOS activity, previous studies have investigated pharmacological strategies to prevent acute hypobaric hypoxia-reoxygenation damage by inhibiting iNOS activity and reducing NO production. Melatonin can suppress hippocampal iNOS, nNOS, and eNOS overexpression, with reduced NO production after acute hypobaric hypoxia-reoxygenation [12]; previous studies indicated that melatonin has an antioxidant and free radical scavenging effect [30]. However, the mechanism of how NOS inhibitors or melatonin prevent NOS expression has not been studied thoroughly. Other studies showed that aminoguanidine could prevent the expression of iNOS of cerebral cortex of rats caused by acute hypobaric hypoxia-reoxygenation, improving retrograde memory disorders [11]. However, aminoguanidine also has some selectivity over nNOS and eNOS, and whether nNOS and eNOS play a role in the hypoxia-reoxygenation process remains to be determined.

Based on previous research strategies, we aimed to determine the source of NO and the impact of a selective inhibitor on NOS expression and NO release through in vitro and in vivo experiments. We chose the highly selective iNOS inhibitor N-[3-(Aminomethyl)benzyl]-acetamidine Dihydrochloride (1400W), which is more effective in preventing iNOS expression and activity than are eNOS and nNOS (5000 and 200 times higher respectively), and at a conventional dose, 1400W has no effect on the biological activity of eNOS and nNOS [31-33]. In the present study, rats were pretreated with 1400W before exposure to hypobaric hypoxia-reoxygenation to determine whether 1400W could improve the spatial memory deficits typically seen after this hypoxic treatment, and whether 1400W could inhibit iNOS overexpression in microglia cells, and the subsequent excessive NO levels causing oxidative stress and neuronal cell apoptosis in the rat cerebral cortex. We then utilized primary

microglia in in vitro experiments to observe the effect of 1400W pretreatment on the transcription and expression of NOS, the release of NO and the impact on oxidative stress and cell apoptosis after hypoxia-reoxygenation.

2. Materials and Methods

2.1 Animal source and ethics statement

Adult Sprague–Dawley (SD) rats (200 – 250 g) were used for the in vivo experiments and for obtaining primary microglia. Rats were maintained in polypropylene cages with a 12 h light/12 h dark schedule and were provided with food and water ad libitum. Animals were housed at the Laboratory of Animals in the Medical Research Center, Southwest Hospital, Third Military Medical University. All experiments were carried out in accordance with the Provisions and General Recommendation of the Chinese Experimental Animals Administration Legislation, which were approved by the Animal Ethics Committee of Chongqing.

2.2 In vivo hypobaric hypoxia/reoxygenation (HH/R)

2.2.1 HH/R and drug treatment

Animals were randomly assigned to one of four experimental groups: vehicle-treated normoxia group, 1400W-treated normoxia group, vehicle-treated hypoxia group, and 1400W-treated hypoxia group. The 1400W-treated groups were pretreated with ip injections of 1400W (20 mg/kg, optimum dose; Code No. W4262, SIGMA-ALDRICH LLC) at 12 h intervals as previously described ^[34]. 1400W was dissolved in sterile distilled water at a concentration of 20 mg/ml. Vehicle-treated groups were pretreated with ip injections of an equal volume of sterile distilled water. Two hours after administration of vehicle or 1400W, normoxia groups were maintained in a normoxic environment while hypoxia groups were exposed to simulated hypobaric hypoxia (HH) and reoxygenation as previously described ^[11,12]. In brief, rats were exposed to simulated HH for 12 h at 8000 m (267 Torr) in an animal decompression chamber (Aviation Industry Corporation of China, China) with the temperature and humidity maintained at $22 \pm 2^{\circ}\text{C}$ and $30 \pm 5\%$, and animals were

provided with food and water ad libitum. After 12 h of HH, the hypoxia groups were brought down to sea level. Subjects from each experimental group were assessed at 0, 1 or 3 days post-HH with behavioral experiments or by resection of the cerebral cortex for embedding in paraffin and preparing tissue homogenate. Treatment of all 1400W treated animals was stopped prior to spatial memory retention trial or resection of the cerebral cortex.

2.2.2 Behavioral experiments

2.2.2.1 Morris Water Maze (MWM) test

Spatial memory was tested using a MWM ^[35]. The water maze apparatus consisted of a circular pool 150 cm in diameter, 60 cm deep, filled to the height of 30 cm with water (temperature 22–24°C) to cover a platform (diameter 10 cm). The platform was submerged approximately 2 cm below the surface of the water, and was camouflaged by adding non-toxic white paint to the water. The rat's head was painted yellow using picric acid, and an overhead camera and computerized video imaging analysis system (Chengdu TME Technology Co., Ltd, China) were used to record the swimming paths of the marked rat in the maze.

Before the HH insult, rats were trained in the MWM for 7 days. The platform was always placed into the water in the center of the north quadrant; the pool wall from the nearest platform posted a visual cue ^[4]. Each rat was trained to find the platform with three trials a day from three different fixed locations at east, south, and west quadrants. On each trial, the rat was lowered gently into the water facing the pool wall at one of the three fixed locations, and allowed to find the hidden platform. In cases where the rat did not succeed in finding the platform within 60 s, it was placed onto the platform. Once on the platform, the rats were allowed to remain on there for 30 s, whether they had found the platform on their own or not. There was a 60 s recovery period between trials. On Day 7, all rats were treated with vehicle or 1400W and exposed to either normoxia or simulated HH for 12 h. After the HH insult, rats were subjected to a spatial memory retention trial using MWM without the platform at 0, 1 or 3 days post-insult. The 60 s memory retention trial (one trial

without the platform) was assessed; the rat was placed into the water in the south quadrant, and the number of times the rat crossed the former location of the platform and the time spent in the former platform quadrant were recorded for 60 s.

2.2.2.2 Y-maze test

Spatial memory of animals was further investigated using a Y-maze. This test is based on the innate tendency of rodents to explore novel environments ^[36]. The apparatus consists of three horizontal arms 50 cm long and 10 cm wide, with walls 20 cm high, symmetrically disposed at 120° to each other and made of black plastic. Each arm had different cues for distinction from each other. Y-maze testing consisted of two trials separated by an interval of 1h ^[37,38]. In the first trial (training), the animal was placed at the end of one arm and allowed access to that arm and another arm for 10 min. The third arm (the novel arm) was blocked by a guillotine door. The rat was then removed from the maze and returned to its home cage. After 1 h, in the second trial (retention), the mouse was placed back in the maze and given free access to all three arms for 8 min. The number of entries and the time spent in each arm were recorded. The percentage of number of novel arm visits and duration of novel arm visits were calculated. To avoid the presence of olfactory cues, maze arms were thoroughly cleaned between tests. Rats that distinguished the unfamiliar arm show exploratory behavior, and spend more time and enter more frequently in to the novel arm in comparison with the familiar arms.

2.2.3 Preparation of cerebral cortex tissues homogenate

Experimental group rats at 0, 1, and 3 days post-HH and normoxia group rats were sacrificed by cervical dislocation, and the cerebral cortex was resected. A 10% (w/v) homogenate of the cerebral cortex tissue was prepared in 0.154 M KCl solution and was centrifuged at $10,000 \times g$ for 10 min at 4°C. Aliquots of supernatant were used for further biochemical analysis. The protein contents of the samples were estimated by the Bradford method ^[39].

2.3 In vitro hypoxia and reoxygenation (H/R)

2.3.1 Isolation and culture of primary adult microglia

Primary adult microglia were prepared from adult SD rat cerebral cortices as previously described ^[40]. The rats were sacrificed by decapitation and the forebrain was rapidly dissected, the cerebellum and olfactory bulbs were removed, and the meninges and blood vessels were carefully stripped off using a stereomicroscope. The tissue was cut into 1-3 mm³ fragments in pre-cooled phosphate-buffered saline (PBS), and then incubated with 4 ml 0.25% trypsin in a 37°C water bath for 15 min. Then 5 ml complete Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Gibco, Rockville, MD, USA) medium containing 10% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin was added to terminate digestion. The digest was gently pipetted several times, then filtered through a 70-µm mesh. The filtrate was centrifuged at 800 × g for 5 min at 4°C. The supernatant was removed and added to an appropriate amount of complete DMEM/F12 medium to seed the cells at a density of 4×10⁴ cells/cm². Cells were treated with M-CSF (10 ng/ml; R&D Systems, Minneapolis, MN, USA), and GM-CSF (20 ng/ml; R&D Systems) from 1 day post-seeding to promote microglial proliferation ^[41]. Microglia were isolated using the shaking method. The media containing the detached and floating microglia was collected and centrifuged at 800 × g for 6 min. The pelleted isolated microglia were resuspended in complete DMEM/F12 medium and seeded in culture flasks at a density of 4×10⁴ cells/cm². As determined by flow cytometry for a microglia marker (CD11b), the isolated microglia cultures were 92.7% pure.

2.3.2 Drug treatment and H/R

Exposure to hypoxia/reoxygenation (H/R) was performed as previously described ^[42]. Briefly, cells were cultured in complete DMEM/F12 medium supplemented with 500 µM arginine, and placed in a hypoxic humidified incubator flushed with a gas mixture of 5% CO₂: 1% O₂: 94% N₂ (model HF100; HealForce Ltd, Shanghai, China). Before beginning the hypoxia experiments, the incubator was equilibrated to the desired hypoxic condition, and the culture medium was placed

under this hypoxic condition to equilibrate the dissolved oxygen concentration. After 12 h hypoxia, cells were cultured in complete DMEM/F12 medium and normoxia condition for reoxygenation for 0, 3, 6, 12 or 24 h. 1400W (60 μ M, optimum dose) dissolved in PBS was added to cell cultures 1 h before H/R, whereas control cultures received only vehicle (PBS).

2.3.3 Cytotoxicity assay

Cell viability was evaluated using an MTT assay as previously described ^[43]. Cells were seeded into 96 well plates and maintained at 37°C for 24 h. The cells were exposed to various concentrations of 1400W (20, 40, 60, 80, and 100 μ M). After 24 h exposure, 0.5 mg/ml MTT in DPBS was added to each well and incubated for further 4 h. Then 150 μ l of DMSO was added to the wells to dissolve the formazan crystals, and absorbance was measured at 490 nm using the Thermo Scientific Varioskan Flash microplate reader. The cellular viability was determined from the absorbance value and compared with that of the untreated control group.

2.3.4 Detection of apoptosis using flow cytometry

Cells were seeded into 96-well plates at a density of 4×10^4 cells/cm² and maintained at 37°C for 24 h. Cells were then cultured in complete DMEM/F12 medium supplemented with 500 μ M arginine, and placed in a hypoxic humidified incubator (1% O₂). After 12 h hypoxia, cells were cultured in normoxic conditions for reoxygenation for 0, 6, or 24 h. 1400W (60 μ M) dissolved in PBS was added to cell cultures 1 h before H/R, and control cultures received only vehicle (PBS). After H/R, cells were harvested and washed three times with ice-cold PBS. Cells were resuspended at a concentration of 4×10^5 cells per 500 μ l binding buffer, and incubated with Annexin V-FITC (Code No. 556420, BD, San Diego, CA, USA) and propidium iodide (PI) in the dark for 15 min at room temperature. The samples were analyzed using BD FACSCanto II flow cytometer (San Diego, CA, USA). Apoptosis ratio was defined as the ratio between Annexin V positive/PI negative cells (right lower

quadrant) and total cells.

2.4 NO production assay

The nitrate/nitrite concentration was considered an indicator of NO production and was measured as previously described ^[44] using a commercially available Nitric Oxide Fluorometric Assay Kit (K252-200, Biovision, Milpitas, CA, USA) according to the manufacturer's instructions. Fluorescence was measured at 360 nm excitation/450 nm emission using the Thermo Scientific Varioskan Flash fluorescence reader. The fluorescence was an indicator of the concentration of sodium nitrite in the solution, and sodium nitrite concentrations were used to draw a standard curve, from which the concentration of nitrite was calculated. Microglia culture medium and cerebral cortex tissues homogenate were used to assess NO production. The values of NO production were expressed in nmol/mg protein.

2.5 3-Nitrotyrosine assay

3-Nitrotyrosine (3-NT) was determined by Enzyme-linked immunosorbent assay (ELISA) for the quantitative measurement of 3-NT in cerebral cortex tissue homogenate and microglia lysates as previously described ^[45] using a commercially kit (ab116691, Abcam). The assay employs an antibody specific to 3-NT coated on a 96-well plate. Standards and samples were pipetted into the wells and 3-NT present in the sample was bound to the wells by the immobilized antibody. The wells were washed and a biotin-labeled anti-3-NT detector antibody was added. After washing away unbound detector antibody, HRP-conjugated streptavidin specific for the biotin labeled detector antibody was pipetted into the wells. The wells were again washed, an HRP substrate solution (TMB) was added to the wells, and color developed in proportion to the amount of 3-NT bound. After adding hydrochloric acid to stop the reaction, the absorbance was measured at 450 nm using the Thermo Scientific Varioskan Flash microplate reader. The values of 3-NT production were expressed as

changes in percentage among the control group.

2.6 Lipid Peroxidation assay

Lipid peroxidation was determined spectrophotometrically by measuring the malondialdehyde (MDA) levels produced as previously described ^[46] using a commercially available Lipid Peroxidation MDA Assay Kit (S0131, Beyotime, Haimen, China) according to the manufacturer's instructions. Each molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) to form a colored MDA-TBA complex that can be quantified spectrophotometrically at 532 nm using a spectrophotometer. Microglia lysates and cerebral cortex tissues homogenate were used to assay MDA. The values were expressed in nmol/mg protein.

2.7 Quantitative real-time reverse transcriptase PCR

Total RNA was extracted from cerebral cortex and cultured microglia using a MiniBEST Universal RNA Extraction Kit (Code No. 9767, Takara, Dalian, China) according to the manufacturer's instructions. Both the amount and purity of the RNA preparation were confirmed by measuring the absorbance ratio at 260/280 nm. Total RNA (1 µg) was converted to cDNA using the PrimeScript™ RT reagent Kit (RR037A, Takara, Dalian, China). Quantitative real-time reverse transcriptase PCR (RT-PCR) analysis for iNOS, nNOS, eNOS, and β-actin was performed using a Roche LightCycler® 480 System. Quantitative PCR was conducted in 0.2 ml PCR tubes with the appropriate forward and reverse primers and the SYBR® Premix Ex Taq™ II working solution (RR071A, Takara, Dalian, China), using a custom PCR master mix under the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The primers used were: iNOS, 5'-GTTCTCAGCCCAACAATACAAGA-3' (F) and 5'-GTGGACGGGTCGATGTCAC-3' (R); nNOS, 5'-CTGGTGAAGGAACGGGTCAG-3' (F) and

5'-CCGATCATTGACGGCGAGAAT-3' (R); eNOS,
 5'-GGCTGGGTTTAGGGCTGTG-3' (F) and 5'
 -CTGAGGGTGTCTAGGTGATG-3' (R); and β -actin,
 5'-GGCTGTATTCCCCTCCATCG-3' (F) and 5'-CCAGTTGGTAACAA
 TGCCATGT-3' (R). β -actin expression was used as the internal reference. For
 quantification, mRNA expression data were normalized to the β -actin signal by using
 the $2^{-\Delta\Delta CT}$ method.

2.8 Western blot analysis

Microglia cells were washed with ice-cold DPBS and suspended in 200 μ l of lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS supplemented with protease inhibitor cocktail [Sigma, USA]) and incubated at 4°C for 5 min. Samples were then sonicated for 15 s on ice and centrifuged at $13,000 \times g$ for 10 min at 4°C. The supernatant was collected, and the protein content of the lysates was estimated by the Bradford method.

Cerebral cortex tissues were resected to prepare tissue homogenate as follows: tissues were homogenized in ice-cold lysis buffer [10 mM Tris-HCl (pH 7.6), 140 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 1% Nonidet P-40, 0.5% deoxycholate, 2% β -mercaptoethanol, 10 mg/ml pepstatin A and 10 mg/ml aprotinin] and kept at 4 °C for 30 min. The homogenate was centrifuged at $13,000 \times g$ for 20 min at 4°C. The supernatant was collected, and protein concentration was determined by the Bradford method.

Loading buffer was added to protein from each sample and heated to 100°C for 5 min. Cells protein samples (20 μ g) or cerebral cortex protein samples (50 μ g) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes using a Bio-Rad Trans-Blot® module. Non-specific protein binding was blocked by saturating the PVDF membranes with 5% bovine serum albumin (BSA) at room temperature for 1 h. Membranes were then incubated with primary antibodies against iNOS (1:1000,

ab49999, Abcam, Cambridge, MA, USA), nNOS (1:1000, ab76067, Abcam), eNOS (1:500, ab50010, Abcam), caspase-3 (1:500, SC-7272, Santa Cruz), Bax (1:500, SC-7480, Santa Cruz), or β -actin (1:2000, ab3280, Abcam) overnight at 4°C. After three washes with Tris-buffered saline solution containing 0.1% Tween 20 (TBST), the membranes were incubated at room temperature for 1 h with HRP-conjugated goat anti-rabbit IgG antibody (1:5000, ab6721, Abcam) or HRP-conjugated rabbit anti-mouse IgG antibody (1:5000, ab6728, Abcam), and then developed using Pierce enhanced chemiluminescence (ECL) detection reagent (32016, Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The densities of the bands were measured using the Bio-Rad ChemiDocTM XRS luminescent image analyzer and Gel-Pro analyzer 4.0 software.

2.8 Immunofluorescence analysis

After administration of vehicle or 1400W and acute HH exposure for 12 h, followed by 0 or 1 day of reoxygenation, the rats were anesthetized (pentobarbital-sodium, 50 mg/kg, i.p.) and perfused intracardially with heparinized (10 U/ml) 0.1 M/L PBS (pH 7.4) followed by 4% paraformaldehyde in PBS. The whole brain was then harvested. The cerebral cortex was resected and fixed in 4% paraformaldehyde and paraffin embedded. Sections 10 μ m thick were taken from different regions. They were deparaffinized in xylene, hydrated in a decreasing gradient of ethanol and rinsed with PBS and distilled water. Sections were incubated for 20 min in citrate buffer (pH 6.0), which was pre-heated in a water bath to 96°C. Sections were washed, blocked with PBS containing 10% normal goat serum, 1% BSA, and 0.1% sodium azide, and permeabilized with 0.3% Triton X-100 for 1 h with gentle rocking. Primary antibodies used for immunofluorescence were: anti-NeuN (1:300, ab177487, Abcam), anti-caspase-3 (1:200, SC-7272, Santa Cruz), anti-GFAP (1:500, ab7260, Abcam), anti-Iba-1 (1:200, ab178680, Abcam), and anti-iNOS (1:200, ab49999, Abcam). Sections were incubated with primary antibody in PBS with 1% BSA overnight at 4°C, followed by washing and application of secondary antibodies.

Secondary antibodies used were: Alexa Fluor[®] 488 goat anti-rabbit for Iba-1, GFAP and goat anti-mouse caspase-3 (ab150077, ab150113, Abcam), Alexa Fluor[®] 647 goat anti-rabbit for NeuN, and goat anti-mouse for iNOS (ab150079, ab150115, Abcam). We performed double labeling for caspase-3/NeuN to detect neuronal apoptosis, and Iba-1/iNOS to detect microglia activation and iNOS expression. 4',6-diamidino-2-phenylindole (DAPI) was used to counterstain nuclei. After final washing, sections were protected with cover slips with anti-fading mounting medium, sealed with nail polish, and stored at 4°C. Finally, the cover slips were mounted and images were viewed by fluorescence microscopy (Olympus BX51, Tokyo, Japan) and photo collection was performed using a charge-coupled device (CCD) camera. Acquisition parameters were consistent for all sections from each staining session. Five random images from the right and left sides of each section were photographed for a total of 10 images. For double-stained sections, images from the same area were taken under the 2 appropriate wavelengths. Neuronal apoptosis ratio was defined as the ratio between (caspase-3⁺ and NeuN⁺)/NeuN⁺ cells. Microglia activation was indicated as green fluorescence mean intensity (Iba-1 positive) and iNOS expression was indicated as red fluorescence mean intensity (iNOS positive) using the Image J software (National Institutes of Health [NIH], Bethesda, USA).

2.9 Statistical analysis

All values are presented as mean $\pm$ SD. Student's t test and one-way ANOVA with the least-significant difference (LSD) multiple group comparison was used. P values less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. Effect of 1400W supplementation during acute hypobaric hypoxia and reoxygenation on spatial memory performance

All rats had 7 days training on the MWM, and there were no significant differences in the average escape latency and average total swimming distance of the

different groups after training. MWM performance was assessed after exposure to normoxia or HH followed by 0, 1 or 3 days of reoxygenation. The number of times the animal crossed the platform location and the percentage of swimming time in the target quadrant was not altered between, the HH and normoxia group immediately following HH (Figure 1A, 1B). The number of crossings of the former platform location and the percentage of swimming time in the target quadrant was significantly lower in the HH-reoxygenation groups at 1 day of reoxygenation ($P < 0.01$, $P < 0.01$ respectively, Figure 1A, 1B). Treatment with 1400W increased platform crossings and swim time in the target quadrant in the treated HH-exposed groups compared to the vehicle HH group after 1 day of reoxygenation ($P < 0.05$, $P < 0.01$ respectively). At 3 days post-HH, the number of crossings of the former platform location and the percentage of swimming time in the target quadrant was decreased significantly compared to the normoxia group ($P < 0.05$, $P < 0.01$ respectively), but the number of crossing of the former platform location in the 1400W group was higher than vehicle ($P < 0.01$), and the percentage of swimming time in the target quadrant was not significantly changed.

In the Y-maze experiment, we found similar results to the MWM (Figure 1C, 1D). HH and reoxygenation groups had significantly lower duration of novel arm visits and number of novel arm visits compared with normoxia group ($P < 0.001$ to 0.05). 1400W treatment increased the duration and number of novel arm visits compared to the vehicle-treated group after 1 day of reoxygenation ($P < 0.001$). The 3-day reoxygenation group treated with 1400W was no different than the vehicle for the duration of novel arm visits; however, the number of novel arm visits was significantly higher than the vehicle group ($P < 0.01$).

3.2. Effect of 1400W treatment during acute HH/R on NOS expression, NO, 3-NT, and MDA production, and apoptosis in the cerebral cortex

After acute HH exposure for 12 hours followed by 0, 1, or 3 days of reoxygenation, the cerebral cortex was removed for assessment of biochemical changes and immunochemical analysis. HH without reoxygenation significantly

increased eNOS mRNA ($P < 0.01$, Figure 2C). After 1 day of reoxygenation, levels of iNOS, nNOS and eNOS mRNA were significantly increased ($P < 0.001$, $P < 0.01$, $P < 0.001$ respectively, Figures 2A-2C). With 3 days of reoxygenation, iNOS and nNOS mRNA levels were still elevated ($P < 0.001$, $P < 0.05$ respectively, Figure 2A, 2B), but eNOS mRNA was not significantly changed. Treatment with 1400W significantly inhibited the increase in iNOS at 1 and 3 days of reoxygenation ($P < 0.01$, $P < 0.05$ respectively, Figure 2A), but did not have any significant effect on mRNA levels of nNOS or eNOS (Figure 2B, 2C).

Protein expression of iNOS, nNOS, and eNOS was significantly increased by HH without reoxygenation ($P < 0.001$, $P < 0.05$, $P < 0.05$, Figure 2D-2G). After 1 day of reoxygenation, iNOS and nNOS protein levels increased further ($P < 0.001$, $P < 0.01$, Figure 2E, 2F). After 3 days of reoxygenation, the iNOS and nNOS expression were significantly increased ($P < 0.001$, $P < 0.05$). Treatment with 1400W significantly inhibited the induction of iNOS expression after HH, and after 1 and 3 days of reoxygenation ($P < 0.05$, $P < 0.001$, $P < 0.01$, Figure 2D, 2E).

After acute HH exposure followed by 1 day of reoxygenation, Iba-1⁺ cell fluorescence intensity in rat cerebral cortex were higher than that in the HH alone group ($P < 0.001$, Figure 3A, 3B). This increase was inhibited by 1400W treatment ($P < 0.05$). However, Iba-1⁺ cell fluorescence intensities in the 1400W treatment group were higher than those in the HH alone group ($P < 0.01$). In addition, iNOS⁺ cell fluorescence intensities in rat cerebral cortex were higher than those in the HH alone group ($P < 0.001$, Figure 3A, 3C). This increase was inhibited by 1400W treatment ($P < 0.001$). However, iNOS⁺ cell fluorescence intensity in the 1400W treatment group were higher than those in the HH alone group ($P < 0.001$). Most importantly, iNOS expression was mainly located in Iba-1⁺ cells.

NO levels in the HH group was lower compared with the control group (ctl, $P < 0.05$, Figure 4A). After reoxygenation of 1 or 3 days, the NO level increased ($P < 0.001$, $P < 0.001$); this increase was inhibited by treatment with 1400W ($P < 0.001$, $P < 0.001$). 3-NT levels were higher in the hypoxia group after 1 or 3 days of reoxygenation compared to the control ($P < 0.001$, $P < 0.01$, Figure 4B); this increase

was inhibited by 1400W treatment ($P < 0.001$, $P < 0.01$). MDA levels were higher in the hypoxia group after 0, 1 or 3 days of reoxygenation compared to the control ($P < 0.05$, $P < 0.001$, $P < 0.01$, Figure 4C). This increase in MDA was inhibited by 1400W treatment after 1 day of reoxygenation ($P < 0.01$).

Caspase-3 and Bax expression levels were higher after HH treatment as compared to control ($P < 0.001$ to 0.05 , Figure 4D-4F). With 1 and 3 days of reoxygenation, caspase-3 levels in the 1400W group were lower than the vehicle group ($P < 0.01$, $P < 0.05$ respectively, Figure 4D, 4E). After 0 or, 1 day reoxygenation, Bax levels of the 1400W group were lower than the vehicle group ($P < 0.05$, $P < 0.01$, Figure 4D, 4F).

After acute HH exposure followed by 1 day of reoxygenation, the number of Caspase-3⁺ neuronal cells in the rat cerebral cortex was higher than that in the HH alone group ($P < 0.001$, Figure 5A, 5B). This increase was inhibited by 1400W treatment ($P < 0.01$). However, the number of Caspase-3⁺ neuronal cells in the 1400W treatment group remained higher than that in the HH alone group ($P < 0.01$). In addition, besides neuronal cells, other cell types also expressed Caspase-3.

3.3. Effect of 1400W treatment during H/R on NOS expression, NO, 3-NT, and MDA production, and apoptosis in primary adult microglia

We assessed primary microglia viability after treatment with 1400W (20, 40, 60, 80, and 100 μ M) using an MTT assay. At concentrations of up to 100 μ M of 1400W had no significant impact on the viability of microglia (Figure 6A). We then examined the effect of 1400W pretreatment on NOS mRNA expression in primary microglia after 12h hypoxia and after 3 or 12 h of reoxygenation. The level of iNOS mRNA after hypoxia (0 h) or after hypoxia with reoxygenation for 3 or 12 h was significantly increased ($P < 0.05$, $P < 0.001$, $P < 0.001$ respectively, Figure 6B). Levels of nNOS mRNA were significantly increased at the same time points ($P < 0.01$, $P < 0.001$, $P < 0.001$ respectively, Figure 6C). Levels of eNOS mRNA were not statistically different (data not shown).

Treatment with 1400W prevented the increase in iNOS mRNA level after hypoxia

or H/R for 3 or 12 h ($P < 0.05$, $P < 0.01$, $P < 0.01$ respectively, Figure 3B), but did not affect eNOS mRNA levels (Figure 6C).

Hypoxia (0 h) or after H/R for 6 or 24h, significantly increased iNOS ($P < 0.01$, $P < 0.001$, $P < 0.001$ respectively, Figures 6D, 6E), and nNOS expression ($P < 0.01$, $P < 0.05$, $P < 0.05$ respectively, Figures 6D, 6F). Expression of eNOS protein was not statistically different (data not shown). Treatment with 1400W prevented the increase in iNOS protein ($P < 0.05$, $P < 0.001$, $P < 0.001$ respectively, Figures 6D, 6E) but had no effect on nNOS levels (Figure 6F).

Hypoxia alone reduced NO levels in primary microglia ($P < 0.01$, Figure 7A); after 6 or 24 h reoxygenation the NO level increased ($P < 0.001$). Treatment with 1400W significantly reduced NO levels after reoxygenation ($P < 0.001$). 3-NT levels after hypoxia were higher than those in the control group after 6 and 24 h of reoxygenation ($P < 0.001$, Figure 7B); 1400W treatment significantly reduced 3-NT levels ($P < 0.01$, $P < 0.001$). MDA levels after hypoxia were higher than the control group after 6 and 24 h of reoxygenation ($P < 0.001$, Figure 7C); 1400W treatment significantly reduced MDA levels ($P < 0.001$).

After hypoxia or 6 or 24 h of reoxygenation, apoptosis in hypoxia groups was higher than the control group ($P < 0.05$, $P < 0.001$, $P < 0.001$, Figure 7D, 7E). Treatment with 1400W significantly reduced cell apoptosis at these time points ($P < 0.05$). With hypoxia or H/R 6 or 24 h, caspase-3 expression increased compared to the control group ($P < 0.001$, Figure 7F-7H); 1400W treatment prevented this increase ($P < 0.05$, $P < 0.01$, $P < 0.01$ respectively). Bax protein was significantly increased after reoxygenation for 6 or 24 ($P < 0.001$), but Bax protein levels decreased after treatment with 1400W ($P < 0.001$, $P < 0.01$ respectively).

4. Discussion

In the present study, we used acute hypobaric hypoxia-reoxygenation (HH/R) to generate a spatial memory deficit model. Rats exposed to acute HH/R exhibited spatial memory deficits in a MWM test, and reduced exploratory behavior in a Y-maze test. This indicated that after hypoxia-reoxygenation, the spatial memory and

discriminatory ability of the brain was lowered. This impairment appeared after 1 day of reoxygenation, similar to previous reports ^[11,47]. Pretreatment with the highly selective iNOS inhibitor 1400W improved the spatial memory deficit in rats caused by acute HH/R, similar to the result that iNOS inhibitor aminoguanidine improves rat cerebral retrograde memory disorders caused by acute hypobaric hypoxia reported in the literature ^[11]. Furthermore, we investigated the transcription and translation of iNOS, nNOS, and eNOS in rat cerebral cortex after acute HH/R. We found that the iNOS, nNOS and eNOS mRNA level reached a peak 1 d after acute HH/R and then began to decrease; only eNOS mRNA was restored to the normoxic level after 3 days of reoxygenation. The expression of iNOS protein reached a peak 1 day after acute HH/R, and then began to decrease; nNOS protein levels responded similarly to iNOS, but the change in nNOS protein was smaller. The eNOS protein level reached a peak during hypoxia and then dropped to the normoxic level with reoxygenation. Although 1400W pretreatment significantly decreased iNOS mRNA and protein levels, it had no significant inhibitive effect on nNOS or eNOS mRNA and protein expression. Further experiments showed that cerebral cortex microglial cells were activated by acute HH/R, and iNOS protein expression was mainly in activated microglial cells, which indicated that the increased expression of iNOS in the cerebral cortex induced by acute HH/R is mainly due to activated microglia cells. 1400W could inhibit but not completely suppress microglia activation and iNOS expression, which is consistent with the results from western blot analysis. The reason for these observations could be an insufficient dose of 1400W that did not completely inhibit iNOS overexpression.

We investigated the level of NO produced in cerebral cortex after acute HH/R. NO levels were initially reduced by hypoxia, which may be because insufficient oxygen levels reduce the formation of NO from L-arginine. After 1 day of reoxygenation, the level of NO was significantly increased, similarly to results reported in previous studies ^[11,12]. 1400W pretreatment significantly reduced the levels of NO in rat cerebral cortex tissue after 1 day of reoxygenation back to baseline levels, although it was slightly higher compared the normoxic group, it was not statistically different. Because of the highly selective inhibition of 1400W on iNOS,

conventional doses will not affect the biological activity of nNOS and eNOS, so the experiment results showed that NO produced from acute hypobaric HH/R mainly came from iNOS activity, as which was the same as those reported in previously literature reported ^[14,20,21].

We then investigated the effect of acute HH/R on oxidative stress and apoptosis in cerebral cortex of rats. In the oxidative stress response, 3-NT modification of proteins is a marker of protein damage by ONOO⁻, which is formed by the reaction of nitric oxide. MDA is a product of membrane lipid peroxidation. In the present study, levels of 3-NT and MDA increased gradually after acute HH/R compared with those in the normoxic group, as reported in previous studies ^[12,13,48-51]. 1400W pretreatment could significantly lower the 3-NT and MDA level in cortical tissue after 1 day of reoxygenation, which indicated that inhibiting NO generation interrupted production of ONOO⁻, thereby reducing ONOO⁻ related damage to the proteins and cell membrane. However, it was interesting that after 3 days of reoxygenation, there was a high level of MDA in the cortical tissue that was not reduced by 1400W. This may be due other oxygen free radicals besides ONOO⁻. We also found that the caspase-3 and Bax levels, and caspase-3⁺ neuronal cells after acute HH/R increased gradually compared to the control group, consistent with previous reports ^[8,52]. Our study also found that, in addition to neuronal cells, other cell types in the cerebral cortex also underwent apoptosis, which was ameliorated by 1400W treatment. These results demonstrated that acute HH/R could induce cell apoptosis in the cerebral cortex, especially in neuronal cells, and that 1400W pretreatment significantly inhibited apoptosis, indicating that inhibition of NO generation interrupted ONOO⁻ emergence as well as reducing nucleic acid damage and apoptosis induced by ONOO⁻.

Activated microglia have been proposed as the largest source of endogenous NO in the brain after hypoxia-reoxygenation ^[20,21], so we established a primary microglia hypoxia-reoxygenation model to observe the impact of 1400W on NOS expression and NO generation. We found that the iNOS and nNOS mRNA levels increased after hypoxia, and began to decrease after 3 h of reoxygenation. We found that iNOS protein increased after hypoxic exposure and kept increasing after reoxygenation;

nNOS protein followed a similar trend with a smaller response. The changes in the transcription of iNOS and nNOS and their protein expression in microglia after hypoxia-reoxygenation induction observed were consistent with previous results with the hypoxia-reoxygenation model, the OGD/R model and the intermittent hypoxia model [24,53-55]. At the same time, we observed that eNOS was not expressed in microglia, supporting findings in the literature [56]. 1400W pretreatment significantly lowered iNOS mRNA transcription and protein expression, but had no effect on nNOS mRNA or protein expression level, once again supporting a highly selective effect of 1400W on iNOS [57-59].

Hypoxia reduced production of NO through reduced synthesis from arginine, and the level of NO was significantly increased after reoxygenation. 1400W pretreatment significantly reduced the level of NO products in microglia after reoxygenation. Although it was slightly higher compared with the normoxic group, the NO level was not statistically different, supporting the hypothesis that microglial activation by hypoxia-reoxygenation results in production of NO by iNOS activity.

We further investigated the effect of hypoxia-reoxygenation on the oxidative stress and cell apoptosis of primary microglia cells. We found that the 3-NT, MDA level, and apoptosis rate, and the levels of apoptosis factors caspase-3 and Bax increased gradually after hypoxia-reoxygenation compared with the normoxic group; these increases were inhibited by 1400W treatment, indicating that inhibiting the production of NO reduced the generation of ONOO⁻ and the cellular damage caused by excessive ONOO⁻. Interestingly, after 1400W pretreatment, caspase-3 and Bax levels decreased to the level of normoxic group, but the apoptosis rate was still higher than the normoxic group. This seems contradictory, and requires further exploration in future studies. In summary, the excessive NO generated from microglia during hypoxia-reoxygenation could cause oxidative damage and apoptosis. 1400W pretreatment largely prevented such cellular damage by inhibiting iNOS overexpression and excessive NO generation.

Of the currently available iNOS inhibitors, 1400W is likely the most potent and selective, and is a small, BBB permeable molecule [60,61]. 1400W could suppress

abnormal levels of NO in many models of rodent brain injury such as focal cerebral ischaemia ^[34,62], traumatic brain injury ^[63], and temporal lobe epilepsy ^[61]. In our preliminary studies, we tested different doses of 1400W in rats, by using the Morris water maze for cognitive functions, and confirmed that 20 mg/kg 1400W significantly improved the spatial memory deficit caused by acute HH/R. At this dose, 1400W had no effect on the biological activity of eNOS and nNOS. Thus, we concluded that 1400W has potential neuroprotective effects in acute HH/R.

The preliminary working model is that excessive NO is generated in microglia during hypoxia-reoxygenation. The NO released by microglia would spread to nearby cells around and cause oxidative stress, leading to apoptosis. In future studies, the impact of microglial-generated NO on the neurovascular unit (NVU), including cerebral microvascular endothelial cells, astrocytes, and neurons, thereby providing an analysis of the multiple mechanisms memory deficit after hypoxia-reoxygenation. In addition, this study also ignored the role of nNOS and eNOS in various cells in brain tissue after hypoxia-reoxygenation; we found that the iNOS inhibitor 1400W did not completely resolve oxidative stress and apoptosis, perhaps due to other sources for NO production. We also found that 1400W did not completely inhibit microglial activation after hypoxia-reoxygenation, This could be due to an insufficient dose of 1400W that did not completely suppress the catalytic generation of excessive NO by iNOS. Another possible explanation is that there are other sources of oxygen free-radical production such as superoxide anions or OH radical, which may play a role in the acute HH/R process. In addition, memory is closely related to the function of hippocampal tissue. During our research, no changes of iNOS expressions were observed in the hippocampal tissue; which needs further in-depth study.

Based on the above findings, we now suggest that acute HH/R can cause spatial memory deficits, and the damage mechanism may be microglial iNOS overexpression during hypoxia-reoxygenation, leading to excessive NO production and subsequent oxidative stress and neuronal cell apoptosis in rat cerebral cortex. The highly selective iNOS inhibitor 1400W inhibited overexpression of iNOS in microglia, and to reduce the generation of NO, mitigating oxidative stress and reducing neuronal cell

apoptosis, leading to improved spatial memory after acute hypobaric hypoxia-reoxygenation.

References

- [1] Basnyat B, Murdoch DR, High-altitude illness, *Lancet* 361(2003) 1967-1974.
- [2] de Aquino Lemos V, Antunes HK, dos Santos RV, Lira FS, Tufik S, de Mello MT, High altitude exposure impairs sleep patterns, mood, and cognitive functions, *Psychophysiology* 49(2012) 1298-1306.
- [3] Kramer AF, Coyne JT, Strayer DL, Cognitive function at high altitude, *Hum Factors* 35(1993) 329-344.
- [4] Pavlicek V, Schirlo C, Nebel A, Regard M, Koller EA, Brugger P, Cognitive and emotional processing at high altitude, *Aviat Space Environ Med* 76(2005) 28-33.
- [5] Yan X, Cognitive impairments at high altitudes and adaptation, *High Alt Med Biol* 15(2014) 141-145.
- [6] Maiti P, Singh SB, Mallick B, Muthuraju S, Ilavazhagan G, High altitude memory impairment is due to neuronal apoptosis in hippocampus, cortex and striatum, *J Chem Neuroanat* 36(2008) 227-238.
- [7] Jayalakshmi K, Singh SB, Kalpana B, Sairam M, Muthuraju S, Ilavazhagan G, N-acetyl cysteine supplementation prevents impairment of spatial working memory functions in rats following exposure to hypobaric hypoxia, *Physiol Behav* 92(2007) 643-650.
- [8] Zhou B, Li M, Cao X, Zhang Q, Liu Y, Ma Q, Qiu Y, Luan F, Wang X, Phenylethanoid glycosides of *Pedicularis muscicola* Maxim ameliorate high altitude-induced memory impairment, *Physiol Behav* 157(2016) 39-46.
- [9] Vornicescu C, Bosca B, Crisan D, Yacoob S, Stan N, Filip A, Sovrea A, Neuroprotective effect of melatonin in experimentally induced hypobaric hypoxia, *Rom J Morphol Embryol* 54(2013) 1097-1106.
- [10] Maiti P, Singh SB, Ilavazhagan G, Nitric oxide system is involved in hypobaric hypoxia-induced oxidative stress in rat brain, *Acta Histochem* 112(2010) 222-232.
- [11] Udayabanu M, Kumaran D, Nair RU, Srinivas P, Bhagat N, Aneja R, Katyal A, Nitric oxide associated with iNOS expression inhibits acetylcholinesterase activity and induces memory impairment during acute hypobaric hypoxia, *Brain Res* 1230(2008) 138-149.
- [12] Huang CC, Lai CJ, Tsai MH, Wu YC, Chen KT, Jou MJ, Fu PI, Wu CH, Wei IH, Effects of melatonin on the nitric oxide system and protein nitration in the hypobaric hypoxic rat hippocampus, *BMC Neurosci* 16(2015) 61.
- [13] Encinas JM, Serrano J, Bentura ML, Castro-Blanco S, Fernandez AP, Rodrigo J, Nitric oxide system and protein nitration are modified by an acute hypobaric hypoxia in the adult rat hippocampus, *J Neuropathol Exp Neurol* 62(2003) 863-877.
- [14] Serrano J, Encinas JM, Fernandez AP, Rodrigo J, Martinez A, Effects of acute hypobaric hypoxia on the nitric oxide system of the rat cerebral cortex: Protective role of nitric oxide inhibitors, *Neuroscience* 142(2006) 799-808.
- [15] Garthwaite J, Glutamate, nitric oxide and cell-cell signalling in the nervous system, *Trends Neurosci* 14(1991) 60-67.

- [16] Prast H, Philippu A, Nitric oxide as modulator of neuronal function, *Prog Neurobiol* 64(2001) 51-68.
- [17] Haley JE, Wilcox GL, Chapman PF, The role of nitric oxide in hippocampal long-term potentiation, *Neuron* 8(1992) 211-216.
- [18] Snyder SH, Bredt DS, Biological roles of nitric oxide, *Sci Am* 266(1992) 68-71, 74-67.
- [19] Brune B, Zhou J, Nitric oxide and superoxide: interference with hypoxic signaling, *Cardiovasc Res* 75(2007) 275-282.
- [20] Al-Saleh SS, Kaur C, Ling EA, Response of neurons and microglia/macrophages in the area postrema of adult rats following exposure to hypobaric hypoxia, *Neurosci Lett* 346(2003) 77-80.
- [21] Dheen ST, Kaur C, Ling EA, Microglial activation and its implications in the brain diseases, *Curr Med Chem* 14(2007) 1189-1197.
- [22] Chhor V, Le Charpentier T, Lebon S, Ore MV, Celador IL, Josserand J, Degos V, Jacotot E, Hagberg H, Savman K, Mallard C, Gressens P, Fleiss B, Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro, *Brain Behav Immun* 32(2013) 70-85.
- [23] Hu X, Li P, Guo Y, Wang H, Leak RK, Chen S, Gao Y, Chen J, Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia, *Stroke* 43(2013) 3063-3070.
- [24] Park SY, Lee H, Hur J, Kim SY, Kim H, Park JH, Cha S, Kang SS, Cho GJ, Choi WS, Suk K, Hypoxia induces nitric oxide production in mouse microglia via p38 mitogen-activated protein kinase pathway, *Brain Res Mol Brain Res* 107(2002) 9-16.
- [25] Lu DY, Liou HC, Tang CH, Fu WM, Hypoxia-induced iNOS expression in microglia is regulated by the PI3-kinase/Akt/mTOR signaling pathway and activation of hypoxia inducible factor-1alpha, *Biochem Pharmacol* 72(2006) 992-1000.
- [26] Pryor WA, Squadrito GL, The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide, *Am J Physiol* 268(1995) L699-722.
- [27] Wei T, Chen C, Zhao B, Xin W, Mori A, EPC-K1 attenuates peroxynitrite-induced apoptosis in cerebellar granule cells, *Biochem Mol Biol Int* 46(1998) 89-97.
- [28] Boczkowski J, Lisdero CL, Lanone S, Carreras MC, Aubier M, Poderoso JJ, Peroxynitrite-mediated mitochondrial dysfunction, *Biol Signals Recept* 10(2001) 66-80.
- [29] Brown GC, Borutaite V, Nitric oxide, cytochrome c and mitochondria, *Biochem Soc Symp* 66(1999) 17-25.
- [30] Siu AW, Maldonado M, Sanchez-Hidalgo M, Tan DX, Reiter RJ, Protective effects of melatonin in experimental free radical-related ocular diseases, *J Pineal Res* 40(2006) 101-109.
- [31] Garvey EP, Oplinger JA, Furfine ES, Kiff RJ, Laszlo F, Whittle BJ, Knowles RG, 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo, *J Biol Chem* 272(1997) 4959-4963.
- [32] Boer R, Ulrich WR, Klein T, Mirau B, Haas S, Baur I, The inhibitory potency and selectivity of arginine substrate site nitric-oxide synthase inhibitors is solely determined by their affinity toward the different isoenzymes, *Mol Pharmacol* 58(2000) 1026-1034.
- [33] Pigott B, Bartus K, Garthwaite J, On the selectivity of neuronal NOS inhibitors, *Br J Pharmacol* 168(2013) 1255-1265.
- [34] Parmentier S, Bohme GA, Lerouet D, Damour D, Stutzmann JM, Margail I, Plotkine M,

- Selective inhibition of inducible nitric oxide synthase prevents ischaemic brain injury, *Br J Pharmacol* 127(1999) 546-552.
- [35] Morris R, Developments of a water-maze procedure for studying spatial learning in the rat, *J Neurosci Methods* 11(1984) 47-60.
 - [36] Javadi-Paydar M, Zakeri M, Norouzi A, Rastegar H, Mirazi N, Dehpour AR, Involvement of nitric oxide in granisetron improving effect on scopolamine-induced memory impairment in mice, *Brain Res* 1429(2012) 61-71.
 - [37] Hansen F, Pandolfo P, Galland F, Torres FV, Dutra MF, Batassini C, Guerra MC, Leite MC, Goncalves CA, Methylglyoxal can mediate behavioral and neurochemical alterations in rat brain, *Physiol Behav* 164(Pt A)(2016) 93-101.
 - [38] Mohammadi F, Rahimian R, Fakhraei N, Sorkhabadi SM, Paydar MJ, Dehpour AR, Afshari K, Ejtemaei-Mehr S, Effect of glatiramer acetate on short-term memory impairment induced by lipopolysaccharide in male mice, *Fundam Clin Pharmacol* [Epub ahead of print](2016).
 - [39] Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal Biochem* 72(1976) 248-254.
 - [40] Kinsella S, Konig HG, Prehn JH, Bid Promotes K63-Linked Polyubiquitination of Tumor Necrosis Factor Receptor Associated Factor 6 (TRAF6) and Sensitizes to Mutant SOD1-Induced Proinflammatory Signaling in Microglia, *eNeuro* 3(2016) pii: ENEURO.0099-0015.2016.
 - [41] Suzumura A, Sawada M, Yamamoto H, Marunouchi T, Effects of colony stimulating factors on isolated microglia in vitro, *J Neuroimmunol* 30(1990) 111-120.
 - [42] Huang J, Zhang X, Qin F, Li Y, Duan X, Jian J, Chen J, Huang R, Protective effects of *Millettia pulchra* flavonoids on myocardial ischemia in vitro and in vivo, *Cell Physiol Biochem* 35(2015) 516-528.
 - [43] Fu J, Cao H, Wang N, Zheng X, Lu Y, Liu X, Yang D, Li B, Zheng J, Zhou H, An anti-sepsis monomer, 2',5,6',7-tetrahydroxyflavanonol (THF), identified from *Scutellaria baicalensis* Georgi neutralizes lipopolysaccharide in vitro and in vivo, *Int Immunopharmacol* 8(2008) 1652-1657.
 - [44] Nussler AK, Glanemann M, Schirmeier A, Liu L, Nussler NC, Fluorometric measurement of nitrite/nitrate by 2,3-diaminonaphthalene, *Nat Protoc* 1(2006) 2223-2226.
 - [45] de Oliveira MR, Lorenzi R, Schnorr CE, Morrone M, Moreira JC, Increased 3-nitrotyrosine levels in mitochondrial membranes and impaired respiratory chain activity in brain regions of adult female rats submitted to daily vitamin A supplementation for 2 months, *Brain Res Bull* 86(2011) 246-253.
 - [46] Dousset JC, Trouilh M, Foglietti MJ, Plasma malonaldehyde levels during myocardial infarction, *Clin Chim Acta* 129(1983) 319-322.
 - [47] Shukitt-Hale B, Stillman MJ, Welch DI, Levy A, Devine JA, Lieberman HR, Hypobaric hypoxia impairs spatial memory in an elevation-dependent fashion, *Behav Neural Biol* 62(1994) 244-252.
 - [48] Serrano J, Encinas JM, Salas E, Fernandez AP, Castro-Blanco S, Fernandez-Vizarra P, Bentura ML, Rodrigo J, Hypobaric hypoxia modifies constitutive nitric oxide synthase activity and protein nitration in the rat cerebellum, *Brain Res* 976(2003) 109-119.
 - [49] Shrivastava K, Shukla D, Bansal A, Sairam M, Banerjee PK, Ilavazhagan G, Neuroprotective effect of cobalt chloride on hypobaric hypoxia-induced oxidative stress, *Neurochem Int*

52(2008) 368-375.

- [50] Barhwal K, Singh SB, Hota SK, Jayalakshmi K, Ilavazhagan G, Acetyl-L-carnitine ameliorates hypobaric hypoxic impairment and spatial memory deficits in rats, *Eur J Pharmacol* 570(2007) 97-107.
- [51] Maiti P, Singh SB, Sharma AK, Muthuraju S, Banerjee PK, Ilavazhagan G, Hypobaric hypoxia induces oxidative stress in rat brain, *Neurochem Int* 49(2006) 709-716.
- [52] Prasad J, Baitharu I, Sharma AK, Dutta R, Prasad D, Singh SB, Quercetin reverses hypobaric hypoxia-induced hippocampal neurodegeneration and improves memory function in the rat, *High Alt Med Biol* 14(2013) 383-394.
- [53] Mana L, Wang S, Zhu H, Xing Y, Lou L, Wu A, Dong B, Sun Y, Yang S, Wang L, Gao Y, Qingkailing Suppresses the Activation of BV2 Microglial Cells by Inhibiting Hypoxia/Reoxygenation-Induced Inflammatory Responses, *Evid Based Complement Alternat Med* 2014(2014) 696218.
- [54] Chern CM, Liou KT, Wang YH, Liao JF, Yen JC, Shen YC, Andrographolide inhibits PI3K/AKT-dependent NOX2 and iNOS expression protecting mice against hypoxia/ischemia-induced oxidative brain injury, *Planta Med* 77(2011) 1669-1679.
- [55] Smith SM, Friedle SA, Watters JJ, Chronic intermittent hypoxia exerts CNS region-specific effects on rat microglial inflammatory and TLR4 gene expression, *PLoS One* 8(2013) e81584.
- [56] Ding J, Li QY, Wang X, Sun CH, Lu CZ, Xiao BG, Fasudil protects hippocampal neurons against hypoxia-reoxygenation injury by suppressing microglial inflammatory responses in mice, *J Neurochem* 114(2010) 1619-1629.
- [57] Patel P, Qi WN, Allen DM, Chen LE, Seaber AV, Stamler JS, Urbaniak JR, Inhibition of iNOS with 1400W improves contractile function and alters nos gene and protein expression in reperfused skeletal muscle, *Microsurgery* 24(2004) 324-331.
- [58] Murugan M, Sivakumar V, Lu J, Ling EA, Kaur C, Expression of N-methyl D-aspartate receptor subunits in amoeboid microglia mediates production of nitric oxide via NF-kappaB signaling pathway and oligodendrocyte cell death in hypoxic postnatal rats, *Glia* 59(2011) 521-539.
- [59] He YF, Chen HJ, Qian LH, Chen GY, Effect of 1400W on blocking lipopolysaccharide-induced microglial toxicity to preoligodendrocytes, *World J Pediatr* 6(2010) 249-254.
- [60] Babu BR, Griffith OW, Design of isoform-selective inhibitors of nitric oxide synthase, *Curr Opin Chem Biol* 2(1998) 491-500.
- [61] Puttachary S, Sharma S, Verma S, Yang Y, Putra M, Thippeswamy A, Luo D, Thippeswamy T, 1400W, a highly selective inducible nitric oxide synthase inhibitor is a potential disease modifier in the rat kainate model of temporal lobe epilepsy, *Neurobiol Dis* 93(2016) 184-200.
- [62] Perez-Asensio FJ, Hurtado O, Burguete MC, Moro MA, Salom JB, Lizasoain I, Torregrosa G, Leza JC, Alborch E, Castillo J, Knowles RG, Lorenzo P, Inhibition of iNOS activity by 1400W decreases glutamate release and ameliorates stroke outcome after experimental ischemia, *Neurobiol Dis* 18(2005) 375-384.
- [63] Jafarian-Tehrani M, Louin G, Royo NC, Besson VC, Bohme GA, Plotkine M, Marchand-Verrecchia C, 1400W, a potent selective inducible NOS inhibitor, improves histopathological outcome following traumatic brain injury in rats, *Nitric Oxide* 12(2005) 61-69.

Figure legends:

Figure 1: 1400W ameliorated spatial memory deficits in Morris water maze and Y-maze after hypobaric hypoxia and reoxygenation.

Representative Morris water maze (MWM) analyses for number of times of crossing the former platform location (A) and percentage of swim time in the target quadrant (B) are shown. Representative Y-maze analyses for duration of novel arm visits (C) and number of novel arm visits (D) are shown. Black columns indicate vehicle pretreatment, and grey column indicate 1400W pretreatment. Data are expressed as mean $\pm$ SD (n = 8 for each group). * P < 0.05, ** P < 0.01 and *** P < 0.001 vs. the vehicle group or the indicated group. # P < 0.05, ## P < 0.01 and ### P < 0.001 vs. the vehicle normoxic (ctl) group.

Figure 2: 1400W inhibited iNOS expression after hypobaric hypoxia and reoxygenation.

Representative qRT-PCR analyses for (A) iNOS mRNA, (B) nNOS mRNA, and (C) eNOS mRNA levels are shown. (D) Representative Western blot for iNOS, nNOS, and eNOS expression in lysates from cerebral cortex is shown. Statistical analysis of the relative values of (E) iNOS, (F) nNOS, and (G) eNOS in normoxic (ctl) and HH/R group. Black columns indicate vehicle pretreatment, and grey column indicate 1400W pretreatment. Data are expressed as mean $\pm$ SD. * P < 0.05, ** P < 0.01 and *** P < 0.001 vs. the indicated group. # P < 0.05, ## P < 0.01 and ### P < 0.001 vs. the vehicle normoxic (ctl) group.

Figure 3: 1400W inhibited microglial cell activation and iNOS expression after hypobaric hypoxia and reoxygenation.

(A) Immunocytochemical staining of Iba-1 and iNOS in rat cerebral cortex treated with vehicle or 1400W, and exposed to acute HH or HH with 1 day of reoxygenation. Statistical analysis of the mean intensity of (B) Iba-1⁺ green fluorescence and (C) iNOS⁺ red fluorescence in the HH, HH/R, and 1400W+HH/R groups. DAPI was used

to counterstain nuclei. Data are expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. the HH group. # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs. the indicated group.

Figure 4: 1400W reduced NO, 3-NT, and MDA production, and prevented apoptosis after hypobaric hypoxia and reoxygenation.

Representative fluorometric analysis for (A) NO production levels and colorimetric analysis for (B) 3-NT, (C) MDA production levels in homogenates of rat cerebral cortex are shown. (D) Representative western blot for caspase-3 and Bax expression in cerebral cortex tissue lysates. Statistical analysis of the relative values of (E) caspase-3 and (F) Bax in the normoxic and HH/R groups. Black columns indicate vehicle pretreatment and grey columns indicate 1400W pretreatment. Data are expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. the indicated group. # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ vs. the vehicle normoxic (ctl) group.

Figure 5: 1400W inhibited neuronal cell apoptosis after hypobaric hypoxia and reoxygenation.

(A) Immunocytochemical staining of caspase-3 and NeuN in rat cerebral cortex treated with vehicle or 1400W, and exposed to acute HH or HH with 1 day of reoxygenation. (B) Statistical analysis of the ratio between (caspase-3⁺ and NeuN⁺)/NeuN⁺ cells in the HH, HH/R, and 1400W+HH/R groups. Data are expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. the HH group. # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ vs. the indicated group.

Figure 6: 1400W inhibited iNOS expression in primary adult microglia exposed to normoxia or hypoxia and reoxygenation.

(A) Cell viability of primary microglia incubated in the presence or absence of 1400W (20, 40, 60, 80, and 100 μ M) for 24 h was measured using an MTT assay. 1400W did not show any microglia cytotoxicity at concentrations of up to 100 μ M.

Representative qRT-PCR analyses for (A) iNOS mRNA and (B) nNOS mRNA levels are shown. (D) Representative Western blot for iNOS and nNOS expression in lysates from microglia is shown. (E, F) Statistical analysis of the relative values of (E) iNOS and (F) nNOS in ctl and H/R group. Black columns indicate vehicle pretreatment, and grey column indicate 1400W pretreatment. Each data point represents the mean $\pm$ SD of triplicate samples from one experiment. The experiment was repeated three times on different cell preparations with similar results. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. the indicated group. # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ vs. the vehicle normoxic (ctl) group.

Figure 7: 1400W reduced NO, 3-NT, and MDA production, and inhibited apoptosis in primary adult microglia exposed to normoxia or hypoxia and reoxygenation.

(A) Representative fluorometric analysis for NO production levels and colorimetric analysis for (B) 3-NT, and (C) MDA production levels in cell lysates from microglia. (D) Representative flow cytometry plots with Annexin V/FITC and PI staining analyses for apoptosis. (E) Statistical analysis of the percentage of apoptosis in ctl and H/R group. Apoptosis ratio was defined as the ratio between Annexin V positive/PI negative cells (right lower quadrant) and the total cells. (F) Representative western blot for caspase-3 and Bax expression in microglia lysates. Statistical analysis of the relative values of (G) Caspase-3 and (H) Bax in ctl and H/R groups. Black columns indicate vehicle pretreatment and grey columns indicate 1400W pretreatment. Each data point represents the mean $\pm$ SD of triplicate samples from one experiment. The experiment was repeated three times with different cell preparations with similar results. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. the indicated group. # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ vs. the vehicle normoxic (ctl) group.

Figure 1

A

B

C

D

Figure 2

Figure 3

B

C

Figure 4

Figure 5

Figure 6

Figure 7