Neurochemistry International 129 (2019) 104461

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Neuroprotection mediated by remote preconditioning is associated with a decrease in systemic oxidative stress and changes in brain and blood glutamate concentration

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ARTICLE INFO

Keywords:
Brain ischemia
Remote ischemic preconditioning
Glutamate
Systemic oxidative stress
Blood
Neuroprotection

ABSTRACT

It has been shown that ischemia of remote organs can generate resistance to ischemic conditions within sensitive brain tissues. However, only limited information about its mechanism is available. In the present paper, we used hind-limb ischemia by tourniquet to generate early remote ischemic tolerance in rats. The main objective was to investigate the role of glutamate in the process of neuroprotection and discover parameters that are affected in the blood of ischemia-affected animals. Our results showed that pretreatment with a hind-limb tourniquet caused a decrease in neurodegeneration by about 30%. However, we did not observe neurological deficit recovery. When compared to ischemia, glutamate concentration decreased in all observed brain regions (cortex, CA1 and dentate gyrus of hippocampus), regardless of their sensitivity to blood restrictions. In contrast to this, the blood levels raised significantly from 26% to 29% during the first four days of postischemic reperfusion. Pretreatment of animals reduced systemic oxidative stress—as represented by lymphocytic DNA damage—by about 80%, while changes in blood antioxidant enzymes (catalase, superoxide dismutase) were not detected.

With these data we can further hypothesize that hind-limb-tourniquet preconditioning could accelerate brain-to-blood efflux of glutamate which could positively impact neuronal survival of ischemia-affected brain regions. Moreover, remote preconditioning improved systemic oxidative stress and did not seem to be affected by enzymatic antioxidant defenses in the blood.

1. Introduction

In Europe, 1.1 million people suffer from strokes each year. The most common subtype of stroke is ischemic, which accounted for 80% of the all cases (Bejot et al., 2016). The effort to find a clinical solution to negative stroke outcomes is considerable. The Internet Stroke Center at Washington University's School of Medicine registered more than 5,000 records in the stroke trial registry (Internet Stroke Center, 2018). The number of experimental stroke studies is even higher. However, there is currently no definitive therapy for a stroke victim.

Recently, induction of ischemic tolerance—i.e., application of sublethal stresses of different natures—has shown great potential in the improvement of brain ischemia outcomes in several animal models. Tolerance-inducing stress in the form of sublethal ischemia, hypothermia, hind-limb ischemia and other techniques could be applied before (pre-), during (per-) or after (post-) lethal ischemia conditioning, followed by significantly improved neuronal survival (reviewed in Zhao et al., 2012). Remote ischemic tolerance (RIT) is very attractive for clinical practice because the tolerance of the brain to ischemic conditions is induced by ischemia of remote organs, usually skeletal muscles in limbs. This potential for conditioning patients is even higher, considering the finding that blood cells are the source of the tolerance and stimulated blood could be transferred from a donor with ischemic tolerance to an at-risk recipient to ensure the initiation of the neuroprotective mechanism in the target patient (Bonova and Gottlieb, 2015). Improved neuronal survival in conditioned animals is associated with a massive decline in excitotoxicity and oxidative stress. Enzymatic antioxidant defenses are preserved in animals with induced tolerance (Danielisova et al., 2005). Similarly, the glutamate content in the hippocampus and cortex drops dramatically after induction of ischemic tolerance, regardless of different levels of sensitivity to ischemia (Bonova et al., 2013a).

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https://doi.org/10.1016/j.neuint.2019.05.005
Received 26 March 2019; Received in revised form 30 April 2019; Accepted 7 May 2019
Available online 13 May 2019
0197-0186/

It is well known that ischemia generates changes that are detectable in peripheral blood. The blood level of glutamate rises significantly, even in the early stages of post-ischemic reperfusion (Kravcukova et al., 2009); this could be responsible for the increase in ROS production. Elevated levels of ROS in the blood was confirmed by an increased rate of lipid peroxidation (Ljubisavljevic et al., 2016; Lorente et al., 2016) and DNA damage in peripheral lymphocytes (Kravcukova et al., 2009, 2010; Sivonova et al., 2008). Antioxidant defenses in the blood were altered, reflecting increased oxidative stress in the animal models (Devyatov et al., 2017; Kravcukova et al., 2009), as well as patients (Zitnanova et al., 2016).

Whereas most of the preclinical treatments reported substantial improvements for these subjects, clinical interventions were much less successful. The poor translation of preclinical stroke studies to clinical therapies is attributed to the precise definition of the time window of treatment, the use of relevant biomarker end points and good laboratory practice (Fisher et al., 2009). Clinical use of ischemic tolerance induction is restricted due to the limited information about its mechanism. In the present paper, we used a model of early remote preconditioning by hind-limb ischemia in order to improve the translational potential of this technique. Similar to brain ischemia's reflection in blood findings, it is logical to theorize that induction of ischemic tolerance or any kind of therapy would reflect on the level of systemic circulation as well. With an effort to identify those valuable biomarkers, we focused mainly on investigating peripheral blood parameters of oxidative stress and enzymatic antioxidant defense. Moreover, the level of glutamate in blood and brain tissue was determined to clarify its role in ischemic tolerance.

2. Materials and methods

The experiments were carried out in accordance with the protocol for animal care approved by the European Communities Council Directive (2010/63/EU) with permission of the State Veterinary and Food Administration of the Slovak Republic (4451/14–221 and 4247/15–221) under the supervision of the ethical council of the Institute of Neurobiology SAS. Every effort was made to minimize animal suffering and reduce the number of animals used. Adult male albino Wistar rats (bred at a certified vivarium of the Institute of Neurobiology SAS originating from Velaz, Czech Republic) weighing 330–350 g were maintained on a 12 h light/dark cycle and given food and water *ad libitum*. Food was withdrawn one day before surgery.

2.1. Design of experiments

Experimental outline (summarized in Fig. 1) was divided into the two parts. The first was focused on analysis of biochemical parameters in the blood after 10 min long transient global ischemia (ischemia, n = 8) and preconditioning (ischemic tolerance, n = 8) (Fig. 1A). Blood was collected after 1 h (R1h) and every day from the first to the seventh day of post-ischemic reperfusion (R1d- R7d). Sham controls (SHC) were collected directly before ischemia. The blood samples were analyzed to determine activity of antioxidant enzymes (superoxide dismutase and catalase) and blood concentration of glutamic acid.

The second part of experiment was dedicated to analyzing glutamate levels and neurodegeneration in brain tissue, and systemic oxidative stress after global ischemia (ischemia) and preconditioning (ischemic tolerance) (Fig. 1B). Three groups with 3 time intervals of survival (one day- R1d; five days- R5d and seven days- R7d) were designed. Two of them were subjected to 10 min of global ischemia, followed by a relevant number of days of reperfusion, with (ischemic tolerance, n = 8 animals for each time interval) or without hind-limb tourniquet preconditioning (ischemia; n = 8 animals for each time interval). The third group represents the sham control counterparts to the ischemia and ischemic tolerance groups (SHC, n = 8 for each time interval). The samples of the brain tissue were used for determination of brain glutamate concentration (cortex, CA1 and dentate gyrus of hippocampus), neurodegeneration (Fluoro-Jade B staining of CA1 hippocampal region) and the blood samples for evaluation of systemic oxidative stress by Comet assay. Behavioural testing was performed on the animals that survived 7 days.

2.2. Ischemia model

For induction of transient forebrain (global) ischemia we used the model of four-vessel occlusion by Pulsinelli and Brierley (1979). Briefly, the rats were anaesthetized with 4% isoflurane in the anaesthetic cage and then maintained during surgery with 1.5% isoflurane. Both vertebral arteries were permanently occluded by electrocoagulation through each alar foramen on the first day. The next day, global ischemia was completed by occlusion of the common carotid arteries with atraumatic clips. After 10 min of ischemia, blood flow was restored by releasing the clips; blood flow was verified visually. Normothermic conditions (approximately 37 °C) were maintained by a feedback-controlled heating lamp and pad during all surgical procedures. Criteria for forebrain ischemia were bilateral loss of the righting reflex within 20 s after clip tightening, paw extension and mydriasis. Animals which did

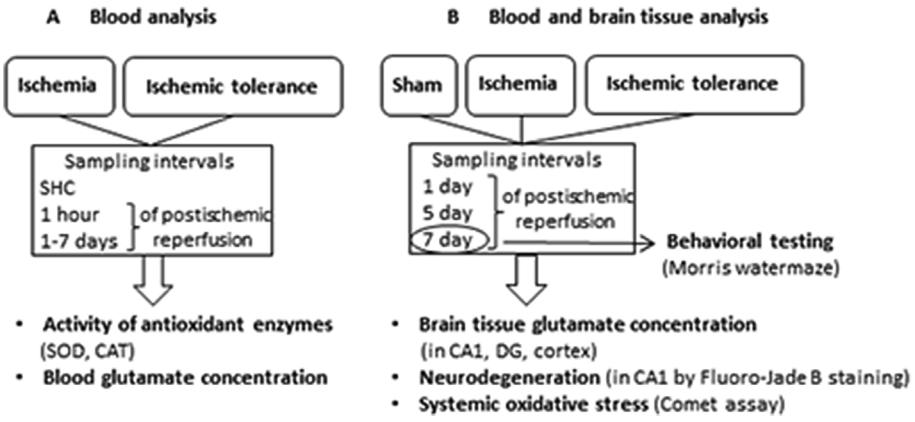

Fig. 1. Schematic drawing of the experimental design. A) Blood analysis. Two groups of animals were subjected to 10 min of global ischemia followed by seven days of reperfusion with (ischemic tolerance; n = 8) or without hind-limb tourniquet preconditioning (ischemia; n = 8). Blood was collected after 1 h and every day from the first to the seventh day of post-ischemic reperfusion (R1h, R1–7d). Sham controls (SHC) were collected directly before ischemia. The blood samples were analyzed to determine activity of antioxidant enzymes (superoxid dismutase- SOD; catalase- CAT) and blood concentration of glutamic acid. B) Blood and brain tissue analysis. Three groups with 3 time intervals of survival (one day- R1d; five days- R5d and seven days- R7d) were designed in this part of experiment. Two of them were subjected to 10 min of global ischemia, followed by a relevant number of days of reperfusion, with (ischemic tolerance, n = 8 animals for each time interval) or without hind-limb tourniquet preconditioning (ischemia; n = 8 animals for each time interval). The third group represents the sham control counterparts to the ischemia and ischemic tolerance groups (sham, n = 8 for each time interval). The samples of the brain tissue were used for determination of brain glutamate concentration (cortex, CA1 and dentate gyrus of hippocampus), neurodegeneration (Fluoro-Jade B staining of CA1 hippocampal region) and the blood samples for evaluation of systemic oxidative stress by Comet assay. Behavioural testing was performed on the animals that survived 7 days.

not fully lose their righting reflexes or who developed seizures after carotid artery occlusion were excluded from the study.

2.3. Tolerance induction

To induce early, remote, preischemic tolerance in experimental animals, a modified hind-limb-tourniquet model based on the work by Hu et al. (2012) was used. One hour before global cerebral ischemia, an elastic rubber-band tourniquet was placed on the proximal part of the right hind-limb for 5 min, followed by 5 min reperfusion during which the tourniquet was untied. The procedure was performed for three cycles.

2.4. Blood sample collection and processing

Samples of whole blood were obtained from the tail vein one hour after induction of ischemia and every day afterward. Sham control samples were collected one day after irreversible occlusion of vertebral arteries, directly before carotid occlusion.

Glutamate was measured in the whole blood. Samples were deproteinized by adding ice-cold 1M perchloric acid in a 1:1 ratio, precipitated for 10 min on the ice and centrifuged at 12000 rpm for 10 min at 4 °C. The supernatant was collected and stored at −80 °C for later analysis.

Blood samples for the measurement of superoxid dismutase (SOD) and catalase (CAT) activity were centrifuged at 12000 rpm for 10 min at 4 °C to separate plasma and blood cells. CAT activity was measured in plasma, and SOD activity was measured in blood cell lysates. The pellet of blood cells was resuspended in distilled water in the volume equivalent to the collected plasma. Samples were rapidly frozen (10 min, −20 °C) and defrosted at 37 °C in three cycles. Samples were centrifuged at 12000 rpm for 10 min at 4 °C. The supernatant representing the blood cell lysate and the plasma samples were stored at −80 °C until analysis.

Lymphocytes assigned for Comet assay analysis were isolated from whole blood (100 µl) and diluted in phosphate buffered saline (PBS) in a ratio of 1:4. Isolation was based on density centrifugation (2000 rpm, 5 min, 4 °C) on Ficoll-PaqueTM Plus gradient. The lymphocyte layer was collected, rewashed in PBS (1:4) and directly used for DNA damage assessment (Single-Cell Gel Electrophoresis).

2.5. Brain tissue processing

The rats survived for 1, 5 and 7 days. Then, animals were anaesthetized by chloral hydrate (300 mg/kg, i.p.) and transcardially perfused with ice-cold saline solution. Their brains were quickly removed and chopped into 2 mm coronal slices. The fourth slice contained hippocampus and was fixed with 10% formaline and used for Fluoro-Jade B staining; the remaining slices were used for the biochemical analysis of the cortical and hippocampal regions. To compare the selectively vulnerable cornu ammonis 1 (CA1) versus the relatively resistant dentate gyrus (DG) and cornu ammonis (CA3) regions, the hippocampus was divided into the CA1 region and the rest of the hippocampus. After the tissue was collected from the cortex, CA1 and DG were weighted, homogenized in homogenization buffer (20 mM Tris–HCl pH 7.5 containing 1 mM DTT, 50 mM magnesium acetate, 140 mM KCl, 1 mM EDTA, 2 mM EGTA with addition of Protease Inhibitor Cocktail Tablets, Roche, Germany) and centrifuged at 12,000 rpm (15 min, 4 °C). Total protein concentrations in the samples were determined using the method described by (Bradford, 1976), and BSA was used to establish a standard curve. Post-mitochondrial supernatant was aliquoted and stored at −80 °C.

For the tissue analysis of glutamate, post-mitochondrial supernatants from the cortical and hippocampal regions were precipitated with 1M ice-cold perchloric acid (1:20) for 10 min at 4 °C, then centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was collected and stored at −80 °C for later analysis.

2.6. Glutamate concentration

The glutamate concentration in the whole blood and brain tissue was measured by the enzymatic–fluorimetric method described by Graham and Aprison (1966) with modifications for the microplate reader (Kravcukova et al., 2009). This method is based on the fluorometric detection of NADH, resulting from the reaction of glutamate and NAD^+ catalyzed by glutamate dehydrogenase. Glutamate concentration is directly proportional to the concentration of NADH in a reaction. Briefly, 10 µl of supernatant was mixed with 190 µl of reaction buffer (0.25 mol/L hydrazine hydrate/0.3 mol/L glycine buffer, pH 8.6) containing 200 nmol/L of NAD^+ and 15 U of glutamate dehydrogenase. After 30 min of incubation at room temperature, fluorescence intensity of the final product of NADH was read in a SynergyTM 2 Multi-mode microplate reader (BioTek) at 460 nm with an excitation wavelength of 360 nm. Blood level of glutamic acid was expressed in µmol per liter of blood (µmol/L); the brain tissue concentration was normalized according to protein content (µmol/mg protein).

2.7. SOD activity

The SOD activity assay was based on the indirect inhibition assay developed by Sun et al. (1988) with modifications for a microplate reader (Kravcukova et al., 2009). This assay involves inhibition of nitro blue tetrazolium (NBT, p-nitrotetrazolium blue grade III, Sigma) reduction by superoxide, with xanthine-xanthine oxidase used as a superoxide generator. It measures absorbance obtained from NBT reduction to blue formazan by superoxide at 560 nm spectrophotometrically at room temperature. SOD in the sample decomposes superoxide and inhibits the rate of blue formazan formation. The standard assay substrate mixture contained in 0.2 ml is as follows: 1 mol/L xanthine (Sigma), 0.1 mol/L EDTA, 5.6×10^2 mol/L NBT and 1 mg/ml BSA (bovine serum albumin, Fluka) in 0.1 mol/L sodium phosphate (pH 7.8). One unit of SOD activity in blood cells was defined as the amount that reduced the absorbance change by 50%. Results were expressed in kU per liter of blood.

2.8. CAT activity

The blood plasma activity of CAT was measured by the spectrophotometric method described by Goth (1991), based on the formation of a stable complex of hydrogen peroxide with ammonium molybdate. Briefly, 20 µl of samples were incubated in 100 µl of substrate (65 µmol/ml hydrogen peroxide in 60 mmol/L sodium–potassium phosphate buffer, pH 7.4) at 37 °C for 60 s. The enzymatic reaction was stopped with 100 µl of 32.4 mmol/L ammonium molybdate, and the yellow complex of molybdate and hydrogen peroxide was measured at 405 nm. One unit of catalase decomposes 1 µmol of hydrogen peroxide in l min under these conditions. CAT activity was expressed in kU per liter of blood.

2.9. Comet assay

DNA damage of the circulating lymphocytes was assessed by single-cell gel electrophoresis (SCGE) according to Singh et al. (1988) with minor modifications. Isolated lymphocytes were mixed with 1% low-melting-point agarose in PBS (pH 7.4) and added onto microscope slides precoated with 1% normal-melting-point agarose in PBS (pH 7.4). The suspension was covered by coverslips and allowed to solidify in a refrigerator for 20 min. The coverslips were removed, and the slides were submerged in lysing solution (2.5 mol/L NaCl, 100 mmol/L Na_2EDTA, 10 mmol/L Tris, 1% Triton X-100, 10% DMSO, pH 10) for 1 h. The slides were then placed in the electrophoresis buffer (5 mol/L NaOH, 200 mmol/L Na_2EDTA, pH 13) for 20 min. For the assessment of

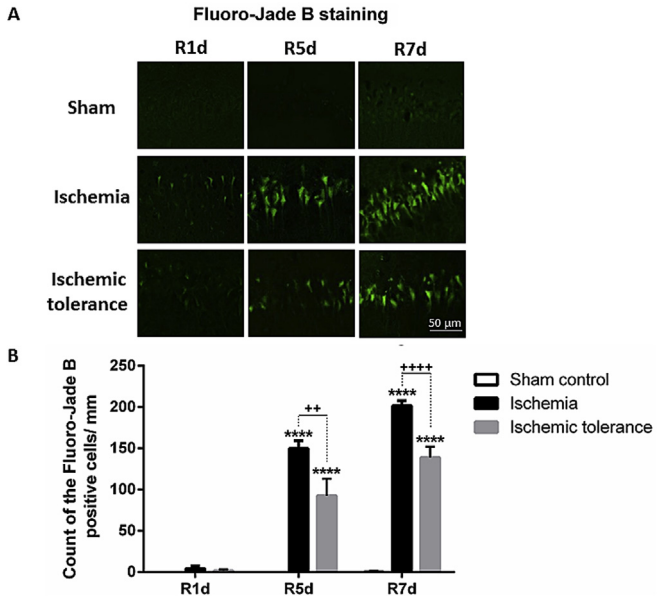

Fig. 2. Brain tissue response to ischemia and pre-ischemic tolerance induction: Presence of CA1 hippocampal neurodegeneration. (A) Representative micrographs of degenerating neurons. Bar = 50 µm; (B) Graphically expressed count of the Fluoro-Jade B positive cells in CA1 region of hippocampus. Mean ± SEM, (++) $p < 0.01$, (****, ++++) $p < 0.0001$. Sign "*" represents statistically significant control group vs ischemia/tolerant group difference, "+" represents ischemia vs tolerant group difference. R = reperfusion, d = day.

single-strand breaks (SSBs) of lymphocyte DNA, an alkaline pH is necessary to unwind the DNA spiral. Electrophoresis was carried out using the same solution for 25 min at 25 V. After electrophoresis, the slides were neutralized with neutralization buffer (400 mmol/L Tris, pH 7.5, 15 min). All previously described steps were carried out in a refrigerator (4 °C). Slides were stained with a SYBR GOLD nucleic acid gel stain directly before imaging under a fluorescence microscope (Olympus BX51, ex.f. 485, em.f. 520 nm) equipped with a camera (Olympus DP50). Microphotographs of Comets (software Olympus DP image) were processed using the CometScoreTM 1.5 image analysis system (TriTekCorp., USA). Lymphocyte DNA damage was examined by the parameter "% DNA in tail" (100% of cell fluorescence intensity minus intensity of the head % DNA). For each individual sample, two slides were prepared and cells were randomly selected for analysis (total 100 cells per sample).

2.10. Fluoro-Jade B staining

Fluoro-Jade B staining was used for observation of CA1 hippocampal neurodegeneration (Schmued and Hopkins, 2000). The fixed coronal slices were cut into 10 µm sections and subsequently mounted on gelatinized microscope slides. These were allowed to air dry, then they were placed in absolute alcohol, 70% ethanol and distilled water for 3 min. The sections were oxidized by soaking in a solution of 0.06% $KMnO_4$ for 15 min, then they were washed in distilled water for 2 min and stained in 0.001% Fluoro-Jade B (Histo-Chem Inc., USA) in 0.1% acetic acid for 20 min. The slides were subsequently washed 3 times in distilled water for 1 min at each wash and allowed to dry at room temperature. The dried sections were cleared by xylene and coverslipped with DPX Mountant for histology (Fluka Chemie AG, Switzerland). The slides were examined using an Olympus BX51 microscope with a camera (Olympus DP50). Fluoro-Jade B-positive neurons of the CA1 hippocampus region were counted by an investigator blinded to the study using ImageJ software and expressed per 1 mm of tissue.

2.11. Morris water maze test

For the assessment of neurological deficits after global ischemia and ischemic tolerance induction, we used the Morris water maze test developed by Richard Morris (1984). The test is based on learning the location of a hidden platform using only spatial memory. A large circular pool (120 cm in diameter and 70 cm deep) was filled with water; milk was added to make it opaque. A submerged escape platform (20 cm tall and 15 cm diameter) was located in the single fixed location of the maze. A variety of visual cues were visible within the maze. Testing started on the fourth day after ischemia induction and lasted for 4 days. Each animal received 4 timed trials per day. They were placed into the water facing the side walls of the pool at different start positions across trials. The rats were allowed 60 s to locate the platform. If after 60 s they did not find the escape platform, they were guided there by the experimenter and allowed to remain on the platform for 10 s. The inter-trial interval for each animal was 5 min, during which time the rat remained in its home cage. On day 4, we conducted a trial to test memory after 3 days of learning the location. All rats started from the same start position, namely opposite the quadrant where the escape platform had been positioned during acquisition. The escape latencies—the time each subject required to locate the hidden platform after being released—for each subject were measured.

2.12. Statistical analyses

Data are expressed as a mean ± SEM. Statistical analysis was performed with one-way and two-way ANOVA, followed by the Dunnett post hoc test. The baseline representing the sham control value is set to 0. The value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Determination of neuronal degeneration and behavioural influence

Our results showed that hind-limb-tourniquet pretreatment caused significant improvement in neuronal survival (Fig. 2). It is generally known that global ischemia leads to delayed death in neurons located in the CA1 area of the hippocampus. In our experiment, we observed degenerating neurons since the fifth day of post-ischemic reperfusion.

On the fifth day, 149.7 ± 9.54 degenerating neurons/mm were detected in the ischemia group, and this count rose by about 25.8%–201.62 ± 6.03 degenerating neurons/mm two days later (i.e., 7 days of post-ischemic reperfusion). In contrast to this, hind-limb preconditioning improves neuronal survival significantly. As Fig. 2 shows, at five days of postischemic reperfusion the number of degenerating neurons decreases by about 38% (i.e., 92.54 ± 20.53 degenerating neurons/mm), and decreases by about 31% by day seven (i.e., 138.84 ± 13.13 degenerating neurons/mm) compared to the ischemia group (Fig. 2).

Although hind-limb pretreatment decreased neuronal degeneration, it was not reflected in neurological deficit improvement. Learning ability of the preconditioned animals was comparable to the ischemic group of rats (Fig. 3A). The same results were observed in the reference memory evaluation (Fig. 3B).

3.2. Systemic oxidative stress and antioxidant defenses in peripheral blood

Systemic oxidative stress in animals subjected to brain ischemia and hind-limb-tourniquet preconditioned rats was evaluated using an alkaline Comet assay, which is a method for detecting the amount of single-strand breaks (SSBs) in the DNA of peripheral lymphocytes. Our results showed that induction of ischemic tolerance caused significant reduction of oxidative stress in treated animals (Fig. 4). Although global cerebral ischemia led to significant elevation of lymphocyte DNA damage on the first and fifth day of post-ischemic reperfusion

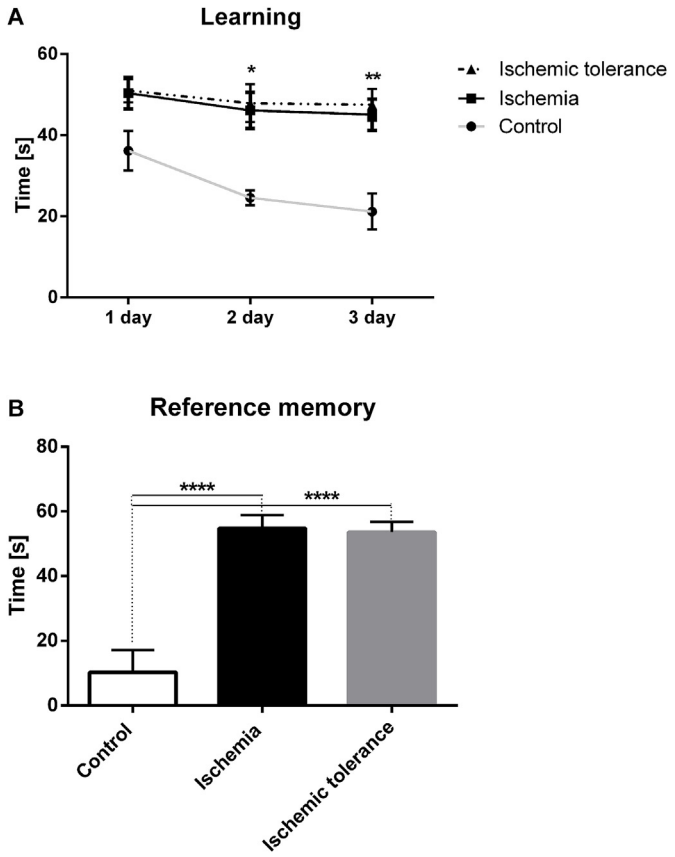

Fig. 3. Behavioral influence of brain ischemia and pre-ischemic tolerance induction. Ability to learn (A) and reference memory (B). Mean ± SEM, (*) $p < 0.05$, (**) $p < 0.01$, (****) $p < 0.001$. Sign "*" represents statistically significant control group vs ischemia/tolerant group difference. s = second.

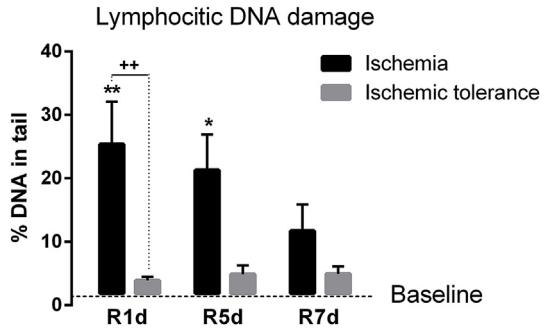

Fig. 4. Peripheral lymphocyte DNA damage. Single-strands breaks (SSBs) in the DNA of peripheral blood lymphocytes after global ischemia with or without pre-ischemic tolerance induction were measured by a Comet assay. Mean ± SEM, (*) $p < 0.05$, (**, ++) $p < 0.01$. Sign "*" represents statistically significant control group vs ischemia/tolerant group difference, "+" represents ischemia vs tolerant group difference. R = reperfusion, d = days.

(25.4 ± 6.7% of DNA in the tail and 21.3 ± 5.6% of DNA in the tail, respectively), animals subjected to pre-ischemic hind-limb tourniquets did not demonstrate significantly increased systemic oxidative stress. The most evident divergence between the ischemia and ischemic tolerance group was detected one day after the attack. The changes between those two experimental groups reached 80% (3.9 ± 0.6% of DNA in the tail).

We measured the enzymatic antioxidant defenses of the peripheral blood—blood cell SOD and plasma CAT activity—as a response to elevated systemic oxidative stress. We did not detect changes in SOD activity in either of the groups studied (Fig. 5). When compared to the

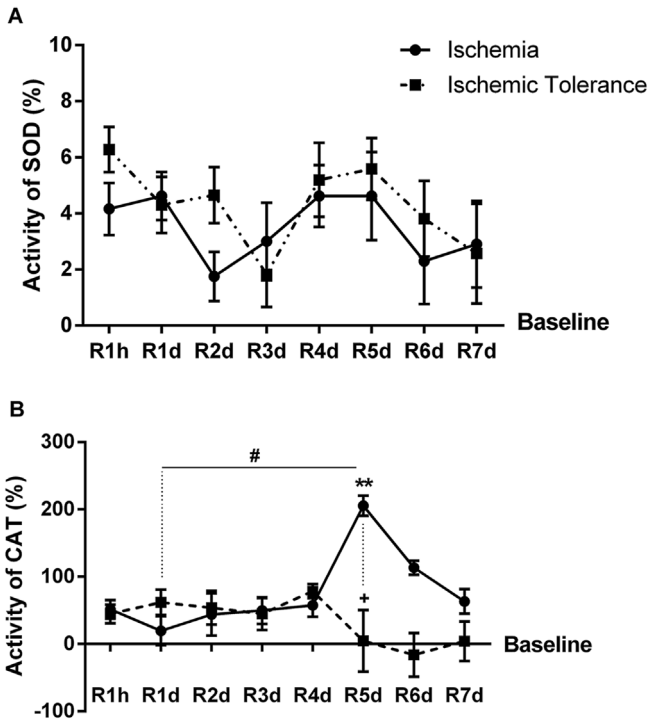

Fig. 5. Enzymatic antioxidant defense activity in animals subjected to brain ischemia with or without pre-ischemic tolerance induction. Activity of SOD in the blood cells (A) and CAT in plasma (B) expressed as a percentage of the control group values. The baseline represents enzyme activity in the SHC group and is set to 0%. Mean ± SEM, (+, #) $p < 0.05$, (**) $p < 0.01$. Sign "*" represents statistically significant control group vs ischemia/tolerant group difference, "#" represents difference between days of reperfusion within one group. SHC = sham control, SOD = superoxide dismutase, CAT = catalase, R = reperfusion, d = days.

control (55.2 ± 13 kU/l of blood), CAT activity of ischemic and tolerant rats stayed unchanged up to the fourth day of postischemic recovery. On the fifth day, brain ischemia significantly increased by about 205.6 ± 15% (168.7 ± 25.28 kU/L of blood) above the baseline and was elevated by about 200% compared to hind-limb-tourniquet treated rats (Fig. 5). In the next two days, plasma CAT activity of the ischemia group declined gradually to baseline.

3.3. Glutamate level in brain tissue and peripheral blood

Extracellular increase in glutamate is one of the first events that happen in ischemia-affected brain tissue. The restoration of blood supply in our experiment influenced all the studied brain regions. Regardless of their sensitivity to ischemic conditions, significant increases were detected on the first day of post-ischemic recovery (Fig. 6A and C). The most evident changes were recorded in the cortex (Fig. 6A), where the glutamate concentration rose by about 113.8 ± 15.7% (0.248 ± 0.039 μmol/mg of protein) compared to the sham control (0.116 ± 0.01 μmol/mg of protein) at the first day. The peak was reduced with ongoing reperfusion by about 48.8%% on the fifth day (to 0.127 ± 0.01 μmol/mg of protein) and by about 53.2% on the seventh day after the attack (to 0.116 ± 0.02 μmol/mg of protein). In the CA1 (0.224 ± 0.06 μmol/mg of protein; i.e., 130.93 ± 28.2% over the control) and dentate gyrus region of hippocampus (0.203 ± 0.06 μmol/mg of protein; i.e., 89.72 ± 30.4% over the control) glutamate levels peaked within the first day of reperfusion as well, then decreased to the baseline over the next few days (Fig. 6B and C). When compared to the ischemia group, pretreatment of animals with hind-limb ischemia reduced brain tissue glutamate at the first day by about 33.5% in cortex (to 0.165 ± 0.03 μmol/mg of protein), by

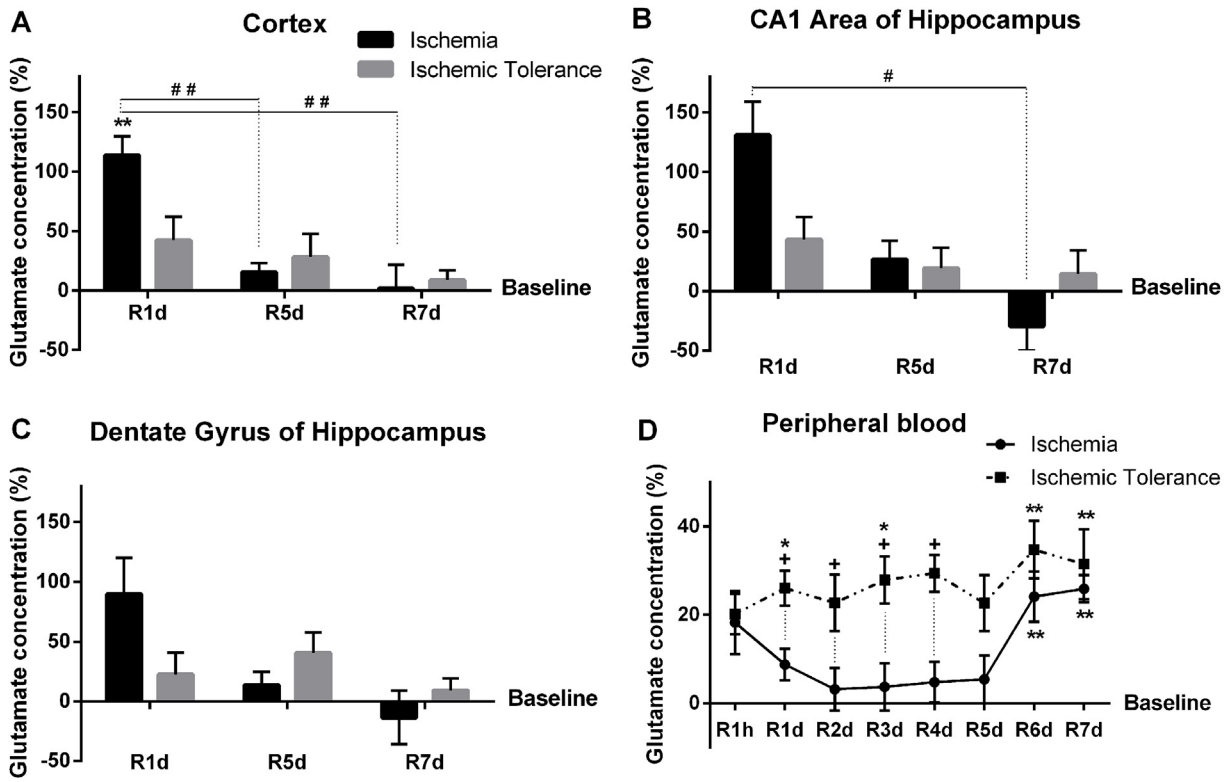

Fig. 6. Effect of ischemia and pre-ischemic tolerance induction on the glutamate concentration in the brain tissue of regions with different sensitivities to ischemic conditions and in peripheral blood. Glutamate concentration in *cortex* (A), *CA1* (B), *dentate gyrus* region (C) of the *hippocampus* and peripheral blood (D). Glutamate levels are expressed as a percentage of the control. The baseline represents glutamate concentration in the SHC group and is set to 0%. Mean ± SEM, (#, +) $p < 0.05$, (**, ##) $p < 0.01$. The sign "*" represents a statistically significant control group vs ischemia/tolerant group difference, "+" represents an ischemia vs tolerant group difference and "#" represents a difference between days of reperfusion within one group. SHC = sham control, R = reperfusion, d = day.

about 38% in CA1 (0.139 ± 0.03 μmol/mg of protein) and 35.5% in DG of hippocampus (0.131 ± 0.02 μmol/mg of protein), but not significantly. Similarly, with ongoing reperfusion the brain tissue glutamate did not significantly differ compared to the ischemia group as well as the control. (Fig. 6A–C).

In contrast, blood levels of this excitatory amino acid in the tolerant rats rose when compare to the control animals as well as the ischemia group (Fig. 6D). We did not detect changes in blood glutamate caused by pre-ischemic hind-limb-tourniquet treatment (tolerance induction) one hour after the brain ischemia event. When compared to the control, a significant rise in this group was detected in the first (108.76 ± 4.11 μmol/L of blood, i.e. 26 ± 3.9%), third (110.39 ± 5.88 μmol/L of blood, i.e. 27.88 ± 5.32%) and last two days of survival (116.3 ± 7.59 μmol/L of blood, i.e. 34.73 ± 6.52% at the sixth and 113.49 ± 8.02 μmol/L of blood, i.e. 31.48 ± 7.92% at the seventh day, respectively). Interestingly, this hind-limb pretreatment of ischemic animals evoked significantly higher blood glutamate during the first four days compared to the ischemia group (elevation from 108.76 ± 4.12 μmol/L of blood to 111.7 ± 4.69 μmol/L of blood, i.e. form 26 ± 3.9% to 29.4 ± 4.2% compared to the ischemia group). Difference between glutamate levels in those two groups disappeared in the last two days; however, both stayed significantly increased from the baseline (Fig. 6D).

4. Discussion

Remote ischemic tolerance (RIT) induction by transient limb ischemia is a non-invasive, safe and attractive approach to improve neuronal survival. Significant reduction of cerebral damage after RIT induction was confirmed in several animal models of focal (Bonova and Gottlieb, 2015; Ren et al., 2008) and global cerebral ischemia (Jensen et al., 2011). However, clear evidence about its deleterious effects remains unknown. In the present paper, we investigated the response of early remote preconditioning on brain tissue and circulating blood using several markers that can be significantly changed in animals subjected to transient forebrain ischemia.

The final effect of blood supply restriction is progressive neurodegeneration that leads to long-term motor and cognitive impairment. While we did not find a recovery from neurological deficits, significant reduction of neuronal degeneration by about 30% was detected in animals pretreated with RIT. In rats, impairments are detected mostly in tasks of spatial learning and spatial working (reviewed in Hodges et al., 1997). There are several reports confirming correlational links between CA1 cell loss following global brain ischemia in rats and behavioral impairments (Nelson et al., 1997a, 1997b), but some findings indicate that at the intermediate level of neurodegeneration these relationships are weak and variable (Moser et al., 1993), suggesting that deficits are more than damage to the CA1 field (Hodges et al., 1997). Ischemia mediated changes contribute to substantial remodeling of neuronal circuits (Schmidt-Kastner and Freund, 1991) and might also contribute to cognitive dysfunction or compensation even when the CA1 cell loss was not detected (Jaspers et al., 1990). Based on available literature data, the rate of tissue protection seems to be dependent on the interval between RIT application and the lethal ischemic insult, as well as the ischemia model itself. For example, only a short reperfusion period (30 min) between sublethal and lethal global ischemia induction can provide significant—albeit temporary—protection against ischemic injury (Perez-Pinzon et al., 1997). In contrast, when the interval is prolonged to 24 h, no improvement in neuronal survival was detected (Saxena et al., 2009).

As described previously in several studies, neuronal degeneration can be assessed with biomarker levels in circulating blood. The

circulating blood is easily accessible biological material, which reflects the situation inside the ischemia-affected brain tissue. In the present paper, we studied enzymatic antioxidant defenses that exhibited completely unchanged SOD activity; however, we observed significantly increased CAT activity at five days of post-ischemic reperfusion which was probably caused by neuronal loss manifesting at that point in time. However, plenty of studies have contradictory results. For example (Aygul et al., 2006), describe an attenuation of antioxidant defenses, while (Devyatov et al., 2017) observed its increase in peripheral blood. Systemic oxidative stress after ischemia (represented as DNA damage in lymphocytes) declined by about 80% in the group pre-treated with a hind-limb tourniquet. No response of the antioxidant enzymes in the blood was detected at the same time, suggesting that such antioxidant enzymes are not the major determinants of RIP-induced neuroprotection. Nevertheless, oxidative stress could contribute to the development of ischemic tolerance in the brain by mechanisms separate from an upregulation of antioxidant enzymes, particularly by producing free radical scavengers or by reducing the ischemia-induced production of free radicals (Puisieux et al., 2004).

The most powerful evidence of the protective effects of RIP and the most likely mechanism involved in RIP neuroprotection comes from our observation of the systemic metabolism of glutamate. It is well known that glutamate excitotoxicity in the brain is elevated in several models of brain ischemia for different brain regions (Bonova et al., 2013a; Qureshi et al., 2003). Moreover, there is strong evidence that ischemia causes an elevation in glutamate concentration in the peripheral blood of animal models of ischemia (Bonova et al., 2013a, b; Kravcukova et al., 2009, 2010) and in stroke patients; the correlation between the size of the infarct and the concentration of glutamate in the blood is detectable (Aliprandi et al., 2005; Castellanos et al., 2008). Determination of peripheral blood glutamate has great potential for monitoring the progression of ischemia and the effectiveness of therapy (Bonova et al., 2013a; Jickling and Sharp, 2011).

Hind-limb pretreatment used for induction of tolerance in this experiment caused marked reduction in the glutamate levels of all observed brain structures similarly to other models of preconditioning (Zhang et al., 2010), postconditioning (Bonova et al., 2013a) and *in vitro* induction of ischemic tolerance (IT) (Pinto et al., 2012). In contrast, blood levels of glutamate rose significantly during the first days of post-ischemic recovery in those animals. Elevated levels of glutamate play an important role in neuroprotection after ischemia. This amino acid represents the main source of glutathione synthesis for red blood cells (RBCs). Glutathione plays a central role in repairing oxidative damage to erythrocytes. However, the erythrocytes' membranes are impermeable to glutamate; consequently, RBCs rely on *de novo* synthesis of glutamate to maintain its intracellular levels (Ellinger et al., 2011).

Brain-to-blood efflux could be accelerated due to the creation of a larger glutamate concentration gradient between the brain fluids, endothelial cells and blood plasma (Gottlieb et al., 2003; Teichberg et al., 2009). This may possibly be achieved by its scavenging in blood by pyruvate and oxaloacetate as it was described previously (Boyko et al., 2014; Zlotnik et al., 2007). We suppose that glutamate scavenging of blood cells via glutamate transporters is involved in the aforementioned mechanism of ischemia tolerance. Three sodium-dependent glutamate transporters (excitatory amino acid transporters 1–3; EAAT1–3) were detected on the surface of platelets and lympho-monocytes (Zoia et al., 2004), and only EAAT3 was detected in red blood cells (Winterberg et al., 2012). Glutamate uptake by those cells could be affected by aging, neurological disorders such as Parkinson's or Alzheimer's disease (Zoia et al., 2004), or it could be activated chemically or by parasitic infection (Winterberg et al., 2012). Our previous papers showed that RIP could stimulate blood cells to produce a bioactive protein substance in ischemia-affected animals (i.e., factor-induced tolerance) which could reduce neuronal loss in treated rats. This neuroprotective stimulus could be transferred via blood to another rat by blood transfusion (Bonova and Gottlieb, 2015; Bonova et al., 2016).Currently, we have not confirmed any connection to EAATs transporters, which will be the target of our next investigation.

5. Conclusion

Taken together, we can assume that hind-limb-tourniquet preconditioning could accelerate brain-to-blood efflux of glutamate that could positively impact neuronal survival in ischemia-affected brain regions; however, this impact does not extend to the neurological outcome. Moreover, remote preconditioning improved systemic oxidative stress and did not seem to be affected by the enzymatic antioxidant defenses in the blood. These results could significantly advance investigations into the clinical applicability of ischemic tolerance caused by remote conditioning.

Acknowledgments

The authors gratefully acknowledge the excellent technical assistance of Dana Jurusova. This study was supported by the Slovak Grant Agencies VEGA 2/0094/18 and VEGA 2/0029/18.

References

Aliprandi, A., Longoni, M., Stanzani, L., Tremolizzo, L., Vaccaro, M., Begni, B., Galimberti, G., Garofolo, R., Ferrarese, C., 2005. Increased plasma glutamate in stroke patients might be linked to altered platelet release and uptake. J. Cereb. Blood Flow Metab. 25, 513–519.

Aygul, R., Kotan, D., Demirbas, F., Ulvi, H., Deniz, O., 2006. Plasma oxidants and antioxidants in acute ischaemic stroke. J. Int. Med. Res. 34, 413–418.

Bejot, Y., Bailly, H., Durier, J., Giroud, M., 2016. Epidemiology of stroke in Europe and trends for the 21st century. Presse Med. 45, e391–e398.

Bonova, P., Burda, J., Danielisova, V., Nemethova, M., Gottlieb, M., 2013a. Delayed postconditioning reduces post-ischemic glutamate level and improves protein synthesis in brain. Neurochem. Int. 62, 854–860.

Bonova, P., Burda, J., Danielisova, V., Nemethova, M., Gottlieb, M., 2013b. Development of a pattern in biochemical parameters in the core and penumbra during infarct evolution after transient MCAO in rats. Neurochem. Int. 62, 8–14.

Bonova, P., Gottlieb, M., 2015. Blood as the carrier of ischemic tolerance in rat brain. J. Neurosci. Res. 93, 1250–1257.

Bonova, P., Nemethova, M., Matiasova, M., Bona, M., Gottlieb, M., 2016. Blood cells serve as a source of factor-inducing rapid ischemic tolerance in brain. Eur. J. Neurosci. 44, 2958–2965.

Boyko, M., Gruenbaum, S.E., Gruenbaum, B.F., Shapira, Y., Zlotnik, A., 2014. Brain to blood glutamate scavenging as a novel therapeutic modality: a review. J. Neural Transm. 121, 971–979.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Castellanos, M., Sobrino, T., Pedraza, S., Moldes, O., Pumar, J.M., Silva, Y., Serena, J., Garcia-Gil, M., Castillo, J., Davalos, A., 2008. High plasma glutamate concentrations are associated with infarct growth in acute ischemic stroke. Neurology 71, 1862–1868.

Danielisova, V., Nemethova, M., Gottlieb, M., Burda, J., 2005. Changes of endogenous antioxidant enzymes during ischemic tolerance acquisition. Neurochem. Res. 30, 559–565.

Devyatov, A.A., Fedorova, T.N., Stvolinskii, S.L., Belousova, M.A., Medvedev, O.S., Tutelyan, V.A., 2017. Assessment of oxidative status of the brain and blood plasma in rats with modeled focal cerebral ischemia/reperfusion injury. Bull. Exp. Biol. Med. 163, 195–198.

Ellinger, J.J., Lewis, I.A., Markley, J.L., 2011. Role of aminotransferases in glutamate metabolism of human erythrocytes. J. Biomol. NMR 49, 221–229.

Fisher, M., Feuerstein, G., Howells, D.W., Hurn, P.D., Kent, T.A., Savitz, S.I., Lo, E.H., 2009. Update of the stroke therapy academic industry roundtable preclinical recommendations. Stroke 40, 2244–2250.

Goth, L., 1991. A simple method for determination of serum catalase activity and revision of reference range. Clin. Chim. Acta 196, 143–151.

Gottlieb, M., Wang, Y., Teichberg, V.I., 2003. Blood-mediated scavenging of cerebrospinal fluid glutamate. J. Neurochem. 87, 119–126.

Graham Jr., L.T., Aprison, M.H., 1966. Fluorometric determination of aspartate, glutamate, and gamma-aminobutyrate in nerve tissue using enzymic methods. Anal. Biochem. 15, 487–497.

Hodges, H., Nelson, A., Virley, D., Kershaw, T.R., Sinden, J.D., 1997. Cognitive deficits induced by global cerebral ischaemia: prospects for transplant therapy. Pharmacol. Biochem. Behav. 56, 763–780.

Hu, S., Dong, H., Zhang, H., Wang, S., Hou, L., Chen, S., Zhang, J., Xiong, L., 2012. Noninvasive limb remote ischemic preconditioning contributes neuroprotective effects via activation of adenosine A1 receptor and redox status after transient focal

cerebral ischemia in rats. Brain Res. 1459, 81–90.
Internet Stroke Center, 2018. Stroke Trials Registry of the Internet Stroke Center at Washington University School of Medicine. http//www.strokecenter.org/trials.
Jaspers, R.M., Block, F., Heim, C., Sontag, K.H., 1990. Spatial learning is affected by transient occlusion of common carotid arteries (2VO): comparison of behavioural and histopathological changes after '2VO' and 'four-vessel-occlusion' in rats. Neurosci. Lett. 117, 149–153.
Jensen, H.A., Loukogeorgakis, S., Yannopoulos, F., Rimpilainen, E., Petzold, A., Tuominen, H., Lepola, P., Macallister, R.J., Deanfield, J.E., Makela, T., Alestalo, K., Kiviluoma, K., Anttila, V., Tsang, V., Juvonen, T., 2011. Remote ischemic preconditioning protects the brain against injury after hypothermic circulatory arrest. Circulation 123, 714–721.
Jickling, G.C., Sharp, F.R., 2011. Blood biomarkers of ischemic stroke. Neurotherapeutics 8, 349–360.
Kravcukova, P., Danielisova, V., Nemethova, M., Burda, J., Gottlieb, M., 2009. Transient forebrain ischemia impact on lymphocyte DNA damage, glutamic acid level, and SOD activity in blood. Cell. Mol. Neurobiol. 29, 887–894.
Kravcukova, P., Danielisova, V., Nemethova, M., Burda, J., Gottlieb, M., 2010. Effects of one-day reperfusion after transient forebrain ischemia on circulatory system in the rat. Gen. Physiol. Biophys. 29, 113–121.
Ljubisavljevic, S., Cvetkovic, T., Zvezdanovic, L., Stojanovic, S., Stojanovic, I., Kocic, G., Zivkovic, M., Paunovic, L., Milenkovic, L., Lukic, D., Stamenovic, J., Pavlovic, D., 2016. The differences in the cellular and plasma antioxidative capacity between transient and defined focal brain ischemia: does it suggest supporting time-dependent neuroprotection therapy? Cell. Mol. Neurobiol. 36, 789–800.
Lorente, L., Martin, M.M., Perez-Cejas, A., Abreu-Gonzalez, P., Ramos, L., Argueso, M., Caceres, J.J., Sole-Violan, J., Jimenez, A., 2016. Association between total antioxidant capacity and mortality in ischemic stroke patients. Ann. Intensive Care 6, 39.
Morris, R., 1984. Developments of a water-maze procedure for studying spatial learning in the rat. J. Neurosci. Methods 11, 47–60.
Moser, E., Moser, M.B., Andersen, P., 1993. Spatial learning impairment parallels the magnitude of dorsal hippocampal lesions, but is hardly present following ventral lesions. J. Neurosci. 13, 3916–3925.
Nelson, A., Lebessi, A., Sowinski, P., Hodges, H., 1997a. Comparison of effects of global cerebral ischaemia on spatial learning in the standard and radial water maze: relationship of hippocampal damage to performance. Behav. Brain Res. 85, 93–115.
Nelson, A., Sowinski, P., Hodges, H., 1997b. Differential effects of global ischemia on delayed matching- and non-matching-to-position tasks in the water maze and Skinner box. Neurobiol. Learn. Mem. 67, 228–247.
Perez-Pinzon, M.A., Xu, G.P., Dietrich, W.D., Rosenthal, M., Sick, T.J., 1997. Rapid preconditioning protects rats against ischemic neuronal damage after 3 but not 7 days of reperfusion following global cerebral ischemia. J. Cereb. Blood Flow Metab. 17, 175–182.
Pinto, M.C., Mourao, F.A., Binda, N.S., Leite, H.R., Gomez, M.V., Massensini, A.R., Gomez, R.S., 2012. Pharmacological induction of ischemic tolerance in hippocampal slices by sarcosine preconditioning. Neurochem. Int. 61, 713–720.
Puisieux, F., Deplanque, D., Bulckaen, H., Maboudou, P., Gele, P., Lhermitte, M., Lebuffe, G., Bordet, R., 2004. Brain ischemic preconditioning is abolished by antioxidant drugs but does not up-regulate superoxide dismutase and glutathion peroxidase. Brain Res. 1027, 30–37.
Pulsinelli, W.A., Brierley, J.B., 1979. A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke 10, 267–272.
Qureshi, A.I., Ali, Z., Suri, M.F., Shuaib, A., Baker, G., Todd, K., Guterman, L.R., Hopkins, L.N., 2003. Extracellular glutamate and other amino acids in experimental intracerebral hemorrhage: an in vivo microdialysis study. Crit. Care Med. 31, 1482–1489.
Ren, C., Gao, X., Steinberg, G.K., Zhao, H., 2008. Limb remote-preconditioning protects against focal ischemia in rats and contradicts the dogma of therapeutic time windows for preconditioning. Neuroscience 151, 1099–1103.
Saxena, P., Bala, A., Campbell, K., Meloni, B., d'Udekem, Y., Konstantinov, I.E., 2009. Does remote ischemic preconditioning prevent delayed hippocampal neuronal death following transient global cerebral ischemia in rats? Perfusion 24, 207–211.
Schmidt-Kastner, R., Freund, T.F., 1991. Selective vulnerability of the hippocampus in brain ischemia. Neuroscience 40, 599–636.
Schmued, L.C., Hopkins, K.J., 2000. Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res. 874, 123–130.
Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.
Sivonova, M., Kaplan, P., Durackova, Z., Dobrota, D., Drgova, A., Tatarkova, Z., Pavlikova, M., Halasova, E., Lehotsky, J., 2008. Time course of peripheral oxidative stress as consequence of global ischaemic brain injury in rats. Cell. Mol. Neurobiol. 28, 431–441.
Sun, Y., Oberley, L.W., Li, Y., 1988. A simple method for clinical assay of superoxide dismutase. Clin. Chem. 34, 497–500.
Teichberg, V.I., Cohen-Kashi-Malina, K., Cooper, I., Zlotnik, A., 2009. Homeostasis of glutamate in brain fluids: an accelerated brain-to-blood efflux of excess glutamate is produced by blood glutamate scavenging and offers protection from neuropathologies. Neuroscience 158, 301–308.
Winterberg, M., Rajendran, E., Baumeister, S., Bietz, S., Kirk, K., Lingelbach, K., 2012. Chemical activation of a high-affinity glutamate transporter in human erythrocytes and its implications for malaria-parasite-induced glutamate uptake. Blood 119, 3604–3612.
Zhang, F., Wu, Y., Jia, J., Hu, Y.S., 2010. Pre-ischemic treadmill training induces tolerance to brain ischemia: involvement of glutamate and ERK1/2. Molecules 15, 5246–5257.
Zhao, H., Ren, C., Chen, X., Shen, J., 2012. From rapid to delayed and remote postconditioning: the evolving concept of ischemic postconditioning in brain ischemia. Curr. Drug Targets 13, 173–187.
Zitnanova, I., Siarnik, P., Kollar, B., Chomova, M., Pazderova, P., Andrezalova, L., Jezovicova, M., Konarikova, K., Laubertova, L., Krivosikova, Z., Slezakova, L., Turcani, P., 2016. Oxidative stress markers and their dynamic changes in patients after acute ischemic stroke. Oxid Med Cell Longev 2016, 9761697.
Zlotnik, A., Gurevich, B., Tkachov, S., Maoz, I., Shapira, Y., Teichberg, V.I., 2007. Brain neuroprotection by scavenging blood glutamate. Exp. Neurol. 203, 213–220.
Zoia, C., Cogliati, T., Tagliabue, E., Cavaletti, G., Sala, G., Galimberti, G., Rivolta, I., Rossi, V., Frattola, L., Ferrarese, C., 2004. Glutamate transporters in platelets: EAAT1 decrease in aging and in Alzheimer's disease. Neurobiol. Aging 25, 149–157.