

# Decrease in Oxidative Stress Parameters after Post-Ischaemic Recombinant Human Erythropoietin Administration in the Hippocampus of Rats Exposed to Focal Cerebral Ischaemia

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**Abstract:** Recombinant human erythropoietin (rhEpo) is a multi-functional drug with antioxidant potential. However, the underlying molecular mechanisms of its action are still unclear. The purpose of this study was to investigate the effects of rhEpo on the brain infarct volume as well as on the levels of the neuronal damage, oxidative stress parameters and active caspase-3, nuclear factor erythroid 2-related factor 2 (Nrf2) and haemeoxygenase-1 (HO-1) expressions in the hippocampi of rats exposed to the right middle cerebral artery occlusion (MCAO) for 1 hr. Ischaemic animals received either vehicle or rhEpo (5000 IU/kg, i.p.) immediately or 3 hr after the induction of ischaemia. Sham-operated, vehicle-treated animals served as the control group. Rats were killed 24 hr after the onset of the ischaemic or sham experimental procedure. MCAO caused ipsilateral brain infarction within the striatum and cortex. In the CA1 region of the hippocampi, we did not find significant neuronal loss, but a statistically significant rise in the active caspase-3 and Nrf2 protein expressions was registered. We detected also significant increases in the hippocampal levels of oxidative stress parameters (thiobarbituric acid-reactive substances, superoxide dismutase, glutathione peroxidase). Post-ischaemic administration of rhEpo significantly reduced the brain infarct volume, decreased levels of all tested oxidative stress parameters and increased the Nrf2 expression level. These findings suggest that decrease in oxidative stress parameters in the hippocampus could be an early indicator of post-ischaemic neuroprotective effect of rhEpo in rats exposed to focal cerebral ischaemia and that this effect could be attributable to additional post-ischaemic activation of Nrf2 endogenous antioxidant system.

Human brain constitutes only 2.3% of body mass, but it consumes about 18% of cardiac output and 20% of the total body oxygen. Such high vascular and metabolic demand and lack of appreciable energy reserves render it uniquely susceptible to alterations of blood supply [1]. Cerebral stroke is a cerebrovascular disorder characterized by rapidly developing clinical signs of focal or global disturbances of cerebral function, caused by impairment in cerebral blood flow. Worldwide, it is ranked as one of the leading causes of death, as a major cause of disability, and is considered to be the second-most frequent cause of projected mortality in 2020 [2]. More than 80% of human strokes are of ischaemic origin, and most of them are caused by middle cerebral artery occlusion (MCAO) [3].

In experimental or clinical conditions, the brain is extremely sensitive to oxidative stress, defined as imbalance between reactive oxygen species (ROS) and endogenous antioxidant enzyme capacities. High oxygen consumption makes the brain the major generator of ROS. Additional favouring conditions for ROS production in the brain are high polyunsaturated lipid content, chemical reactions involving dopamine oxidation or high concentration of redox active metals like copper or iron such as the fact that it is insufficiently equipped with antioxidant defence systems [4]. The antioxidant enzyme activity of the tissue affected by ischaemia/reperfusion (I/R) represents

primary endogenous defence against ROS injury and involves the co-ordinated action of the intracellular antioxidant enzymes, superoxide dismutases (SOD), glutathione peroxidase (GSH-Px) and catalase [5,6]. Briefly, SOD catalyses the dismutation of superoxide anion O<sub>2</sub><sup>-</sup> to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), which is then converted to water by mitochondrial and cytosolic GSH-Px or peroxisomal catalase. Although the beneficial antioxidant role of SOD and GSH-Px is widely recognized, the alterations in their activities after ischaemic neuronal damage are not fully understood. Recently, it was shown that the major role in the regulation of mentioned endogenous antioxidants after brain oxidative stress is exerted by the nuclear factor erythroid 2-related factor 2 (Nrf2). Several authors have demonstrated that after the activation of the Kelch-like ECH-associated protein 1 (Keap1) and its binding to antioxidant response elements (ARE), that is activation of Keap1–Nrf2/ARE pathway, Nrf2 induces transcription of different Nrf2-dependent phase II enzymes including glutathione reductase, haemeoxygenase-1 (HO-1), SOD or GSH-Px, thus directly affecting cellular antioxidant defence [7–9]. At present, treatment options focusing on brain endogenous antioxidant repair mechanisms represent a major focus of stroke research [10] with the Nrf2/ARE pathway as a new potential therapeutic target for treating focal cerebral ischaemia [7].

The beneficial effects of endogenous erythropoietin (Epo) and Epo receptor (EpoR) expression in the central nervous system were recently discovered. Evidence that both are expressed locally in response to the brain ischaemia has raised

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the possibility that exogenous administration of rhEpo could contribute to endogenous neuroprotective response of ischaemic brain tissue [11,12]. Recombinant human erythropoietin (rhEpo) is a clinically approved drug whose indications are currently restricted to the treatment of anaemia in renal failure and cancer [13,14]. Several hypotheses of potential neuroprotective effect of rhEpo were based either on direct cytoprotective mechanisms activated by increase in anti-apoptotic proteins and Bcl-X<sub>L</sub>/Bax ratio or reduction in pro-apoptotic factors like active caspases, suppression of matrix metalloproteinases, inhibition of glutamate excitotoxicity, modulation of nitric oxide synthase and enhancement of the brain's antioxidant defences or on indirect activation via its angiogenic potential and improvement of cerebral blood flow in the ischaemic tissue [15–17]. The capability of rhEpo to cross the blood–brain barrier after systemic administration, its acceptable therapeutic window and good clinical tolerability are additional advantages for its potential application in human stroke therapy [18]. However, the exact mechanisms of its neuroprotection are still not completely understood and outcome from conducted clinical trials are controversial [19,20]. Therefore, the objectives of our study were to examine a) the effects of focal cerebral I/R injury on the extent of the brain infarct and the levels of neuronal loss, the expressions of active caspase-3, Nrf2 and (HO-1), lipid oxidative damage and the SOD and GSH-Px activities in the hippocampi of rats, ipsilaterally to the infarction, and b) possible neuroprotective effect of rhEpo, administered after the induction of MCAO on the above-mentioned parameters. Experimentally induced transient MCAO in rodents is a well-standardized animal model of focal cerebral I/R injury that mimics clinical conditions of early reperfusion after ischaemic stroke [21,22].

### Materials and methods

**Experimental animals.** Male Hannover-Wistar rats, weighing 250–350 g, were used in this study. Rats were maintained on a 12-hr light–dark cycle and allowed free access to food and water. All experiments were performed between 9 a.m. and 6 p.m. in a silent room, at a temperature of 22–24°C. All experimental procedures involving animals were approved by the Faculty Ethical Committee and were carried out in accordance with the Croatian laws and rules (NN 135/06; NN 37/13; 55/13) and with the guidelines set by the European Community Council Directive (2010/63/EU). All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

**Chemicals.** Chemicals and reagents were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA), unless otherwise specified. Recombinant human erythropoietin (Eprex 2000 IU/0.5 ml) was obtained from Janssen Biologics B.V., Leiden, the Netherlands. RANSOD kit for superoxide dismutase (SOD) (EC 1.15.1.1.) and RANSEL kit for glutathione peroxidase (GSH-Px) (EC 1.11.1.9.) determinations were obtained from Randox Laboratories Ltd. (Crumlin, UK) and protein assay from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

**Surgical preparation.** Rats were anaesthetized with 350 mg/kg of chloral hydrate administered intraperitoneally (i.p.). The cerebral ischaemia-reperfusion injury was performed by right MCAO for 1 hr.

MCAO was performed by intraluminal nylon suture occlusion method as described by Longa *et al.* [23] and Belayev *et al.* [24]. Briefly, under an operating microscope, the right common carotid artery was exposed and carefully dissected free from surrounding nerves and fascia. The internal carotid artery was isolated and separated from the adjacent vagus nerve, and the pterygopalatine artery was ligated close to its origin with a 5-0 nylon suture. Next, a 4-0 silk suture was tied loosely around the mobilized external carotid artery stump, and a 4-cm 3-0-monofilament nylon suture (prepared by blunting the tip of the suture by heating it near a flame) was inserted through the proximal external carotid artery into the internal carotid artery and then into the circle of Willis, effectively occluding the middle cerebral artery (MCA). The suture was inserted 18–20 mm from the bifurcation of the common carotid artery after which the neck incision was closed. The body temperature was maintained at 37 ± 0.5°C with a heating pad and rectal probe. After 1 hr of MCAO, the intraluminal suture was carefully removed and the internal carotid artery was reperfused. The internal carotid artery was isolated only in the sham-operated group, but the middle cerebral artery was not occluded. The animals awakened from anaesthesia were returned to their cages to be allowed free access to food and water. Sham-operated, vehicle-treated rats served as control groups. Animals of all experimental groups were killed 24 hr after the induction of MCAO or sham experimental procedure.

**Measurement of infarct volume.** Infarct volume was determined in ischaemic rats treated with vehicle or rhEpo (5000 IU/kg, i.p.), both administered 3 hr after the induction of ischaemia (n = 6 animals per group). Rat brains were removed from the skull, cut into 1-mm-thick coronal sections using a rat brain matrix (Stoelting Co., Wood Dale, IL, USA) and stained in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in normal saline [25] at 37°C for 30 min. With this dye, the vital tissues are stained red while the infarcted area does not stain. Sections were then preserved in 10% formalin, and the images of the TTC-stained sections were acquired (HP Scanjet G3110, Hewlett-Packard Company, Palo Alto, USA). Total infarct volume was determined by image analysis software (ImageJ, NIH, Bethesda, USA). Infarct volume was calculated by a blinded investigator by adding all cross-sectional areas multiplied by 1 mm (thickness of sections). Oedema correction of infarct volume was achieved using the following equation [26]:

$$V_{\text{edi}} = V_{\text{infarct}} \times (1 - (V_{\text{ipsi}} - V_{\text{contra}})/V_{\text{contra}})$$

$V_{\text{edi}}$  = volume of oedema-corrected infarct,  $V_{\text{infarct}}$  = volume of infarct,  $V_{\text{ipsi}}$  = volume of ipsilateral hemisphere,  $V_{\text{contra}}$  = volume of contralateral hemisphere.

**Biochemical analyses.** For biochemical analyses, rats were randomly divided into six experimental groups (n = 8 animals per group). Sham-operated, vehicle-treated animals served as control group. Ischaemic animals were injected with either vehicle or rhEpo (5000 IU/kg, i.p.). Animals of all experimental groups were treated by either vehicle or rhEpo immediately or 3 hr after the onset of ischaemia. At the end of the experiments, rat brains were quickly removed and placed on ice. Hippocampi were dissected, immediately frozen in liquid nitrogen and stored at –80°C until analysis. The levels of thiobarbituric acid-reacting substances (TBARS), SOD or GSH-Px activities were determined spectrophotometrically in the right rat hippocampi of experimental animals.

**Thiobarbituric acid-reactive substances assay.** Tissue samples were weighed, homogenized by brief sonication in (1/10, w/v) ice-cold 20 mM Tris–HCl buffer, pH 7.4 and homogenates used in the lipid peroxidation assay. The extent of lipid oxidative damage in hippocampal homogenates was determined by measuring the levels of

TBARS, using a modification of a method established by Ohkawa *et al.* [27] with minor modifications as suggested by Callaway *et al.* [28]. In this method, sample homogenates (150  $\mu$ l) were mixed with 75  $\mu$ l of 8.1% sodium dodecyl sulphate and incubated at room temperature for 10 min. After the addition of 975  $\mu$ l of 20% acetic acid (pH 3.5 with 5 M NaOH), samples were centrifuged at  $10,000 \times g$  for 15 min., and the 1-ml aliquots of the supernatants were heated with an equal volume of 0.8% thiobarbituric acid solution for 60 min. in water bath at 95°C. After 5-min. cooling on ice and addition of 2 ml of n-butanol:pyridine mixture (15:1, v/v), the TBARS were extracted by centrifugation at  $2200 \times g$  for 10 min. The absorbance of the superior organic layer was read at 532 nm. Values of the blank probe, containing 20 mM Tris-HCl buffer instead of homogenate, were subtracted from the obtained results. Malondialdehyde (MDA) was used as an external standard.

*Antioxidant enzymes' activity assays.* For determinations of the SOD and GSH-Px activities, hippocampal samples were homogenized in ice-cold phosphate-buffered saline, pH 7.4, centrifuged at  $800 \times g$  for 10 min. at 4°C and aliquots of supernatants were used. For the measurement of the hippocampal SOD activity, a method using the ability of xanthine and xanthine oxidase to generate superoxide radicals was applied [29]. Superoxide radicals react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) and consequently a red formazan dye is produced. The inhibition of the production of this dye by the enzyme present in the sample is measured by spectrophotometer at 505 nm. The analysis procedure was as follows: 50  $\mu$ l of sample and 150  $\mu$ l of xanthine oxidase (80 U/l) were added to 1 ml of the substrate mixture (0.05 mM xanthine, 0.025 mM INT, 40 mM CAPS (pH 10.2) and 0.94 mM EDTA), the absorbances were determined after 30 sec. and after additional 3 min., and the SOD activity per minute was calculated according to the calibration curve, which was determined using the purified bovine erythrocyte SOD as standard. All the procedures were conducted at 37°C. The samples were diluted with 0.01 mM phosphate buffer (pH 7.0) to set the inhibition between 30% and 60% of the uninhibited reaction. One unit of enzyme activity was defined as the quantity of SOD required to cause a 50% inhibition of the absorbance change per minute of the blank (uninhibited) reaction.

GSH-Px activity was determined by the method originally established by Paglia and Valentine [30]. In this test, GSH-Px catalyses the reduction of cumene hydroperoxide while using glutathione as a reducing agent. The oxidized glutathione, in the presence of glutathione reductase and NADPH, is immediately recycled to the reduced state with concomitant oxidation of NADPH to NADP<sup>+</sup>. The decrease in the absorbance of the sample during the reactions monitored spectrophotometrically at 340 nm. One ml of the reaction mixture (4 mM glutathione, 0.5 U/l glutathione reductase, 0.34 mM NADPH in 0.05 M phosphate buffer (pH 7.2) with 4.3 mM EDTA), 20  $\mu$ l of sample and 50  $\mu$ l of cumene hydroperoxide (0.18 mM) were mixed, and after 1 min. and an additional 2 min., the absorbances were recorded. The reaction was conducted at 37°C. One unit of the GSH-Px activity was defined as the amount required to cause the formation of 1  $\mu$ mol NADP<sup>+</sup> from NADPH per min., based on the extinction coefficient for NADPH of 6220 M/cm at 340 nm.

*Western blotting.* Active caspase-3, Nrf2 and HO-1 protein expression levels were determined by Western blotting. Whole right hippocampi from the control animals and the ischaemic animals treated with vehicle or rhEpo, 3 hr after the induction of ischaemia, were used (n = 5 animals per group). Hippocampi were prepared as previously described [31]. In brief, tissue samples were homogenized by brief sonication in 1 ml of lysis buffer containing 25 mM Tris-HCl (pH 7.4) as well as phosphatase and protease inhibitors' cocktail. After 1-hr incubation, lysates were centrifuged at  $10,000 \times g$  for 10 min. at 4°C, supernatants collected and stored at -80°C until analysis. Protein samples were

separated by electrophoresis on 10% polyacrylamide gels and blotted to nitrocellulose membranes. After 1-hr blocking step, membranes were incubated with primary antibody solutions overnight at 4°C. Primary antibodies used were rabbit polyclonal anti-active caspase-3 (1:1000) (Cell Signaling Technology, Beverly, MA, USA), rabbit polyclonal anti-Nrf2 (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-HO-1 (Abcam, Cambridge, UK) and mouse monoclonal anti- $\beta$ -actin (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). On the following day, membranes were incubated for 1 hr with appropriate biotinylated secondary antibody solutions (Dako Cytomation, Glostrup, Denmark) after which streptavidin-horseradish peroxidase conjugate was applied for 30 min., all at room temperature. Protein bands were prepared for the visualization by incubation with Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA), and the chemiluminescent signals were detected with Kodak Image Station 440CF. Densitometric analyses of the bands were performed using the Kodak 1D Image Analysis Software (Eastman Kodak, Rochester, NY, USA).

*Immunohistochemical analyses.* Immunohistochemical analyses were performed in the CA1 region of the hippocampi of control animals or ischaemic animals treated with vehicle or rhEpo, 3 hr after the induction of ischaemia (n = 3–4 animals per group).

For these analyses, animals were perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline. Brains were removed and stored in the fixative solution for 20 hr at 4°C and subsequently embedded in paraffin.

For the neuronal loss determination, immunohistochemical detection of NeuN-stained cells was performed on 3- $\mu$ m paraffin sections. Antigen retrieval was achieved by microwaving slides in citric acid buffer (10 mM, pH 6.0). After blocking step with 5% normal rabbit serum and 1% bovine serum albumin in TBS-Triton X-100 (0.025%), sections were incubated with mouse monoclonal anti-NeuN primary antibody (Millipore, Billerica, MA, USA) overnight at 4°C. The next day, biotinylated secondary rabbit antimouse antibody (Dako Cytomation, Glostrup, Denmark) was applied for 2 hr, followed by 30-min. incubation with red fluorescence emitting DyLight 594<sup>TM</sup>-conjugated streptavidin (Vector Laboratories, Burlingame, CA, USA). Images of the sections, that is CA1 regions of the hippocampi, were obtained using an Olympus BX 51 microscope equipped with an Olympus DP 70 digital camera (Olympus, Japan). For the quantification of the number of NeuN-immunostained nuclei, two to four sections per animal were photographed at  $\times 400$  magnification and then analysed employing the aforementioned ImageJ software.

Immunofluorescent detection of active caspase-3 and Nrf-2 was performed on 3- $\mu$ m paraffin sections, which were prepared for immunostaining in the same manner as the sections used for the neuronal loss determination. After the blocking step, hippocampal slices were incubated with rabbit polyclonal anti-active caspase-3 (1:1000; Cell Signaling Technology, Beverly, MA, USA) or rabbit polyclonal anti-Nrf2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, and the next day, biotinylated secondary anti-rabbit antibody was used at 1:200 dilution (Dako Cytomation, Glostrup, Denmark) followed by incubation with DyLight 594<sup>TM</sup>-conjugated streptavidin (Vector Laboratories, Burlingame, CA, USA). As with NeuN staining, microphotographs of the CA1 regions of the hippocampi were made using the Olympus BX 51 microscope equipped with Olympus DP 70 digital camera (Olympus, Japan) at  $\times 400$  magnification.

*Protein content quantification.* Sample protein concentrations were determined according to the method established by Bradford [32], using purified bovine serum albumin as a standard.

*Statistical analyses.* All statistical analyses were performed using the Statistica 12 software (StatSoft Inc., OK, USA). TBARS levels are

expressed as nmol MDA/mg protein, while SOD and GSH-Px activities are expressed as U/mg protein. Statistical significance for the biochemical analyses and for neuronal loss determination was calculated according to the one-way analysis of variance (ANOVA) and Duncan's post hoc test. For the analysis of the effect of focal cerebral ischaemia and rhEpo treatment on the brain infarct volume, Student's *t*-test was used. Results are expressed as means  $\pm$  S.E.M. In all comparisons,  $p < 0.05$  was considered to indicate statistical significance.

## Results

In the present study, we have investigated effects of the treatment with rhEpo on the brain infarct volume, the neuronal damage, oxidative stress parameters as well as on the expressions of active caspase-3, Nrf2 and HO-1 in the hippocampi of rats exposed to the 1-hr MCAO followed by 23 hr of reperfusion.

### Brain infarct volume.

Our results showed that 1-hr MCAO induced irreversible brain injury in the MCAO/Veh animals. Ischaemic injury was represented as infarct core, that is pale tissue areas *versus* non-ischaemic red-coloured tissue (fig. 1A). We found that total infarct of the brain tissue occupied 38.74% of the right hemisphere in MCAO/Veh animals and that striatum and parts of neocortex were predominately affected. It was shown also that administration of rhEpo, 3 hr after the induction of ischaemia, significantly reduced brain infarct volume in comparison with the ischaemic, vehicle-treated animals ( $t = 2.361$ ;  $p = 0.040$ ). Namely, in MCAO/Veh-treated animals, volume of infarcted tissue was  $163.42 \pm 23.89 \text{ mm}^3$  in comparison with  $84.14 \pm 23.6 \text{ mm}^3$  of MCAO/rhEpo animals (fig. 1B).

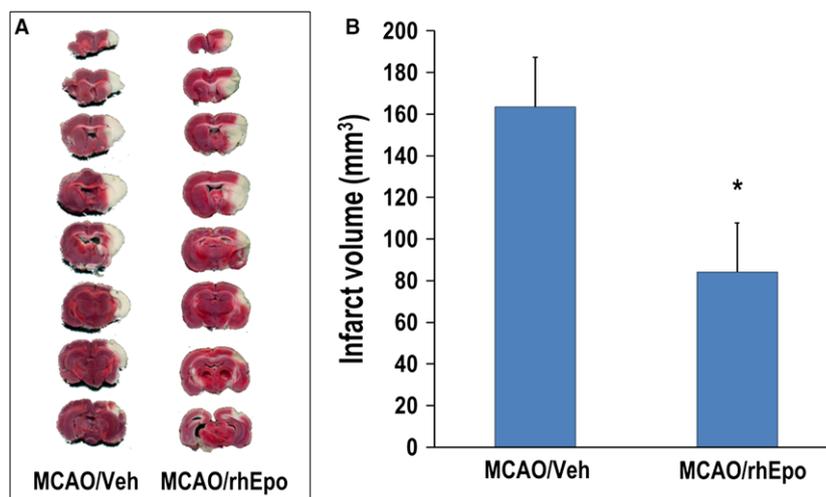


Fig. 1. Brain infarct volume determination in the ischaemic rats treated with recombinant human erythropoietin (rhEpo). (A) TTC staining of the rat brain slices in the ischaemic animals exposed to 1-hr MCAO and treated with either vehicle (MCAO/Veh) or rhEpo (MCAO/rhEpo), 3 hr after the induction of ischaemia. In the MCAO/Veh rats, TTC staining showed severe infarct (pale tissue areas *versus* non-ischaemic red-coloured tissue) at 24 hr after the induction of MCAO. In MCAO/rhEpo animals, significant reduction in infarct size is evident (B) Infarct volume ( $\text{mm}^3$ ) of the MCAO/Veh and MCAO/rhEpo animals. Each value represents the mean of 6 animals  $\pm$  S.E.M. \* $p < 0.05$  significantly different from MCAO/Veh animals.

### Neuronal loss determination.

Fig. 2 shows the NeuN immunoreactivity and the number of NeuN-positive cells in the CA1 region of the hippocampi, in rats of the control group and ischaemic rats treated with either vehicle or rhEpo, 3 hr after the induction of ischaemia. Representative microphotographs showed a decrease in NeuN immunoreactivity in the CA1 hippocampal region of the ischaemic, vehicle-treated animal compared to the rats of the control group. Additionally, rhEpo treatment apparently increased NeuN staining in the examined region of the MCAO exposed animals (fig. 2A). An overall ANOVA did not reveal a statistically significant effect of the treatments on the number of NeuN immunopositive cells in ipsilateral hippocampi, in our experimental conditions [ $F(2,8) = 3.942$ ;  $p = 0.064$ ]. Namely, although the number of NeuN-labelled cells was decreased in MCAO/Veh animals in comparison with the control group ( $443.68 \pm 57.75 \text{ cells/mm}^2$  in the MCAO/Veh animals *versus*  $599.86 \pm 36.42 \text{ cells/mm}^2$  in the control animals), it did not reach statistical significance. In the ischaemic animals treated with rhEpo, the number of NeuN-positive cells was  $513.51 \pm 24.97 \text{ cells/mm}^2$ , and it did not differ significantly in comparison with the control or MCAO/Veh animals (fig. 2B).

### Active caspase-3 protein expression.

In the CA1 hippocampal region of the control rat, weak active caspase-3 immunostaining was detected, mostly within neuronal perikarya (fig. 3A). However, in vehicle-treated ischaemic animals, 24 hr after the MCAO induction, increased active caspase-3 immunostaining was evident within the perikarya and dendrites of the CA1 hippocampal neurons (fig. 3B). In the ischaemic rats treated with rhEpo, active

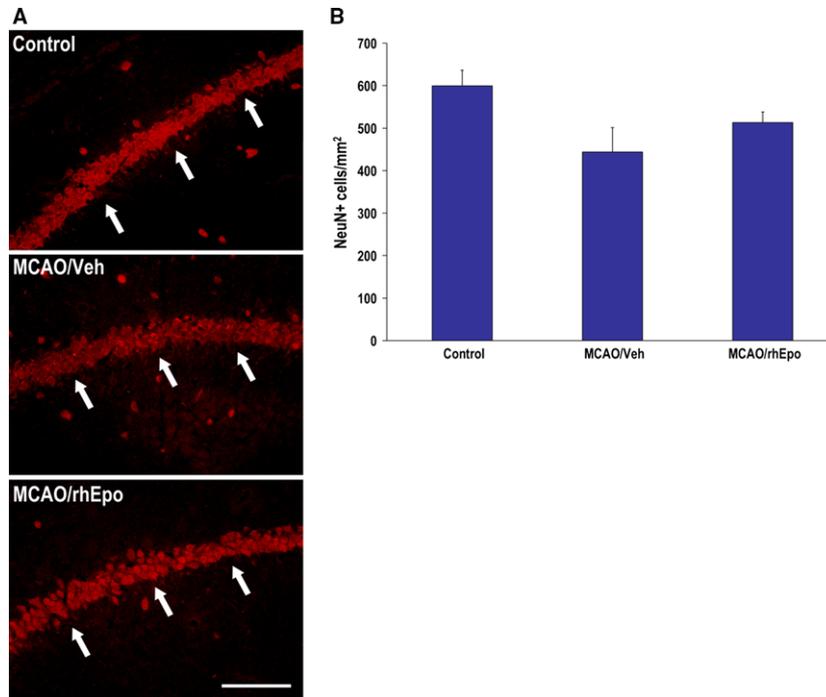


Fig. 2. Neuronal loss measurement in the CA1 hippocampal region of ischaemic rats treated with recombinant human erythropoietin (rhEpo). (A) Representative microphotographs of the NeuN immunohistological staining of neurons, 24 hr after the induction of 1-hr MCAO, in the sham-operated, vehicle-treated animals (Control) and in the ischaemic rats administered with either vehicle (MCAO/Veh) or rhEpo (MCAO/rhEpo), 3 hr after the onset of ischaemic procedure (scale bar: 100  $\mu$ m) (B) Quantification of neuronal loss in the CA1 hippocampal region by counting of NeuN-positive cells. Each value represents the mean of 3–4 animals  $\pm$  S.E.M. White arrows denote NeuN-stained cells.

caspase-3 immunostaining was diminished in the investigated hippocampal region (fig. 3C). Fig. 3D shows the representative active caspase-3 immunoblots and their densitometric analyses for each experimental group. A statistically significant effect of the treatment on mentioned protein's expressions was found [F (2,12) = 5.498,  $p$  = 0.020]. It is evident that the expression level of active caspase-3 in the hippocampi of the ischaemic rats treated with vehicle was significantly higher in relation to the animals of the control group and that there was no significant difference in its expression between ischaemic, vehicle-treated animals and ischaemic animals treated with rhEpo.

#### Parameters of oxidative stress.

To verify the presence of the hippocampal lipid oxidative damage, levels of lipid peroxidation products, TBARS, were measured. Fig. 4 shows TBARS levels in the right hippocampi of the control animals as well as in the ischaemic animals treated with either vehicle (MCAO/Veh) or rhEpo (MCAO/rhEpo), immediately or 3 hr after the induction of MCAO. An overall ANOVA revealed a statistically significant effect of the treatment immediately after [F (2,18) = 9.868,  $p$  = 0.001] or 3 hr after [F (2,20) = 11.069,  $p$  < 0.001] the induction of MCAO. Duncan's multiple range test indicated that hippocampal TBARS levels in the ischaemic animals treated with vehicle immediately after or 3 hr after the induction of MCAO were significantly higher in comparison with the values of the control animals. It was found also that TBARS levels in the

ischaemic animals treated with rhEPO were significantly lower in comparison with the levels of the ischaemic, vehicle-injected animals (fig. 4).

The levels of SOD activities in the right hippocampi of control animals such as ischaemic animals treated with either vehicle (MCAO/Veh) or Epo (MCAO/Epo) in accordance with our experimental protocol are presented in fig. 5. An overall ANOVA revealed a statistically significant effect of the treatment immediately after [F (2,28) = 11.209,  $p$  = 0.003] or 3 hr after [F (2,19) = 7.153,  $p$  = 0.005] the induction of MCAO. It was shown that hippocampal the SOD levels in the ischaemic animals treated with vehicle immediately after or 3 hr after the induction of MCAO were significantly higher in comparison with the levels of the SOD activities in control animals. It was found also that treatment with rhEpo significantly reduced the SOD levels in comparison with the ischaemic, vehicle-injected animals (fig. 5).

An overall ANOVA revealed a statistically significant effect of the treatment on the GSH-Px activities immediately after [F (2,36) = 2.146,  $p$  = 0.132] or 3 hr after [F (2,24) = 9.477,  $p$  < 0.001] induction of MCAO (fig. 6). According to the Duncan's multiple range test, focal cerebral ischaemia caused statistically significant increase in the GSH-Px activities in the right hippocampi of ischaemic animals administered with vehicle both immediately or 3 hr after the induction of MCAO in comparison with the control animals. GSH-Px activities in the ischaemic animals treated with rhEPO were significantly lower in comparison with the level of the ischaemic, vehicle-treated animals.

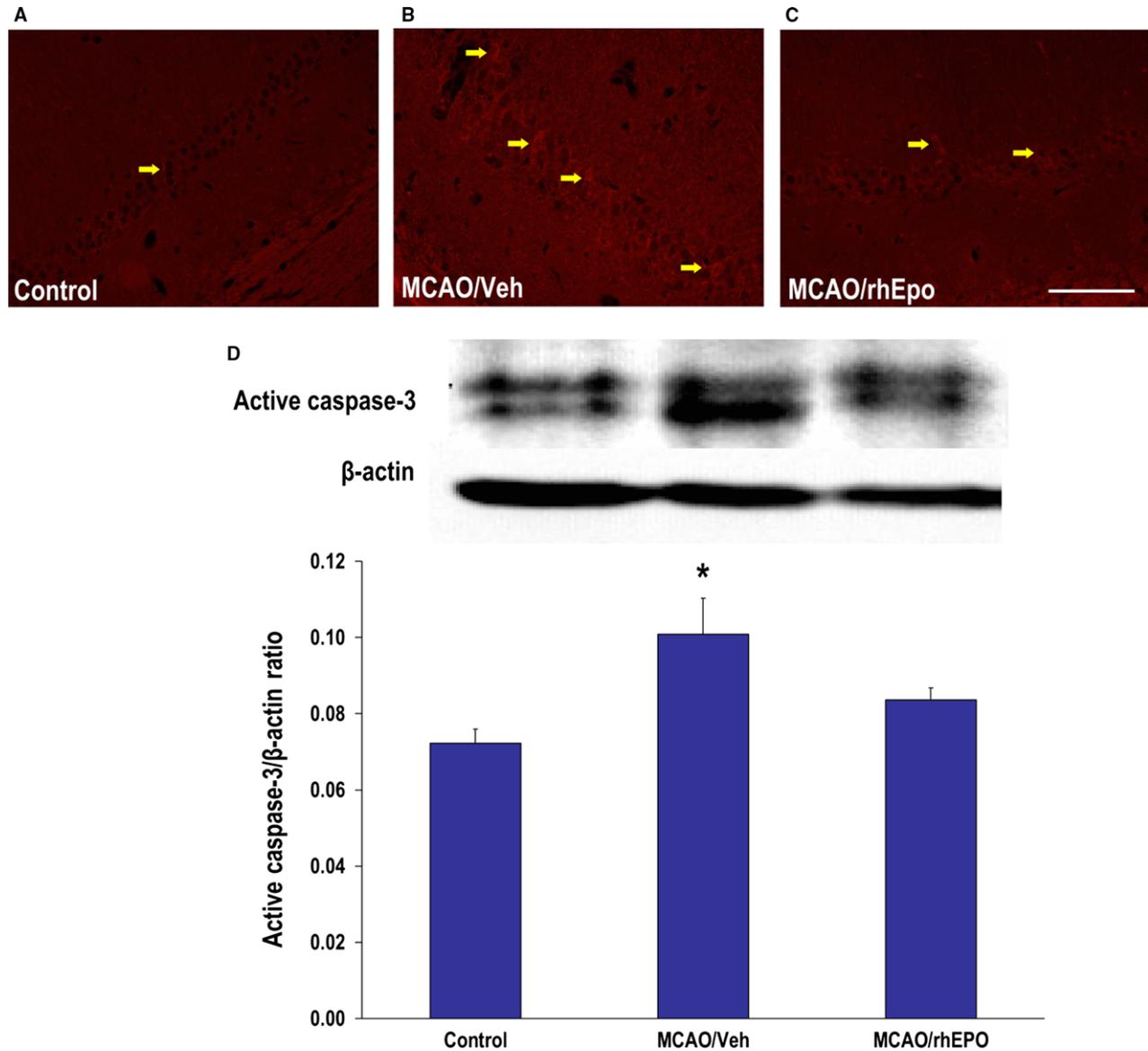


Fig. 3. The influence of the post-ischaemic administration of recombinant human erythropoietin (rhEpo) on the expression of active caspase-3 in the rat hippocampus. Photomicrographs of active caspase-3-stained sections of neurons, 24 hr after the induction of 1-hr MCAO, in the sham-operated, vehicle-treated animals (Control) (A) and in the ischaemic rats administered with vehicle (MCAO/Veh) (B) or rhEpo (MCAO/rhEpo) (C), 3 hr after the onset of the ischaemic procedure. Arrows point to active caspase-3-positive cells (scale bar: 100  $\mu$ m). Representative immunoblots of each group and the active caspase-3 expression levels (caspase-3/ $\beta$ -actin ratio) (D). Data represent mean  $\pm$  S.E.M for 5 rats per group. \* $p < 0.05$ ; significantly different from the control group.

In the control animals, only few Nrf2 immunopositive cells in the CA1 hippocampal region were detected (fig. 7A). In the same brain structure, in the vehicle-treated ischaemic animals, Nrf2 staining intensity was increased (fig 7B) while in the rhEpo-treated ischaemic rats, Nrf2 immunostaining was even more pronounced compared to the vehicle-injected ischaemic animals (fig 7C). To verify the results of the immunohistochemical staining, Western blot assay was used to determine the protein levels of the Nrf2 (fig 7D). An overall ANOVA revealed a statistically significant effect of the treatment [F(2,12) = 13.692,  $p < 0.001$ ]. The results showed that the expression of Nrf2 protein was significantly higher in ischaemic animals treated with vehicle (MCAO/Veh) in comparison

with the control group. Treatment with rhEpo (MCAO/rhEpo) significantly increased the protein levels of Nrf2 in comparison with both the control and MCAO/Veh animals.

The increase in the HO-1 protein expression levels in rhEpo-treated ischaemic animals, compared to both control animals and ischaemic animals treated with vehicle, was not statistically significant (results are not shown).

## Discussion

In the present study, we have investigated the effects of 1-hr MCAO on the extent of the brain infarct volume as well as on the potential neuronal damage, oxidative stress parameters as

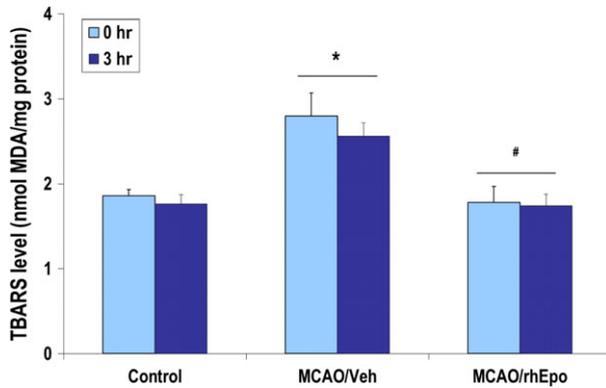


Fig. 4. The influence of the post-ischaemic administration of recombinant human erythropoietin (rhEpo) on the thiobarbituric acid-reacting substances (TBARS) levels in the rat hippocampus. The TBARS levels (nmol MDA/mg protein) in the right hippocampi of the control animals (Control) and of ischaemic animals treated with either vehicle (MCAO/Veh) or rhEpo (MCAO/rhEpo), immediately (0 hr) or 3 hr after the induction of MCAO. The level of TBARS was measured 24 hr after the induction of 1-hr MCAO. Each value represents mean  $\pm$  S.E.M for 8 animals per group. \* $p < 0.05$ ; significantly different from the Control; # $p < 0.05$ ; significantly different from the MCAO/Veh group.

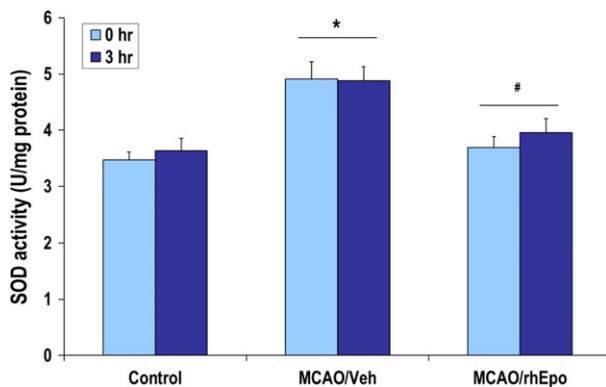


Fig. 5. The influence of the post-ischaemic administration of recombinant human erythropoietin (rhEpo) on superoxide dismutase (SOD) activities in the rat hippocampus. The SOD activities in the right hippocampi of the control rats (Control) and of ischaemic rats treated with either vehicle (MCAO/Veh) or rhEpo (MCAO/rhEpo), immediately after (0 hr) or 3 hr after ischaemia. The SOD activities were measured 24 hr after the induction of 1 hr MCAO. Each value represents mean  $\pm$  S.E.M for eight animals per group. \* $p < 0.05$ ; significantly different from the Control; # $p < 0.05$ ; significantly different from the MCAO/Veh group.

well as on the expressions of active caspase-3, Nrf2 and HO-1 in the hippocampi of rats, ipsilaterally to the infarction. Mentioned parameters were measured 24 hr after the induction of ischaemia. In addition, possible neuroprotective effects of rhEpo (5000 IU/kg, i.p.), administered immediately or 3 hr after the induction of MCAO, on the above-mentioned parameters were examined.

After MCAO, primary ischaemic lesion occurred mostly in the striatum and partly in the cortex, causing a rapid and irreversible neuronal injury [21,33–35]. In this article, we focused

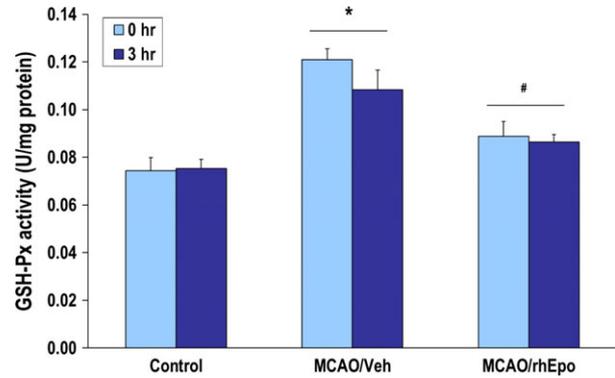


Fig. 6. The influence of the post-ischaemic administration of recombinant human erythropoietin (rhEpo) on glutathione peroxidase (GSH-Px) activities in the rat hippocampus. The GSH-Px activities in the right hippocampi of the control rats (Control) and of ischaemic rats treated with either vehicle (MCAO/Veh) or rhEpo (MCAO/rhEpo), immediately after (0 hr) or 3 hr after ischaemia. The GSH-Px activities were measured 24 hr after the induction of 1-hr MCAO. Each value represents mean  $\pm$  S.E.M for eight animals per group. \* $p < 0.05$ ; significantly different from the Control; # $p < 0.05$ ; significantly different from the MCAO/Veh group.

our interest on the potential damage of the hippocampus although it is not directly supplied by MCA. But, it was shown that it is located within hypoperfused perilesional brain tissue, characterized by processes of delayed neuronal damage. Unlike striatum in which neuronal degeneration begins within 6 hr after MCAO injury, in the hippocampus, it occurs between 12 hr and 7 days after induction of MCAO with neuronal death reaching a peak at 4 days [34,36]. Such delay in progression of tissue damage opens the possibility to salvage affected hippocampal tissue after timely therapeutic intervention and increase possibilities for testing potential molecular mechanisms of neuroprotective drugs. The majority of pre-clinical research in animal models that evaluated the neuroprotective effects of different doses of rhEpo on brain damage, suggested 5000 IU/kg of rhEpo as optimal dosage [37]. It was shown also that rhEpo applied at various time-points ranging from pre-treatment to up to 6 hr or even 24 hr after ischaemia, exerts the best effect when applied within first 3 hr after onset of ischaemia [37–39]. To the best of our knowledge, there are no previously published results that have described the effects of post-ischaemic administration of rhEpo on the SOD and GSH-Px activities such as on Nrf2 expression in the hippocampi of rats exposed to 1-hr MCAO.

The results of our experiments showed that focal cerebral ischaemia induced by right 1-hr MCAO followed by 23 hr of reperfusion causes marked infarction that occupied 38.74% of the right brain hemisphere. The main affected areas included mostly striatum and neocortex due to the lack of collateral blood supply in those regions. Our findings are in good correlation with results of authors who described the spatiotemporal evolution of brain infarct within the area blood supplied by MCA [21,33–35]. It was shown that 30 min.–1 hr of MCAO causes irreversible tissue injury with infarct core development predominantly in the striatum and partially neocortex within first 12–24 hr. Administration of 5000 IU/kg of rhEpo in our

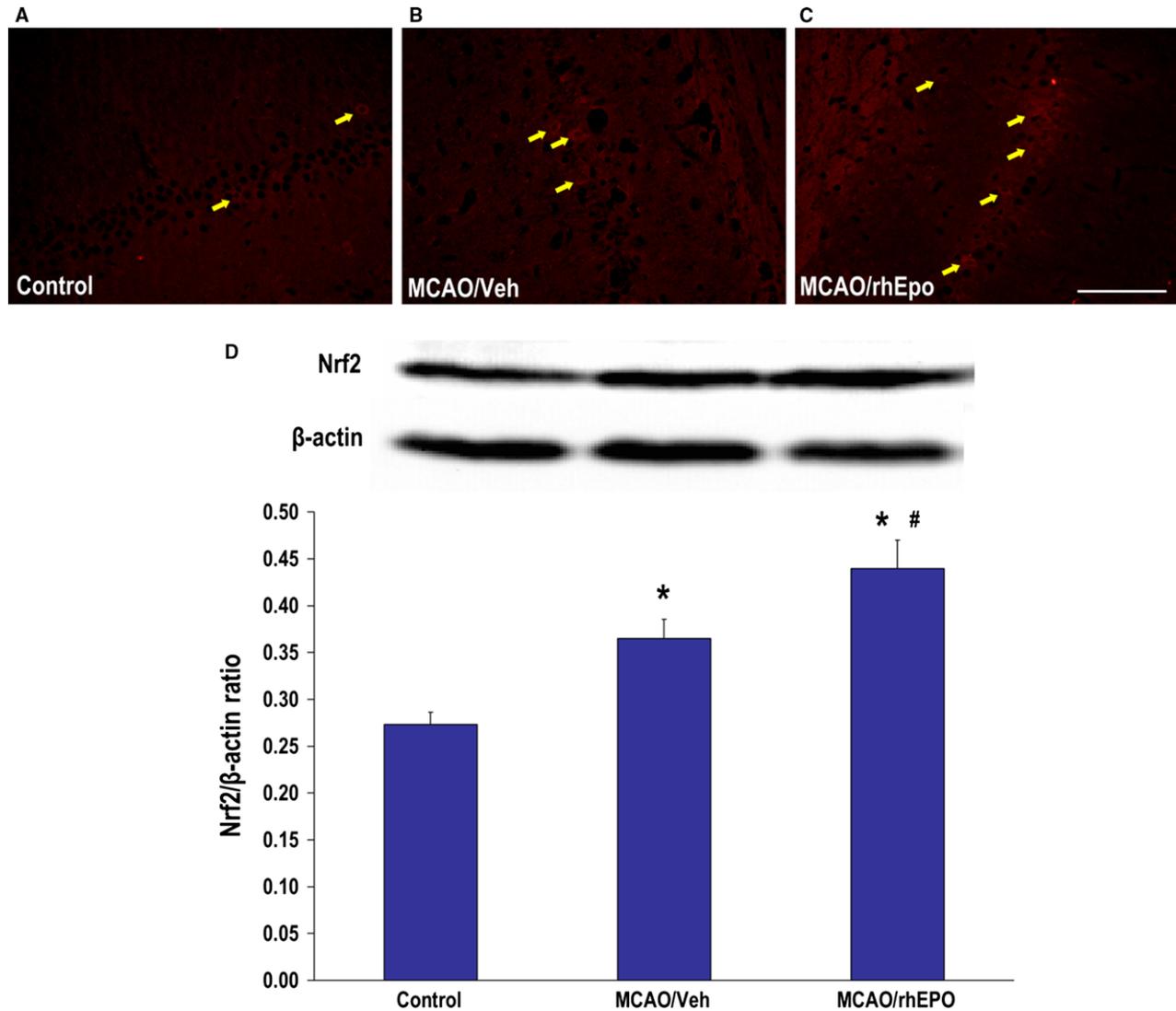


Fig. 7. The influence of the post-ischaemic administration of recombinant human erythropoietin (rhEpo) on the nuclear factor erythroid 2-related factor 2 (Nrf2) expression in the rat hippocampus. Photomicrographs of Nrf2-stained sections of neurons, 24 hr after the induction of 1-hr MCAO, in the sham-operated, vehicle-treated rats (Control) (A) and in the ischaemic animals administered with either vehicle (MCAO/Veh) (B) or rhEpo (MCAO/rhEpo) (C), 3 hr after the onset of the ischaemic procedure. Arrows point to the Nrf2-positive cells (scale bar: 100 μm). Representative immunoblots of each group and the Nrf2 expression levels (Nrf2/β-actin ratio) (D). Data represent mean ± S.E.M for 5 rats per group. \* $p < 0.05$ ; significantly different from the Control group; # $p < 0.05$ ; significantly different from the MCAO/Veh group.

experimental conditions, that is 3 hr after induction of 1-hr MCAO, significantly reduced the extent of the brain infarct volume in the right hemisphere in comparison with ischaemic, vehicle-treated animals. Neuroprotective effect of rhEpo administration was described also by findings of other authors although in different experimental conditions. Prosvirina *et al.* [40] showed that i.p. application of 5000 IU/kg of rhEPO either before or even up to 12 hr after induction of 30 min. MCAO, significantly reduced the size of the brain infarction. Siren *et al.* [38] showed that injection of rhEpo (5000 IU/kg, i.p.) at the beginning of 1-hr MCAO procedure, decreased infarct size by 75% and suppressed apoptosis in the ischaemic penumbra in rats. Application of lower dose of rhEpo (800 IU/kg) via middle cerebral artery infusion also reduced the infarct volume significantly after 2 hr of MCAO

[41]. Recently, two meta-analyses concerning the effects of systemically applied rhEpo in animal models of ischaemic stroke were published, and in both studies, it was concluded that when rhEpo was administered after ischaemia, infarct size was reduced by about 30% and was associated with improved neurobehavioural outcome [19,42]. It was shown also that after MCAO, hippocampal neurons (although not primarily supplied by MCA) could be injured [36]. As TTC staining is not suitable for identification of perilesional affected tissue [43], immunohistochemical marker NeuN could be used to examine a possible neuronal loss in the injured tissue. Immunoreactivity for NeuN has been reported to decrease dramatically after MCAO and peaked between 6 hr and 24 hr of stroke in striatum and cortex [44]. We have examined a potential neuronal loss in the hippocampal tissue 24 hr after

induction of MCAO. Our results indicated that within first 24 hr of reperfusion, the registered decrease in NeuN staining was not statistically significant. According to the literature, immunostaining for NeuN that detected loss of CA1 neurons in hippocampus occurs 2–4 days after global cerebral ischaemia [45,46] while Zhou *et al.* [47] did not find significant CA1 neuronal loss within 10 weeks after 2-hr MCAO in comparison with the control animals. In conclusion, within first 24 hr after 1-hr MCAO, there was no significant neuronal loss of the hippocampal tissue. However, results of our experiments demonstrated a statistically significant increase in the active caspase-3 expression in the CA1 hippocampal neurons of the ischaemic animals. Increased levels of terminal caspases, especially caspase-3, as one of the effector caspase that has a pivotal role in the development of apoptotic neuronal death are often observed at sites of neuronal damage in a number of diseases, including stroke and different neurodegenerative diseases [48]. For example, significant increase in the hippocampal active caspase-3 expression after 2-hr MCAO followed by 24-hr reperfusion or 90-min. MCAO followed by 72-hr reperfusion was described [49,50]. In addition, various authors showed that pharmacological inhibition or molecular blockage of active caspase-3, including rhEpo administration, significantly decreases infarct size or attenuates the loss of hippocampal CA1 neurons after transient cerebral ischaemia [17,48,49]. We found that after administration of rhEpo, decrease in caspase-3 over-expression was not statistically significant, thus indicating that in our experimental conditions, administration of 5000 IU rhEpo was not able to prevent caspase-3-dependent apoptotic cell death.

In addition, we found a significant increase in oxidative stress parameters in hippocampal neurons. The results of our experiments showed that MCAO followed by 23 hr of reperfusion caused statistically significant increase in TBARS levels as indicators of oxidative damage of hippocampal neurons caused by peroxidation of membrane lipids. Similar findings with significant rise in the TBARS levels were reported by results of the authors who analysed the level of TBARS after MCAO of various duration (1–2 hr), followed by reperfusion either in hippocampus [51] or in cortical tissue [52,53]. In addition, Cano *et al.* [54] and Cojocar *et al.* [55] showed also that induction of lipid peroxidation was significantly increased in human ischaemic stroke within the first 24 hr. It is considered that a rise in lipid peroxidation is a valuable early marker of I/R injury developed in a period in which there were still no signs of severe neuronal damage or death [56]. Our results clearly showed that administration of 5000 IU/kg of rhEpo either immediately or 3 hr after the induction of ischaemia, significantly reduced the level of the lipid peroxidation in the ipsilateral hippocampal neurons, suggesting its neuroprotective, antioxidant effect in the MCAO rat model. Although there are a few published data related to rhEpo effect on the TBARS levels under ischaemic conditions caused by intermittent hypoxia-ischaemia or global cerebral ischaemia [57–59], we are the first to report the effect of this drug on the TBARS levels in the hippocampus of animals exposed to oxidative stress caused by 1-hr MCAO. According

to Sakanaka *et al.* [60], rhEpo may inhibit lipid peroxidation in ischaemic tissue either by its inhibitory effect on excitotoxicity, inhibition of  $\text{Ca}^{2+}$  entry, reduction in the NO-mediated formation of free radicals or by its direct free radical scavenging effect.

Enzymatic antioxidant protection of the tissue affected by I/R against ROS and oxidative stress is ensured by cooperative action of SOD, catalase and GSH-Px as dominant endogenous antioxidants [61]. As increase in ROS production occurs within the first 10–15 min. of reperfusion [62], early rise in antioxidant scavenging enzyme activities is particularly important. It is well known that activation of SOD is beneficial only in the presence of sufficient  $\text{H}_2\text{O}_2$ -detoxifying enzyme, GSH-Px [63]. It was shown also that in genetically modified animals, over-expression of both SOD or GSH-Px needs to be maintained at basal or higher level to effectively eliminate harmful ROS effects [64] and that early induction of SOD caused by STAT3 activation is important in reduction of oxidative stress [65]. In our experimental conditions, 24 hr after the induction of cerebral ischaemia, a statistically significant increase in the hippocampal SOD and GSH-Px activities was detected in MCAO/Veh animals in comparison with the control groups. Although different published studies reported decrease in SOD and GSH-Px activities after focal cerebral ischaemia induced by MCAO [66–69], results similar to our findings were described by several authors. They showed that a rise in the SOD and/or GSH-Px activities occur in various models of cerebral ischaemia and in different brain regions. Thus, Bonova *et al.* [70] registered significant increase in total SOD and MnSOD activities 24 hr after 1-hr MCAO in striatum and cortex. In addition, it was shown that within the first hour after permanent MCAO, the SOD activity was increased by 42% in ipsilateral cortex [71]. Focal cerebral ischaemia induced by left common carotid artery occlusion for 0.5, 1, 4 or 6 hr, respectively, caused a significant increase in the total brain SOD and GSH-Px activities 24 hr after induction of 0.5–1 hr of focal cerebral ischaemia [56]. After global cerebral ischaemia, significant increase in the SOD activities was registered after both common carotid arteries occlusion for 10–20 min. and measured from 1 hr up to 7 days of reperfusion [70,72,73]. Viggiano *et al.* [74] showed also that a rise in the SOD activity found 1 hr after the induction of global cerebral ischaemia was followed by a decrease in  $\text{O}_2$  - concentration. It is assumed that such early induction of antioxidant enzyme activities indicates tissue effort to overwhelm oxidative stress due to increase in ROS and to compensate decreased capability of other endogenous mechanisms of tissue neuroprotection [75]. As activation of the Keap1–Nrf2/ARE pathway in the peri-infarcted tissue is considered as a master regulator of endogenous compensatory adaptation against focal cerebral ischaemia and a major protective mechanism against oxidative stress-induced cell damage and death [76], we have analysed the expression of Nrf2 and its downstream enzyme HO-1 in the rat hippocampi, 24 hr after the induction of focal cerebral ischaemia. Results from immunohistochemical stainings of the CA1 hippocampal region in ischaemic rats showed the increase in the Nrf2 expression that was also demonstrated by

Western blot analyses of the hippocampal tissue. These results are in accordance with findings of Takagi *et al.* [77], who have evaluated temporal activation of Nrf2 in the penumbral region, mostly in mice cortical neurons. It was shown that endogenous Nrf2 up-regulation was not detected early after reperfusion but expression peaked 24 hr after ischaemia. Similar findings were reported by Yang *et al.* [78] who revealed a time-dependent increase in Nrf2 expression, starting at 3 hr, peaking at 24 hr and declining after 48–72 hr following permanent MCAO in rat cortex. Although HO-1, as redox-sensitive inducible stress protein is directly modulated by Nrf2, its increase in our experimental conditions was not statistically significant. A possible explanation for such a result could be based on the results of Tanaka *et al.* [76]. According to them, Nrf2 expression in the peri-infarcted region of mouse brain after transient 1-hr MCAO shows different, time-dependent increase in the Nrf2 and HO-1 expressions. While a rise in Nrf2 started at 3 hr with maximal increase at 8–24 hr, the increase in HO-1 expression was delayed, starting from 24 to 72 hr. Our results showed also that after rhEpo administration, both immediately or 3 hr after the induction of focal cerebral ischaemia, the activities of SOD and GSH-Px were significantly reduced in comparison with vehicle-treated ischaemic animals. Various previously published studies described the influence of different doses of rhEpo (1000 IU/or 20 000 IU) on antioxidant enzyme activities like glutathione-S transferase, haem oxygenase-1 or GSH-Px, in a different experimental model of the brain damage (subarachnoid haemorrhage, ethanol intoxication, hyperoxia, inflammation, etc.) [16,79], but to the best of our knowledge, we are the first to report the effects of 5000 IU/kg of rhEpo after its post-ischaemic administration, on the SOD and GSH-Px activities after 1-hr MCAO in rat hippocampus. We assumed that decrease in mentioned antioxidant enzymes activities could be indicator of a decrease in the level of oxidative stress in the hippocampal tissue of the ischaemic animals induced by rhEpo administration. Recently, it was shown that this drug could modulate antioxidant enzyme activities including SOD and GSH-Px by modulating Nrf2 and endothelial nitric oxide synthase (eNOS) pathways [16,79]. Namely, it seems that rhEpo activates Nrf2 by activating Akt and Erk with consequent phosphorylation of eNOS and increase in neuronal nitric oxide (NO). It is responsible for Nrf2 release and activation of antioxidant cytoprotection [79] such as increase in neurovascular protection via improvement of cerebral blood flow [10]. It was shown also that increase in Nrf2 expression leads to the reduction of inflammation-related factors, such as inducible NOS, cyclooxygenase 2 or interleukin-6 [80,81]. To further clarify the possible mechanisms of its antioxidant action, the effect of rhEpo on the expression of Nrf2 and HO-1 was tested. We have found that administration of rhEpo caused additional statistically significant increase in Nrf2 expression in hippocampi of ischaemic animals exposed to 1-hr MCAO while the increase in HO-1 was not statistically significant. The increase in neuronal antioxidant capacities modulated by rhEpo-regulated Nrf2 pathway was reported by several authors in different experimental models of cerebral ischaemia [16], but to the

best of our knowledge, we are the first who evaluated the effects of post-ischaemic administration of rhEpo on the Nrf2 expression in CA1 hippocampal neurons of rats exposed to 1-hr MCAO. Increase in the Nrf2 expression found in our experimental conditions indicated that the endogenous antioxidant system was activated in perilesional hippocampal tissue 24 hr after the induction of MCAO and that a possible beneficial effect of rhEpo could be attributable to additional activation of Nrf2. Decreased activities of antioxidant enzymes SOD and GSH-Px in hippocampal tissue indicated that 24 hr after MCAO, the level of oxidative stress is reduced after rhEpo administration. Although further research is necessary to elucidate these findings, our results could be a valuable contribution to understanding the complex changes in antioxidant enzyme activities in perilesional hippocampal tissue under ischaemic conditions such as possible neuroprotective effects of rhEpo after ischaemic stroke.

In conclusion, this is the first study to demonstrate that reductions in the levels of lipid oxidative damage and antioxidant enzymes of SOD and GSH-Px activities after rhEpo post-ischaemic administration could be indicators of decreased oxidative stress in hippocampal neurons and eventual neuroprotective effect of rhEpo in the MCAO model of cerebral ischaemia. To clarify other possible mechanisms of rhEpo antioxidant action in our experimental conditions, further experiments are necessary.

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#### Conflict of interest

The authors declare that there is no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within 3 years of beginning the submitted work that could inappropriately influence, or be perceived to influence their work.

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