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The influence of erythropoietin treatment on oxidative stress parameters in cortex of rats exposed to transient middle cerebral artery occlusion

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Abstract

Background and Purpose: *Erythropoietin (Epo) plays a central role in process of erythropoiesis. Recently, its neuroprotective potential was reported in various experimental models of brain damage. However, the mechanism of Epo protection is still unclear. In the present study, we examined the effect of Epo administration on lipid peroxidation levels and antioxidant enzymes' (superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px)) activities in rat cortex following focal cerebral ischemia.*

Material and Methods: *Focal cerebral ischemia was induced in male Hanover Wistar rats (250–350 g) by right middle cerebral artery occlusion (MCAO) model for 1 hr. After 23 hrs of reperfusion, ischemic animals were sacrificed and the levels of lipid peroxidation, SOD and GSH-Px activities were determined spectrophotometrically in the right cortex. Ischemic animals received either vehicle or Epo (5000 IU/kg, intraperitoneally) immediately or 3 hrs after MCAO, and were sacrificed 23 hrs or 21 hrs later, respectively. Sham operated, vehicle treated animals served as the control group.*

Results and Conclusion: *Focal cerebral ischemia significantly increased the level of oxidative lipid damage parameters in the right cortex as compared to the control group without affecting SOD and GSH-Px activities. The decrease in level of lipid peroxidation after Epo treatment was registered but it was not statistically significant. Our results indicate that focal cerebral ischemia caused neuronal damage in the right cortex and that Epo treatment was not effective in preventing above mentioned alteration.*

INTRODUCTION

Stroke is the second leading cause of death and a major cause of disability worldwide, but treatment possibilities are still very limited *(1)*. Almost 80% of human strokes are of ischemic origin *(2)* and most of them are caused by middle cerebral artery occlusion (MCAO). So, in experimental conditions, MCAO model is widely used to closely mimic the changes that occur during and after human ischemic stroke. Experimentally and clinically, oxidative stress is one of the most important causative factors that contributes to neuronal damage induced by cere-

bral ischemia/reperfusion. Namely, cerebral ischemia induces an imbalance of endogenous oxidants and antioxidants and overproduction of toxic free radicals *(3)*. Initial brain damage is further potentiated by massive production of reactive oxygene species (ROS) and reactive nitrogen species (RNS) during reperfusion period. Accumulation of toxic free radicals therefore not only increase the susceptibility of brain tissues to oxidative damage but also trigger various cascades of ischemic injury leading to either direct injury via membranous lipid peroxidation and protein and DNA oxidation or indirect damage via inflammation and apoptosis *(4)*. For these reasons, antioxidant agents have been the focus of studies for developing neuroprotective drugs to prevent and treat stroke. Certain brain regions, such as the striatum, cortex and particularly the hippocampus, are more susceptible to oxidative stress and ischemic damage *(5)*.

The antioxidant enzyme activity of the tissue affected by ischemia/reperfusion represents the primary endogenous defense against oxygen free radicals *(6)* and involves the cooperative action of the main intracellular antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). Although the neuroprotective role of SOD and GSH-Px against ischemic neuronal damage is widely recognized, the post-ischemic alterations in their activity are not completely understood.

Erythropoietin (Epo) is glycoprotein hormone i.e. hematopoetic growth factor which is predominantly secreted by the kidneys in response to tissue hypoxia *(7)*. Besides the known role of this substance in the regulation of erythropoiesis stimulation, a significant change in research interest came after it was found that Epo is produced in the central nervous system in response to metabolic/oxidative stress. It was shown that expression of Epo and erythropoietin receptors (EpoR) is prominent in the peripheral zone of the cerebral infarction, penumbra *(8)*. Moreover, despite previous evidence, it is demonstrated that Epo crosses the blood brain barrier in an injured brain and that its administration immediately before or up to 6 hrs after cerebral ischemia reduces cerebral infarction significantly *(9)*. In addition, the results from the administration of recombinant human erythropoietin (rhEpo) in acute stroke patients confirmed its effectivenes in protecting cerebral tissue from ischemia *(10)*. It is assumed that its benefitial effect is the consequence of activation of various biochemical pathways that provide antiapoptotic, antioxidative, and anti-inflammatory response to ischemia *(11)* but still the exact mechanism of its neuroprotection is not completely understood.

The objectives of our study were to examine the effects of a) focal cerebral ischemia/reperfusion on the levels of lipid peroxidation (TBARS) and antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in rats exposed to transient middle cerebral artery occlusion and b) systemic administration of Epo on above mentioned parametars of oxydative stress.

MATERIAL AND METHODS

Animals

Male Hannover-Wistar rats from our breeding stock, weighing 250–350 g were used in this study. Rats were maintained on a 12 h 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 °C–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 19/99; NN 176/04) and with the guidelines set by the European Community Council Directive of 24 November 1986 (86/609/EEC).

Animals were divided into several experimental groups, consisting of eight animals randomly assigned to each group. Ischemic animals were exposed to focal cerebral ischemia by right MCAO for 1 hr. After 23 hrs of reperfusion, animals were sacrificed and the levels of lipid peroxidation, SOD and GSH-Px activities were determined spectrophotometrically in the right cortex. Ischemic animals received either vehicle or Epo (5000 IU/kg, intraperitoneally, i.p.) immediately or 3 hrs after MCAO, and were sacrificed 23 hrs or 21 hrs later, respectively. Sham operated, vehicle treated animals served as the control group.

Materials

Erythropoetin (Eprex 2000 IU/0,5 mL) was obtained from Cilag AG, Switzerland. Chemicals and reagents were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA), unless otherwise specified. 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 intraperitoneally (i.p.) with 350 mg/kg of chloral hydrate. The cerebral ischaemia-reperfusion injury was performed by right middle cerebral artery occlusion (MCAO) for 1 h. MCAO was performed by intraluminal nylon suture occlusion method as described by Longa *(12)* and Belayev *(13)*. Briefly, under an operating microscope, the right common carotid artery was exposed and was carefully dissected free from surrounding nerves and fascia. The internal carotid artery was isolated and carefully 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 length of 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 thence into the circle of Willis, effectively occluding the middle cerebral artery. The suture was inserted 18–20 mm from the bifurcation of the

common carotid artery after which the neck incision was closed. After 1 h 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 anesthesia were returned to their cages to be allowed free access to food and water.

Sample preparation

The brains of experimental animals were quickly removed and placed on ice. Neocortex was dissected, immediately frozen in liquid nitrogen and stored at –80 °C until the analyses. Tissue samples were weighed, homogenized by brief sonication in (1/10, w/v) ice-cold 20 mM Tris-HCl buffer, pH 7.4 and aliquots of the homogenates used in the lipid peroxidation assay. For determinations of the SOD and GSH-Px activities, homogenates were centrifuged at 800 × g for 10 min at 4 °C and aliquots of supernatants were used.

Lipid peroxidation assay

Lipid peroxidation levels were determined by measurement of thiobarbituric acid-reacting substances (TBARS) content, using a modification of a method described by Ohkawa *et al. (14),* with some modifications. Tissue homogenates (150 μL) were mixed with 75 μL of 8.1% sodium dodecyl sulphate and incubated at room temperature for 10 min. Following the addition of 975 μL of 20 % acetic acid (pH 3.5 with 5 M NaOH), samples were centrifuged at 10,000 × g for 15 min. Supernatant aliquots (1 mL) were then mixed with an equal volume of 0.8% thiobarbituric acid solution and heated in water bath at 95 °C for 60 min. Reaction was stopped by cooling the samples on ice and the pink-stained TBARS were extracted by addition of 2 mL of n-butanol:pyridine mixture (15:1, v/v) and by centrifugation at 2,200 × g for 10 min. The upper organic layer was used for spectrophotometric absorbance measurement at 532 nm. Sample buffer was used as blank and malondialdehyde (MDA) was used as an external standard.

SOD activity

Method employing xanthine and xanthine oxidase to generate superoxide radicals was used in order to measure the SOD activity *(15)*. These superoxide radicals react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) thus producing a red formazan dye. The enzyme present in the sample competes with the INT for superoxide radicals consequently inhibiting the production of the formazan dye. The reaction is monitored spectrophotometrically at 505 nm. The standard assay substrate mixture contained. The sample aliquot (50 μL) and xanthine oxidase (150 μL, 80 U/L) were added to 1 mL of the preheated (37 °C) substrate mixture (0.05 mM xanthine, 0.025 mM INT, 40 mM CAPS (pH 10.2) and 0.94 mM EDTA) and the initial absorbance was recorded after 30 s, and the timer was started. Final absorbance was recorded after additional 3 min. Purified bovine erythrocyte SOD was used in order to obtain a calibration curve. All samples were diluted with 0.01 mM phosphate buffer pH 7.0 to cause an 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

GSH-Px activity was determined by the method described by Paglia and Valentine *(16)*. This assay employs the ability of GSH-Px from the test samples to catalyze the reduction of cumene hydroperoxide, using glutathione as a reducing agent. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately recycled to its reduced state with simultaneous oxidation of NADPH to $NADP^+$. This results in decrease in absorbance which is monitored spectrophotometrically at 340 nm. The reaction mixture (1 mL, containing 4 mM glutathione, 0.5 U/L glutathione reductase and 0.34 mM NADPH in 0.05 M phosphate buffer (pH 7.2) with 4.3 mM EDTA) was preheated (37 °C) and the reaction was initiated by the addition of 20 μL of sample aliquot and 50 μL of cumene hydroperoxide (0.18 mM). Initial absorbance was recorded after 1 min and its decrease was observed again after further 2 min. One unit of the GSH-Px activity was defined as the amount required to cause the formation of 1 μmol $NADP^+$ from NADPH per min, based on the extinction coefficient for NADPH of 6220 $(M\ cm)^{-1}$ at 340 nm.

Protein content quantification

Protein concentrations were determined according to Bradford *(17)* using purified bovine serum albumin as a standard.

Statistical analyses

All statistical analyses were performed using Statistica 8.0 software (StatSoft Inc., OK, USA). To investigate possible differences in biochemical parameters tested analyses of variance (ANOVA) and Duncan's post hoc test were used. In all comparisons $P < 0.05$ was considered to indicate statistical significance.

RESULTS

We examined the effects of focal cerebral ischemia/reperfusion such as the influence of i.p. administration of 5000 IU/kg Epo (injected immediately after and 3 hrs after ischemic procedure) on the parameters of oxidative stress i.e. the levels of lipid peroxidation (TBARS) and activities of antioxidant enzymes SOD and GSH-Px in right cortex of experimental animals.

As indicators of the brain lipid oxidative damage, TBARS levels were measured in the right cortex of control animals such as ischemic animals treated either with vehicle or Epo, immediately after or 3 hrs after MCAO. Statistically significant effect of the focal cerebral ische-

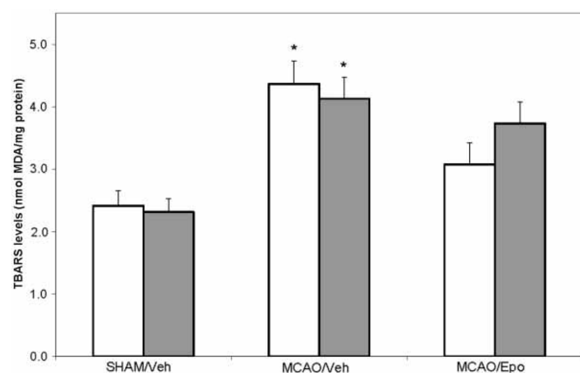

Figure 1. *The level of TBARS in the right cortex of the sham operated animals and ischemic animals (MCAO) treated with vehicle or Epo (5000 IU/kg, i.p.), respectively. Animals were treated immediately (□) or 3 h (■) after reperfusion. Each value represents the mean±S.E.M. *<0.05: significantly different from the sham-operated animals.*

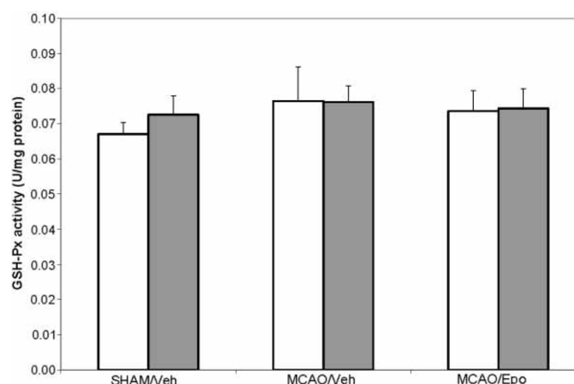

Figure 3. *GSH-Px activity in the right cortex of the sham operated animals and ischemic animals (MCAO) treated with vehicle or Epo (5000 IU/kg, i.p.), respectively. Animals were treated immediately (□) or 3 h (■) after reperfusion. Each value represents the mean±S.E.M.*

mia on the brain lipids was found in the right cortex [F (5,42) = 3,29; *P* = 0,013] of rats. TBARS levels were elevated in ischemic animals (178,9 % imediately after and 180,9% 3 hrs after MCAO procedure) comparing to the TBARS values (100%) in the cortex of animals from control groups. Treatment with Epo did not influence significantly the TBARS levels neither immediately after nor 3 hrs after MCAO [F (3,25) = 2,30; *P* = 0,1] (Figure 1.).

Similar findings were represented on Figure 3. Namely, the level of GSH-Px activity in right cortex of ischemic animals treated with vehicle or Epo, immediately after or 3 hrs after MCAO was not changed significantly in comparison to control animals in our experimental condition [F (5,58) = 1,76; *P* = 0,13].

DISCUSSION

The results of presented experiments showed that focal cerebral ischemia induced by MCAO caused statistically significant increase in TBARS level in the right cortex, both immediately after and 3 hrs after ischemic procedure. Our findings, suggesting increase in lipid peroxidation and oxidative cell damage, are supported by different authors who also presented the increase in lipid peroxidation after ischemic insult. Pratap *et al.* *(18)* reported elevation of TBARS following MCAO occlusion for two hours followed by reperfusion for 22 hours. The estimation of increased markers of oxidative stress was registered in the whole brains. Similar findings were described by Kato *et al.* *(19)* who measured the TBARS levels, as indicators of the extent of lipid peroxidation by free radicals in rats subjected to one hour of MCAO and 4, 24, or 72 hours of reperfusion in cortex and hippocampus. Increased lipid peroxidation was noted also after 90-min MCAO in rat cortex, 24 h after reperfusion *(20, 21)*. The activities of SOD and GSH-Px enzymes after focal cerebral ischemia were tested in our study too. SOD which catalyzes the dismutation of O2 to hydrogen peroxide (H2O2), is a major ROS scavenger. There are three isozymes of SOD, and each displays unique subcellular locations, and plays anti-oxidative roles in various compartments. Cu/Zn-SOD is localized in the cytosol and nucleus of all cell types, and functions as the intracellular anti-oxidative system. Manganese SOD (Mn-SOD) is exclusively localized in the mitochondria. The unique extracellular distribution and secretory nature of extracellular SOD (EC-SOD) offer anti-oxidative protection against ROS not only in the cytosol but also in the extracellular space. It was demonstrated that increased expression of the different forms of SOD decreases ischemia/reperfusion-induced lipid peroxidation and increases neuron survival *(22)*. GSH-Px is a ubiquitous antioxidant enzyme that catalyzes the breakdown of hydrogen peroxide into water. *In vitro* studies have demonstrated that glutathione peroxidase is a more potent antioxidant than either SOD or catalase. The potent antioxidant effects of GSH-Px can be attributed to its presence in the cytosol and mitochondria, and the fact that it is able to scavenge lipid peroxides and hydrogen peroxide *(23, 24)*. In our experimental conditions, the activities of antioxidant enzymes SOD and GSH-Px did not changed significantly in right cortex after focal cerebral is-

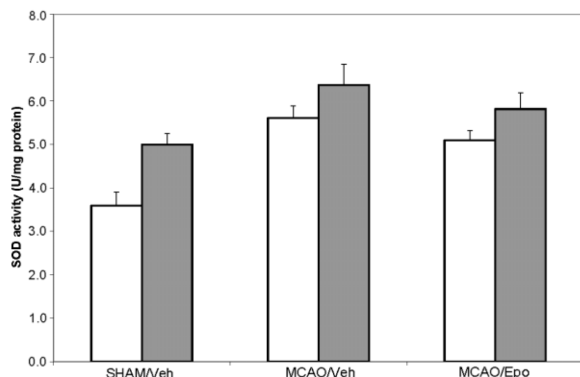

Figure 2. *SOD activity in the right cortex of the sham operated animals and ischemic animals (MCAO) treated with vehicle or Epo (5000 IU/kg, i.p.), respectively. Animals were treated immediately (□) or 3 h (■) after reperfusion. Each value represents the mean±S.E.M.*

chemia. This is in contrast with results of various authors who described alterations in antioxidant enzyme activities following focal or global ischemia *(25, 26)*. Thus, it was shown that activities of SOD and GSH-Px increased after focal cerebral ischemia, possibly as a compensatory mechanism of endogenous antioxidants after increased free radical generation and as a cellular effort to scavenge oxygen free radicals *(22, 23)*. But, as the extent of oxidative stress progresses, the level of SOD and GSH-Px activities decreased *(27)*. Usually, an increased rate of radical production frequently elicits, as a response, an increase in the levels of antioxidant enzymes such as SOD and/or GSH-Px. However, in the initial stages of the insult and/or under marked oxidative stress conditions, enzyme inactivation could prevails and enzymatic activities were not change or were reduced, leading to autocatalysis of the oxidative damage process. Peng et al. showed that after cerebral hypoperfusion, activities of SOD and GSH-Px significantly increased in hippocampus, but they did not registered any significant change in enzymatic activities in cortex *(28)*. According to their findings this may be because the basal levels of SOD and GSH-Px in the cortex of the sham rats are relatively high, and the hypoperfusion only caused a small increase. In addition, the rate of cerebral perfusion and the morphological integrity of the circulatory network of the brain play important role in the maintenance of neuronal function *(29)*. It was demonstrated that in the hypoperfused rats, the level of lipid peroxidation and neuronal damage in the cortex was significantly increased in comparison to the hippocampus i.e that hypoperfusion caused more severe damage to the cortex than to the hippocampus *(28)*. Taking all together, in our experimental condition, the level of oxidative stress probably overwhelmed antioxidant capacity of cortical tissue and consequently activities of SOD and GSH-Px activities were not changed.

In addition, we examined the effect of rhEpo administration on lipid peroxidation levels and antioxidant enzymes SOD and GSH-Px activities in rat cortex following focal cerebral ischemia. Epo is a cytokine commonly associated with its erythropoiesis function. The clinical applications of the rhEpo are currently restricted to the treatment of anemia in renal failure and cancer. Recent studies, however, have suggested a new role for Epo as an anti-inflammatory and neuroprotective drug. Epo and its receptors EpoR are expressed in neurons, glial cells and brain capillary endothelial cells, with pronounced upregulation in conditions of cerebral ischaemia and hypoxia *(30)*. Animal studies indicate that systemic administration of rhEpo exerts neuroprotection in models of stroke. Several hypotheses have been proposed in order to explane the neuroprotective role of erythropoietin indicating that it may exhibits neurotrophic, anti-inflammatory, and antioxidant action *(7)*. It is belived that Epo exerts its antioxidant action directly through scavenging ROS and thus reducing cellular oxidative damage and/or indirectly by activating antioxidant enzymes such as SOD and GSH-Px *(21)*. Sakanaka et al showed that rhEpo may inhibit lipid peroxidation by its inhibitory effect on excitotoxicity and/or inhibition Ca^2 entry and/or by reducing the NO-mediated formation of free radicals or its direct free radical scavenging effect. This finding is supported by studies in which rhEpo was shown to possess a free radical scavenging effect on rat brain and spinal cord homogenates *in vitro (31)*. Administration of Epo (5000 IU/kg, i.p) immediately after or 3 hrs after MCAO in our experimental conditions did not affect increased TBARS level registered in animals exposed to focal cerebral ischemia. In addition, cortical SOD and GSH-Px activities that were relatively unchanged after ischemic insult did not show any significant changes after Epo administration too. According to Byts *et al.,* Epo inhibits lipid peroxidation by increasing the activities of cytosolic antioxidant enzymes such as SOD and GSH-Px *(32)*. Since in our experimental model we did not found activation of antioxidant enzymatic systems after Epo administration, this could be the reason for its failure in decreasing lipid peroxidation levels. One of the reasons for such failure of Epo neuroprotection could be profound oxidative stress in cortical region as already explained above. In addition, it was shown that expression of EpoR is 1.6–3.0 fold lower in cortex in comparison with hippocampus, suggesting that the composition of rhEpo binding sites is not homogenously composed throughout central nervous system *(33)* and consequently that unique brain regional response to Epo therapy could not be expected.

In conclusion, our results showed that focal cerebral ischemia caused by MCAO caused oxidative stress-induced damage of the brain lipids as well as insufficient antioxidant response of SOD and GSH-Px in the right cortex of ischemic rats. In adittion, Epo treatment was not effective in preventing above mentioned alterations.

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