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Oxidative damage following cerebral ischemia depends on reperfusion - a biochemical study in rat

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Abstract

The extent of brain injury during reperfusion appears to depend on the experimental pattern of ischemia/reperfusion. The goals of this study were: first, to identify the rate of free radicals generation and the antioxidant activity during ischemia and reperfusion by means of biochemical measurement of lipid peroxidation (LPO) and both enzymatic (superoxid dismutase - SOD, catalase - CAT, glutathion peroxidase - GPx) and non-enzymatic antioxidants activity (glutathione - GSH); and second, to try to find out how the pattern of reperfusion may influence the balance between free radical production and clearance. Wistar male rats were subject of four-vessel occlusion model (Pulsinelly & Brierley) cerebral blood flow being controlled by means of two atraumatic arterial microclamps placed on carotid arteries. The level of free radicals and the antioxidant activity were measured in ischemic rat brain tissue homogenate using spectrophotometrical techniques. All groups subjected to ischemia shown an increase of LPO and a reduction of the activity of enzymatic antioxidative systems (CAT, GPx, SOD) and non-enzymatic systems (GSH). For both groups subjected to ischemia and reperfusion, results shown an important increase of LPO but less significant than the levels found in the group with ischemia only. Statistically relevant differences ($p<0.01$) between continuous reperfusion and fragmented reperfusion were observed concerning the LPO, CAT, SOD and GSH levels, oxidative aggression during fragmented reperfusion being more important.

Keywords: cerebral ischemia • free radicals • antioxidants • catalase • superoxid dismutase • glutathione peroxidase • glutathione

Introduction

The concept of generation of free radicals during ischemia was first presented by Demopoulos, Flamm and co-workers [1, 2, 3]. They correlated the generation of free radicals with cell damage in cerebral ischemia. Free radicals are highly reactive molecules that initiate radical chain reactions and damage cellular macromolecules, including proteins, DNA, and lipids, ultimately leading to cell death. Free radicals have been implicated in neuronal cell death in acute CNS

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injury and in chronic neurodegenerative diseases [4, 5, 6].

There are a number of potential sources for free radicals generation in the ischemic brain. This comprises leaks from mitochondrial respiratory chain; sequences catalyzed by cyclooxigenase and lipooxigenase, peroxidation of lipid membrane, autooxydation of various small molecules, including cathecolamines, by the microsomal cytochrome P450 reductase system [7] and xanthine oxidase reactions.

The brain and nervous system may be especially prone to oxidant damage for a number of reasons [8]: the membrane lipids are especially rich in polyunsaturated fatty acid side-chains, which are prime targets for free radicals attack; the brain has only moderate amounts of catalase (CAT), superoxid dismutase (SOD) and gluthatione peroxidase (GPx) and also is relatively lacking in vitamin E; some areas of the brain are rich in iron ions which are released from injured cells or from bleeding in the reperfused area and may enhance lipid peroxidation.

One particular role of oxygen free radicals in brain injury appears to involve reperfusion after cerebral ischemia [9]. Reoxygenation during reperfusion provides oxygen to sustain neuronal viability and also provides oxygen as a substrate for numerous enzymatic oxidation reactions that produce reactive oxidants. In addition, reflow after occlusion often causes an increase in oxygen to levels that cannot be utilized by mitochondria under normal physiological flow conditions.

Free radicals species of potential importance in cerebral ischemia include superoxid (O_2^{-}•) and hydroxyl (HO•). The HO• radical is the most reactive and the most toxic of these two molecules. Hydrogen peroxide (H_2O_2) not a free radical *per se* has the potential to generate HO• radicals in reaction with O_2^{-}•, catalyzed by iron (or other transitional metals). Iron can also donate electrons to H_2O_2 to form HO•. Because H_2O_2 is not polar, it readily crosses membranes unlike O_2^{-}•.

Unicellular and multicellular organisms have a variety of defensive systems against free radicals including the low molecular weight scavengers such as alpha-tocopherol, ascorbate and glutathione (GSH). Alpha-tocopherol is lipid soluble and therefore readily crosses the blood-brain barrier and enters cell membranes. It acts like an antioxidant agent by blocking radicalic chain reactions. Ascorbate crosses the blood-brain barrier less easily but is actively transported into cerebrospinal fluid by the choroid plexus and is further concentrated in neuronal cytoplasm by a second active system. After interaction with superoxide (O_2^{-}•) or peroxide (H_2O_2) ascorbate becomes dehydroascorbate. Glutathione is a tripeptide. Its oxidized form is a dimer - GSSG which is involved in the transport of certain aminoacids, is a coenzyme for various enzymes and protects against oxygen radicals and toxic compounds.

Specific enzymes have also evolved to deal with free radicals. Superoxid dismutase, which exists in mithocondrial (Mn-SOD) and cytoplasmic forms (Cu/Zn-SOD), catalyses the conversion of two O_2^{-}• molecules into H_2O_2 and O_2. Because H_2O_2 is a potential source for OH• radicals, two additional protective enzymes: catalase (CAT) and glutathione peroxidase (GPx) transform it. The former converts H_2O_2 molecules into O_2 and H_2O. Catalase activity is largely located in: peroxisomes and mithocondria. The latter catalyzes the oxidation of reduced glutathione by two H_2O_2 molecules. Glutathione peroxidase detoxifies lipid hydroperoxides as well as H_2O_2. Glutathione peroxidase is specific for GSH as a hydrogen donor, but accepts other peroxides as well as H_2O_2.

During reperfusion two opposing mechanisms could be predicted: an increase of free radical production due to contact of oxygenated blood with the lipid membranes weakened by ischemia and a free radicals washing phenomenon due to blood flow restoration.

The goals of this study were: first, to identify the rate of free radicals generation and the antioxidant activity during ischemia and reperfusion by means of biochemical measurement of lipid peroxidation and both enzymatic and non-enzymatic antioxidants activity; and second to try to find out how the efficiency of reperfusion may influence the balance between free radical production and clearance.

Materials and methods

Animals

We used healthy adult Wistar rats (250-300g), with free access to food and water, in agreement with the international rules and regulations concerning animal experiments. To avoid sex-related considerations we used only male rats [10].

All surgical procedures and electrophysiological recordings were performed under deep anesthesia induced with chloral hydrate (CHL) (Merck KCaA) 0.4g/kg (b/w) i.p. The CHL was used due to its minimal influence on the cortical electric activity [11].

EEG monitoring

EEG activity was monitored to document cerebral ischemia duration and effectiveness, knowing that EEG is a valuable method to evaluate the level of neuronal metabolism [12]. Five unipolar gold-plated cylindrical electrodes, 0.5 mm diameter, were placed in direct contact with the external surface of the dura through corresponding stereotaxically drilled holes. A ground electrode was fixed to the scalp in close proximity to the electrodes. Four electrocorticographical (ECoG) leads were obtained using the central electrode as common reference. Each lead measured the corresponding hemispheric potential difference over a 2.5 mm linear distance.

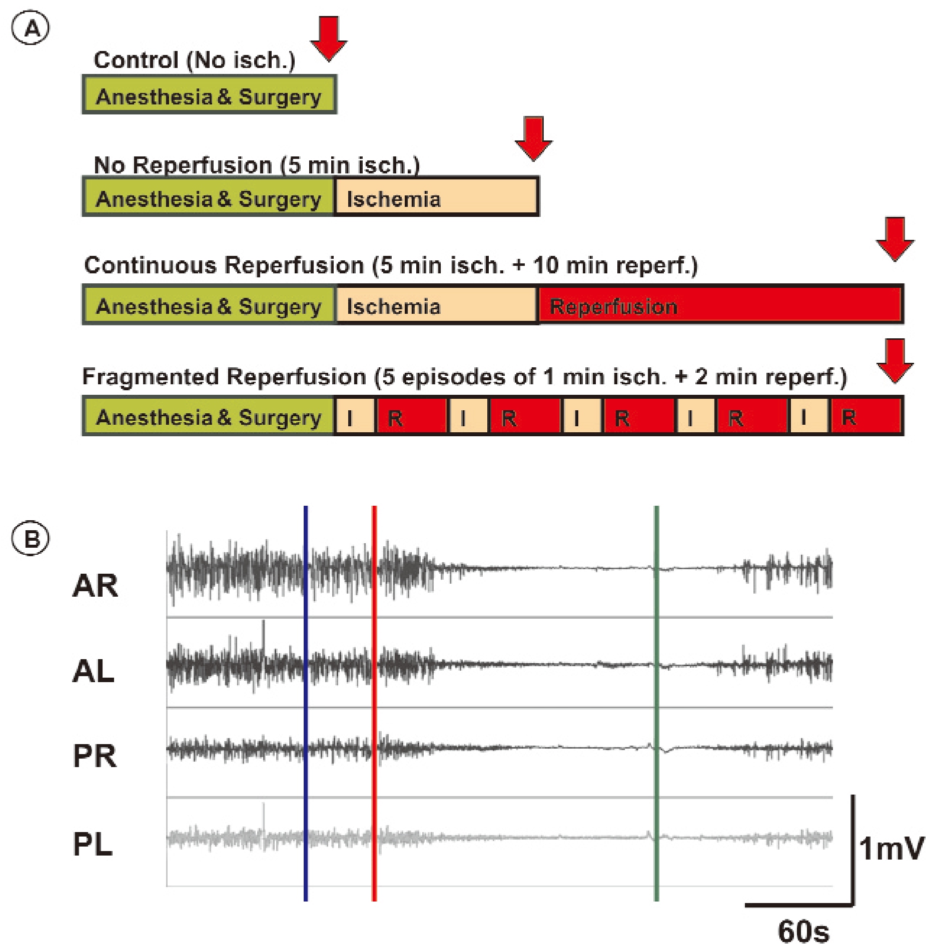

Fig. 1 Material and methods: A. Experimental groups. Red arrow: brain removal. **B. Digital recording showing EEG suppression during ischemia.** Blue line: first common carotid artery being clamped; red line: the second common carotid artery being clamped; green line: vascular clamps removed and reperfusion of the brain tissue allowed. Legend: AR = anterior right, AL = anterior left, PR = posterior right, PL = posterior left.

Table 1 Quantitation in the control group of free radical production estimated by global lipid peroxidation; non-enzymatic antioxidants; enzymatic antioxidant activity.

Parameters	*Estimators*	*Low*	*Mean*	*High*	*Unit*
global lipid peroxidation	**lipid peroxidation (LPO)**	3.37	**3.51**	3.65	nmols/ml
non-enzymatic scavengers	**reduced glutathione (GSH)**	5.20	**5.54**	5.88	mmols/l
antioxidant enzyme activity	**catalase (CAT)**	29.63	**32.56**	35.49	units/mg protein
	superoxiddismutase (SOD)	8.72	**9.40**	10.08	units/mg protein
	glutathione peroxidase (GPx)	57.76	**72.00**	86.24	units/mg protein

The ECoG signal was passed through a 1-35 Hz analogical filter (-3 dB down) and then recorded at 200 samples/second by means of a digital EEG Biopac Systems MP100 (Fig. 1B).

Experimental Protocol

We used a *control* group (N=10); a second group (N=7) with 5 minutes of ischemia and *no reperfusion*, a third group (N=7) with 10 minutes of *continuous reperfusion* after 5 minutes of global transient cerebral ischemia and a fourth group (N=7) with a *fragmented reperfusion*: consisting in 5 minutes of ischemia divided into 5 episodes each one lasting 1 minute separated by 2 minutes of reperfusion between two consecutive ischemic episodes. (Fig. 1A)

Ten minutes of continuous reperfusion were considered as an efficient reperfusion, because it had proved to be sufficient for allowing the electrocortical activity to restore to its preischemic level [13].

Four vessel occlusion surgery

Wistar rats were subjected to four-vessel occlusion (4-VO) model, essentially by the method of Pulsinelli [14, 15]. Briefly, the vertebral arteries were cauterized at the level of the first two cervical vertebrae and the common carotid arteries were then exposed and isolated. Cerebral blood flow was controlled by means of two non-traumatic arterial micro-clamps placed reversibly around the carotids arteries. Assisted ventilation was provided only if respiratory arrest occurred. The body temperature was maintained within normal limits during the experiments.

Ischemic brain preparation

Following ischemia - reperfusion, the brain was removed by craniotomy and transposed into EDTA-buffer (0.02M), pH=7.4, at 4ºC, for removing all other tissue fragments and blood clots. All visible vascular structures were also removed. After washing and fragmentation brain fragments were moved into TRIS-HCl 0,1M pH=7.3 buffer. A brain tissue homogenate was then obtained using TRIS-HCl buffer and brain tissue in a 4:1 ratio. The homogenate was then centrifuged 10 minutes at 2000g and the resulted supernatant was used for biochemical determinations. Enzymatic activity was reported to protein concentration and expressed as specific enzymatic activity.

Biochemical determinations

All biochemical determinations were performed on the supernatant obtained after centrifugation using spectrophotometric methods. In order to evaluate the prooxidant - antioxidant balance we determined the free radicals production by measuring the level of lipid peroxidation, activity of some enzymatic antioxidants (CAT, SOD, GPx) and the level of reduced glutathione (GSH) as a non-enzymatic antioxidant.

Global lipid peroxidation (LPO) was estimated using a technique based on the measurement of the malondialdehyde concentration using thiobarbituric acid (TBA, Sigma), which generates a coloured product. The spectrophotometric detection at λ=532 and 600 nm follows a reaction in an acid environment and incubation at 95ºC [16].

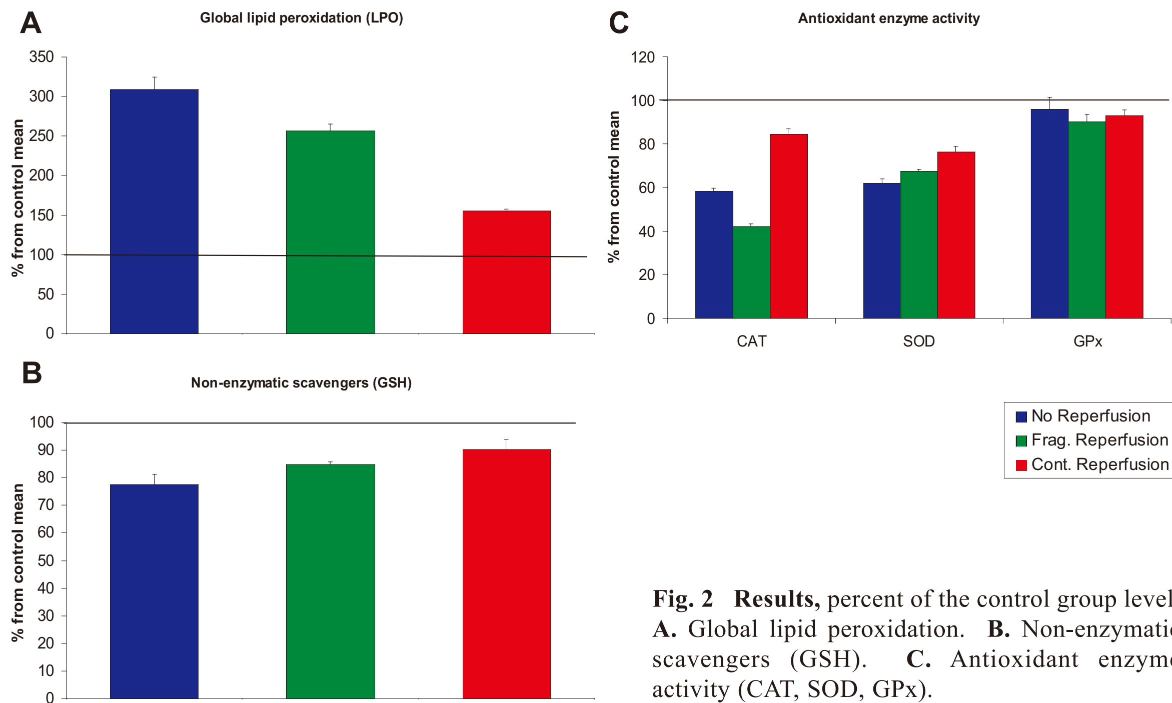

Fig. 2 Results, percent of the control group level. **A.** Global lipid peroxidation. **B.** Non-enzymatic scavengers (GSH). **C.** Antioxidant enzyme activity (CAT, SOD, GPx).

Activity of superoxiddismutase (EC 1.15.1.1) was determined by its inhibitable action on nitroblue tetrazolium (NBT, Sigma) reduction [17].

Glutathione peroxidase assay (EC 1.11.1.19) was based on the reduction of oxidized glutathione generated from the action of GSH peroxidase back to reduced glutathione utilizing the reducing equivalent of NADPH. This method measures the rate of NADPH oxidation at λ=340nm as the indication of GPx activity [18].

Catalase activity (EC 1.11.1.16) was assessed based on measuring the decomposition rate of hydrogen peroxide at λ=240nm [19].

Reduced glutathione was measured using 5,5'-dithiobis-nitrobenzoic acid (DTNB, Sigma), DTNB reacts with GSH to form the highly coloured 5-thio-2-nitrobenzoic acid (TNB) anion and GSSG. λ=420 nm [20].

Results

Experimental protocol

Our experimental setup was adequate to investigate the free radical generation and the level of antioxidants. Its main disadvantage consists in the impossibility to distinguish between the neuronal and glial sufferance on one hand; and on the other hand we couldn't control the variations in the permeability of the blood-brain barrier, plausible during global cerebral ischemia.

Oxidative stress and antioxidants during ischemia and reperfusion

Mean values of the results obtained in control group are presented in Table 1. All other results were expressed as percents of values found in control group. (Fig. 2)

All groups subject of ischemia presented a higher level of lipid peroxidation, the most significantly in the group with ischemia only. Either continuous or repetitive reperfusion played an important role in reducing the level of free radicals. This effect could be explained both by a physical mechanism of free radicals washing and by a possible, at least partial, recovery of metabolism for neurons in penumbra which reduces the rate of free radicals generation.

Antioxidant enzymes with a statistically significant variation (t-Student $p<0.05$) are SOD and CAT; GPx modifications were not significant. CAT and SOD activity during ischemia were reduced at 58.23% respectively 62% of control. During continuous reperfusion these enzymes activities increase at 84.5% and 76.2% compared to

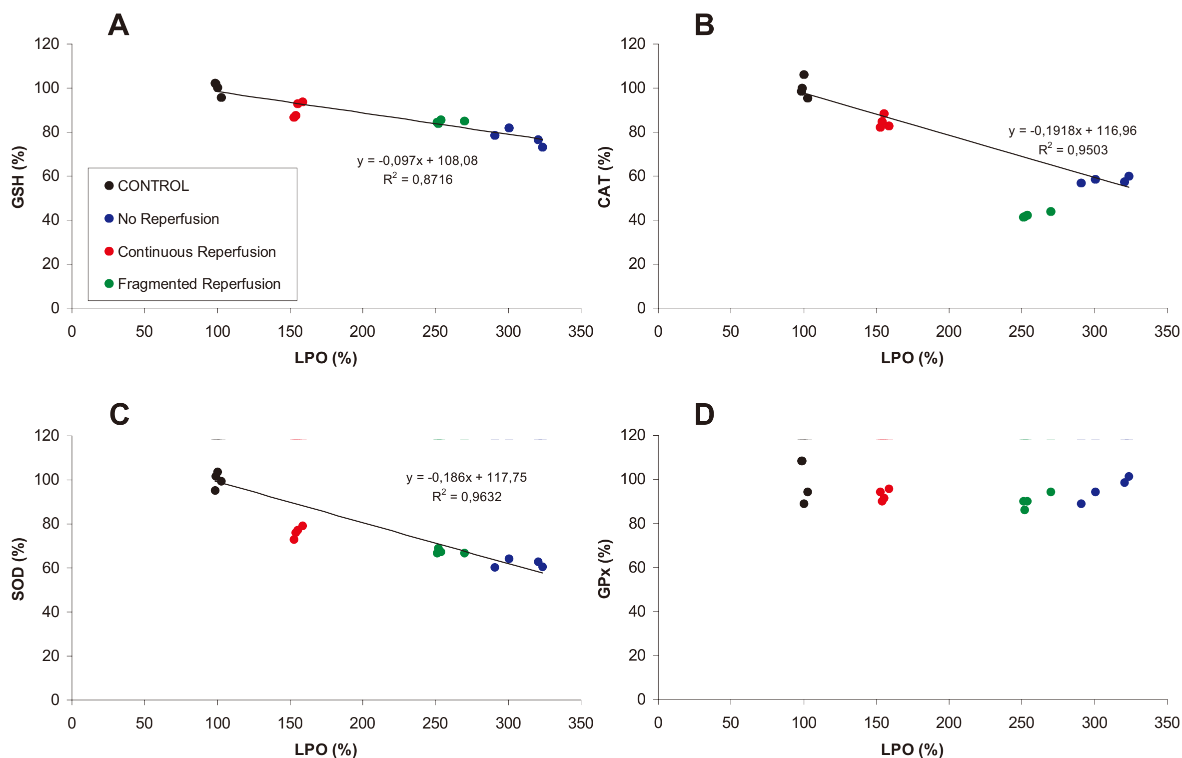

Fig. 3 Results, correlation of analysed parameters versus global peroxidation level, percent of control group levels. **A. GSH level vs. LPO level,** Pearson R^2=0.8716. **B. CAT activity level vs. LPO level,** Pearson R^2=0.9503. **C. SOD activity level vs. LPO level,** Pearson R^2=0.9632. **D. GPx activity level vs. LPO level,** statistically insignificant linear correlation.

controls. The level of reduced glutathione was also diminished during ischemia (77.6%) and slightly increases during reperfusion at 90.25% of control.

Efficient reperfusion vs. inefficient reperfusion

During fragmented reperfusion compared to continuous reperfusion a higher lipid peroxidation amount occurs due to multiple episodes in which oxygenated blood reaches the lipid membranes weakened by ischemia increasing free radicals generation. These differences in LPO amount were statistically significant (t-Student, $p<0.01$).

Antioxidant activity of CAT is dramatically decreased during the repetitive pattern of ischemia/ reperfusion at 42.26% of control, compared with continuous reperfusion, where it was 84.5% of control. SOD activity is also decreased in fragmented reperfusion but only at 67.44% compared with 76.2% in continuous reperfusion.

A possible explanation could involve the level at which CAT act during antioxidant response mechanisms. CAT converts hydrogen peroxide into oxygen and water, but a significant quantity of hydrogen peroxide is produced by the dismutation activity of SOD.

GSH was statistically significant (t-Student, $p<0.05$) diminished during fragmented reperfusion at 84.8% of control, but during continuous reperfusion it was 90.25% of control.

Discussion

One of the mechanisms responsible for delayed neuronal injury after global cerebral ischemia is the membrane alteration. The most important cause of membrane alteration is the lipid peroxidation due to a relative excess of free radicals.

Many mechanisms involving free radical production have been documented both during

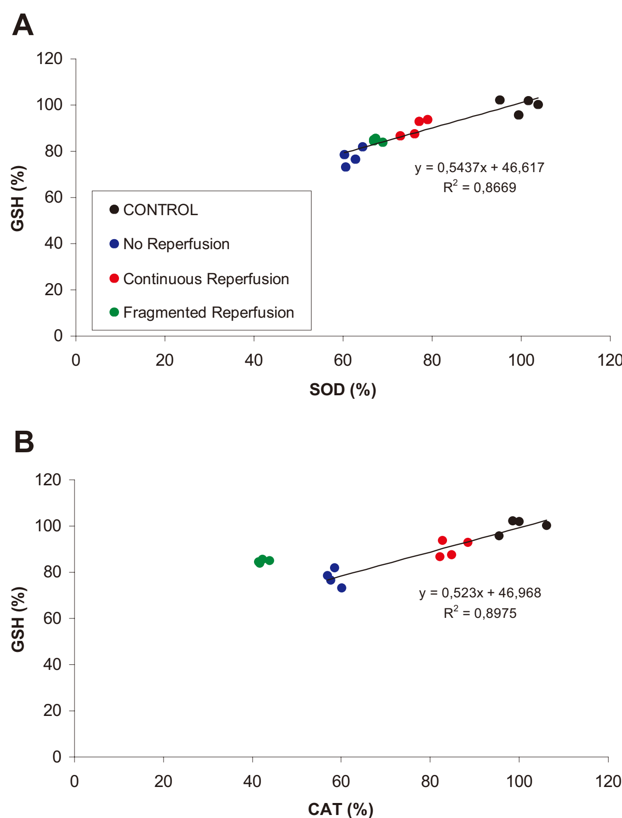

Fig. 4 Results, correlation of nonenzymatic scavengers level (GSH) versus antioxidant enzyme activity level (SOD, CAT). **A. GSH level vs. SOD activity level,** Pearson R^2=0.8669. **B. GSH level vs. CAT activity level,** Pearson R^2=0.8975.

ischemia and reperfusion but their actual importance is still controversial.

In our study we addressed the problem of relative contribution of ischemia and reperfusion to the free radical production estimated by the level of lipid peroxidation.

When free radical production exceeds the buffering capabilities, LPO levels increase while both non-enzymatic scavengers (GSH) and antioxidant enzymes (SOD, GPx, CAT) decrease. A decrease in free radical production was followed by a gradual normalization of LPO and both GSH and antioxidant enzymes.

We found that LPO was markedly increased (3fold) after 5 minutes of continuous ischemia, suggesting that during global cerebral ischemia there is an important production of free radicals.

After 10 minutes of continuous reperfusion all parameters show a clear trend of normalization documenting that most of the postischemic oxidation is produced in the early part of reperfusion. Moreover, the fact that after 5 minutes of ischemia the level of free radicals still exceeds the buffering capabilities suggests that the postischemic oxidative stress can be longer than the ischemic period itself.

By fragmenting the 10 minutes of reperfusion in 5, 2 minutes intervals, the level of LPO almost doubles. Since most of the postischemic free radicals are generated during early reperfusion we may speculate that the same amount of free radicals was produced in 5 minutes of continuous ischemia and 5 episodes of 1 minute ischemia. It may be concluded that, for the same duration, the fragmented ischemia and reperfusion is more damaging than continuous ischemia and reperfusion due to the repetitive reperfusion.

During global ischemia, the free radical production is continuous, proportional with the duration of ischemia while during reperfusion the

free radical production is primarily confined to the early reperfusion when fresh oxygen is supplied to the ischemic region.

Our working hypothesis was that the fairly different mechanisms of intraischemic and postischemic free radical production might be reflected by different buffering systems.

In our study the level of non-enzymatic scavengers (GSH) correlates linearly with LPO for both intra and postischemic groups and this correlation is not affected by fragmentation of reperfusion. (Fig. 3) Thus GSH is unable to distinguish between the different oxidative mechanisms.

The difference was found in the antioxidant enzymes behavior. (Fig. 3)

When the level of GPx is decreased after ischemia the activity levels do not correlate with either the level of LPO or GSH. Nevertheless both SOD and CAT correlate roughly inversely with the level of LPO. Postichemic levels of CAT deviate from this correlation during fragmented reperfusion, while SOD deviates during ischemia, suggesting that these enzymes may be consumed differently according to the source of free radicals.

There is a general correlation between the non-enzymatic scavengers (GSH) and antioxidant enzymes. (Fig. 4) This correlation holds true for SOD under all experimental conditions but LPO is abnormally highly reduced during fragmented reperfusion, enforcing the idea of the important role of CAT in compensating for the postischemic free radical production.

References

1. **Demopoulos H.B., Flamm E.S., Seligman M.L., Mitamura J.A., Ransohoff J.,** Membrane perturbations in central nervous system injury: Theoretical basis of free radical damage and a review of the experimental data. In: Neural Trauma, Raven Press, pp. 63-68, 1979
2. **Demopoulos H.B., Flamm E.S., Pietronigro D.D., Seligman M.L.,** The free radical pathology and the microcirculation in the major central nervous system disorders, *Acta Physiol. Scand. Suppl.*, **492**:91-119, 1980
3. **Flamm E.S., Demopoulos H.B., Seligman M.L., Poser R.G., Ransohoff J.,** Free radicals in cerebral ischemia, *Stroke,* **9**:445-7, 1978
4. **Kontos H.A., George E.,** Brown memorial lecture: oxygen radicals in cerebral vascular injury, *Circ. Res.,* **57**:508-516, 1985
5. **Coyle J.T., Puttfarcken P.,** Oxidative stress, glutamate, and neurodegenerative disorders, *Science,* **262**:689-695, 1993
6. **Chan P.H.,** Oxygen radicals in focal cerebral ischemia, *Brain Pathol.,* **4**:59-65, 1994
7. **Freeman B.A., Crapo J.D.,** Biology of disease: Free radicals and tissue injury, *Lab. Invest.,* **47**:412-426, 1982
8. **Ozben T.,** Mechanisms involved in neuronal damage. In: Free radicals, oxidative stress and antioxidants, NATO ASI series, Plenum Press, New York, London, pp. 163-189, 1998
9. **Chan P.H.,** Role of oxidants in ischemic brain damage, *Stroke,* **27**:1124-1129, 1996
10. **Corsi-Cabrera M, Juarez J, Ponce-de-Leon M, Ramos J, Velazquez PN.,** EEG activity during estral cycle in the rat, *Electroencephalogr. Clin. Neurophysiol.*, **83**:265-269, 1992
11. **Sisson D.F., Sigel J.,** Chloral hydrate anesthesia: EEG power spectrum analysis and effects on VEPs in rat, *Neurotoxicol. Teratol.*, **44**: 61-71, 1998
12. **Zagrean L., Vatasescu R., Oprica M., Nutiu O., Ferechide D.**, A comparative study of EEG suppressions induced by global cerebral ischemia and anoxia, *Rom. J. Physiol.*, **32**: 39-44, 1995
13. **Moldovan M., Munteanu A.M., Nita D. Al., Popa D.P., Spulber St., Zagrean L.**, Ischemic electrocortical suppression - an active mechanism?, *J. Med. Biochem.*, **4**:103-111, 2000
14. **Pulsinelli W.A., Brierley J.B.**, A new model of bilateral hemispheric ischemia in the unanesthetized rat, *Stroke*, **10**: 267-272, 1979
15. **Pulsinelli W.A., Buchan A.M.**, The four-vessel occlusion rat model: method for complete occlusion of vertebral arteries and control of collateral circulation, *Stroke*, **19**: 913-914, 1988
16. **Draper H.H., Squires S.J., Mahmoody H., Agarwal S., Wu J., Hadley M.,** A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials, *Free Radic. Biol. Med.*, **15**, 353, 1993
17. **Nishikimi M., Rao N., Yagi K.,** Reactivity of D-amino acid oxidase with artificial electron acceptors, *Biochim. Biophys. Acta*, **276**:350-62, 1972
18. **Daret K.C., Ching K.C.,** Glutathione peroxidase: activity and steady state level of mRNA. In: Free radicals, Punchard and Kelly eds., Oxford University Press, New York, pp. 227-240, 1993
19. **Aebi H.E.**, Catalase. In: Methods of enzymatic analysis, **3**:273-286, 1983
20. **Anderson M.E.**, Methods in enzymology vol. 113, Meister A. ed., Academic Press, New York, pp. 548-555, 1985