

Behavioral and oxidative metabolism disorders in a model of transient induced cerebral hypoperfusion in rats

✉ Rocío García¹, María E González², Ivette Fernández³, Yuselis Castaño⁴, Mei-Li Díaz⁴, Esteban Alberti¹

¹Department of Neurobiology, International Center for Neurological Restoration, CIREN Ave. 25 #15805, Playa, CP 11 300, Havana, Cuba

²Department of Neurobiochemistry

³Department of Experimental Neurophysiology

⁴Faculty of Biology, University of Havana, UH

E-mail: rociogminiet@infomed.sld.cu

ABSTRACT

The effect of cerebral hypoperfusion on the oxidative metabolism of the hippocampus, cortex, and the striatum remains unknown, and the alterations induced by oligoemia on both reference and work memories have been poorly studied. In this study we induced oligoemia in rats by bilateral occlusion of the common carotid arteries, without hypotension. After 15 days we measured catalase and superoxide dismutase activity, and the concentration of malondialdehyde in homogenates from the hippocampus, cortex, and striatum. Behavioral alterations were evaluated using the Morris water maze test. The ischemic event produced a significant increase ($p < 0.05$) in superoxide dismutase activity and the concentration of malondialdehyde at the cerebral cortex. Animals with brain injury showed a significant decrease in escape latency in finding the platform ($p < 0.01$) and retention ($p < 0.05$). The results of this study shows that the determination of oxidative stress indicators at the cortex and the evaluation of memory and learning are useful tools for estimating the extent of the damage produced by carotid occlusion without hypotension in rats, as well as for the validation of this experimental model.

Keywords: catalase, behavior, cerebral ischemia, Morris water maze, malondialdehyde, oligemia, superoxide dismutase

Biotechnología Aplicada 2008;25:339-344

RESUMEN

Alteraciones conductuales y del metabolismo oxidativo en un modelo de hipoperfusión cerebral transitoria inducido en ratas. El efecto de la hipoperfusión cerebral sobre el metabolismo oxidativo en el hipocampo, la corteza y el cuerpo estriado aún no se conoce. Poco se han estudiado las alteraciones que provoca la oligoemia sobre la memoria de referencia y de trabajo. Mediante oclusión bilateral de las arterias carótidas comunes, y sin maniobras de hipotensión se indujo la oligoemia en ratas. A los 15 días de la lesión se determinó la actividad de la superóxido dismutasa, la catalasa y la concentración de malondialdehído en homogenados del hipocampo, la corteza y el cuerpo estriado. Las alteraciones conductuales se evaluaron mediante la prueba del laberinto acuático de Morris. El evento isquémico indujo un aumento significativo ($p < 0.05$) de la actividad de la superóxido dismutasa y de la concentración de malondialdehído en la corteza cerebral. Los animales lesionados mostraron una disminución significativa de la latencia de escape ($p < 0.01$) y de la retención ($p < 0.05$). Los resultados de este estudio evidencian que las determinaciones de los parámetros oxidativos en la corteza y la evaluación de la memoria y el aprendizaje, son herramientas útiles para evaluar el alcance de la lesión provocada por la oclusión de las carótidas sin maniobras de hipotensión en ratas y validar el modelo experimental.

Palabras clave: catalasa, conducta, isquemia cerebral, laberinto acuático de Morris, malondialdehído, oligoemia, superóxido dismutasa

Introduction

The fact that cerebrovascular disorders still constitute the third leading cause of death worldwide and a major source of disabilities [1] has fostered research into new therapeutic management strategies. This research has, in turn, stimulated the development of animal models for the predictive evaluation of the efficacy of new therapeutics in humans. Although many animal species have been used to obtain models of cerebral ischemia, rats have been preferred in the study of cerebrovascular disorders due to their similarity with humans in the anatomy of intracerebral circulation [2].

The classical method for the global ischemia model in rats is the permanent occlusion of the two vertebral

arteries together with the transient occlusion of the two common carotid arteries (CCA). There are, however, disadvantages because of the complexity of the necessary surgical procedure and its high mortality rate [3, 4]. Another ischemia model affecting the anterior brain involves the bilateral occlusion of the CCA. Taking into account that rats have an extensive collateral circulation due to the existence of the circle of Willis [5], arterial hypotension procedures [6, 7] and genetic selection of hypertensive rat strains [8] have been developed in order to increase the efficiency of ischemic induction.

There are models of bilateral CCA occlusion (transient or permanent) that do not require hypotensive

1. Alexandrova M, Bochev P. Oxidative stress during the chronic phase after stroke. *Free Radic Biol Med* 2005;39:297-316.

2. Peña PG, Díaz AH, Torres JL. Oclusión endovascular permanente de la arteria cerebral media en ratas Wistar: descripción del abordaje a través de la arteria carótida interna. *Rev Neurol* 2004;39:1011-16.

3. López F, Hernández J, López R, Alarcón E, Martínez JF. Actividad de la cobre-zinc superóxido dismutasa en un modelo de lesión cerebral isquémica global sin hipotensión. *Neurocirugía* 2004;15:151-8.

manipulation. But these models are seldom used [5, 9]. Among them, that of transient occlusion has the advantage that it preserves the integrity of the cerebral vasculature, allowing the study of the effects of reperfusion. Additionally, the reduction of the blood flow in the model of double carotid occlusion without hypotension does not result in neuronal death [10], which has led a number of authors to consider it a model of oligoemia, rather than of global ischemia [10-12]. The term "oligoemia" used here refers to a reduction of cerebral blood flow that triggers neurotoxic processes that affect memory and learning; an event that is typical of the threshold zone for cerebral infarction, of vascular disorders associated to Alzheimer's disease and of elderly persons [13].

The brain is particularly sensitive to oxidative damage due to the large amount of oxygen it consumes and the high concentration of iron and other readily oxidizable substrates (such as poly-unsaturated fatty acids and catecholamines) found there [14]. One of the consequences of an ischemic event is a marked increase in reactive oxygen species (ROS) (superoxide anion, hydroxyl radical, hydrogen peroxide and peroxynitrite) which is worsened during reperfusion [1]. The superoxide anion and the hydroxyl radical produce oxidative damage to cell membranes, leading to a progressive decrease in their fluidity and their biochemical and structural integrity that is known as lipid peroxidation [15]. The brain deals with ROS using a defensive anti-oxidative enzyme system that neutralizes these highly reactive species, and depends on the interplay of intracellular enzymes such as superoxide dismutase (SOD) and catalase (CAT) [16].

It has been suggested that the areas of the brain most susceptible to ischemia include the hippocampus, the striatum and the cortex [16]. However, the studies examining the alteration of oxidative metabolism in these areas in animals subjected to CCA occlusion have used cerebral homogenates which have been subjected to a period of reperfusion of at most 24 h [3, 9, 12, 13, 17]. This model has also been validated in previous studies that have evaluated behavioral alterations [9, 13], demonstrating that the deleterious effects of reduced blood flow on memory can be detected 24 h after the lesion and can persist for 13 months [9, 13]. Therefore, our research was aimed at the evaluation of the degree of oxidative stress in the most sensitive areas (hippocampus, striatum and cortex) and the analysis of short-term and long-term effects on memory in rats submitted to bilateral CCA occlusion without hypotensive manipulation.

Materials and methods

Experimental subjects

Rats of the Sprague-Dawley line were used. They were obtained from the National Center for the Production of Laboratory Animals (City of Havana, Cuba) with a body weight ranging from 300 to 450 g. The animals were housed in translucent cages with water and food *ad libitum*, at a relative humidity of $67 \pm 3\%$ and a temperature of $22 \pm 2^\circ\text{C}$ with light/darkness cycles of 12 h. All the experimental procedures complied with the ethical principles established for animal research [18].

Surgical procedure

The experimental group of ischemic animals included 20 rats, which were anesthetized intraperitoneally with 350 mg of chloral hydrate per kilogram body weight. An incision was made at the neck and the CCA were separated from the adjacent tissue and the vagal nerve, after which they were ligated with no. 3 sutures for 60 min. The animals were kept at a controlled temperature of $35 \pm 2^\circ\text{C}$ until their recovery. The control animals ($n = 22$) underwent the same surgical procedure, except for the bilateral occlusion of the CCA.

Study of the alterations of oxidative metabolism

Obtaining the cerebral areas

Fifteen days after the lesion, the animals were anesthetized with chloral hydrate (420 mg/kg of body weight, intra-peritoneally) and decapitated. Their brains were extracted and washed with cold 0.9% NaCl, after which the hippocampus, striatum and cortex were dissected, following the atlas of Paxinos and Watson [19]. The tissue samples were frozen in liquid nitrogen, weighed and stored at -70°C for further analysis. Samples were taken in both the rats with brain injury ($n = 10$) and control ($n = 8$) rats.

Study of oxidative metabolism parameters

For the study of oxidative metabolism parameters, the anatomical samples were homogenized in 1 M Tris/0.25 M sucrose buffer (pH 7.4) at a tissue/buffer volume ratio of 1/5. The homogenates were centrifuged at 14 000 rpm for 15 min, and the supernatant was split into 4 aliquots for SOD and CAT assays, as well as for the determination of malondialdehyde (MDA) and total protein concentrations.

Assays for SOD activity

SOD activity was determined using the Marklund method [20], based on the inhibition by SOD of the pyrogallol reaction (pyrogallol auto-oxidizes yielding pyrogallin, a yellow compound, and the superoxide anion, which in turns auto-catalyzes the decomposition of pyrogallol). The samples were delipidated by adding 150 μL of chloroform and 250 μL of methanol to each 500 μL of the homogenate and shaking vigorously, followed by centrifugation for 20 min at 3000 rpm and the extraction of the supernatant. Starting at 10 s after initiating the reaction, we measured the optical density (OD) at 420 nm of a mixture containing 900 μL of SOD buffer (Tris 0.02 M/HCl 0.2 M, pH 8.2), 50 μL of the delipidated sample and 50 μL of a 0.2 mg/mL pyrogallol solution for a period of 1 min. The assay was performed in triplicate, expressing the results in units of enzymatic activity (UEA). A unit of SOD was defined as the amount of enzyme required to inhibit the auto-oxidation of pyrogallol by 50%.

Assay for CAT activity

CAT activity determinations were performed by spectrophotometry, following the decomposition of hydrogen peroxide (H_2O_2) as described by Aebi [21]. The assay used phosphate buffer at 0.06 M, pH 7.4 and a 60 mM solution of H_2O_2 in phosphate buffer as the

4. Traystman RJ. Animal models of focal and global cerebral ischemia. *ILAR J* 2003;44:85-95.

5. Reinés A, Zárate S, Carmona C, Negri G, Peña C, Rodríguez G. Endobain E, a brain endogenous factor, is present and modulates NMDA receptor in ischemic conditions. *Life Sci* 2005;78:245-52.

6. Al-Majed A, Al-Omar F, Nagi M. Neuroprotective effects of thymoquinone against transient forebrain ischemia in the rat hippocampus. *Eur J Pharmacol* 2006;543:40-7.

7. Hartman RE, Lee JM, Zipfel GJ, Wozniak DF. Characterizing learning deficits and hippocampal neuron loss following transient global cerebral ischemia in rats. *Brain Res* 2005;1043:48-56.

8. Ooboshi H, Ibayashi S, Shichita T, Kumai Y, Takada J, Ago T *et al.* Postischemic gene transfer of interleukin-10 protects against both focal and global brain ischemia. *Circulation* 2005;111:913-9.

9. Maia FD, Pitombeira BS, Araujo DT, Cunha GM, Viana GS. I-Deprenyl Prevents Lipid Peroxidation and Memory Deficits Produced by Cerebral Ischemia in Rats. *Cell Mol Neurobiol* 2004;24:87-100.

10. Liverman CS, Cui L, Yong C, Choudhuri R, Klein RM, Welch KMA, *et al.* Response of the brain to oligemia: gene expression, c-Fos, and Nrf2 localization. *Mol Brain Res* 2004;126:57-66.

11. Franklin LR, Girao MH, Leitao PR. Metabolic evaluation of ischemic and reperfusion brain injury following bilateral occlusion of common carotid arteries: an experimental study in rats. *Acta Cir Bras* 2004;19:529-34.

12. Ghoneim A, Abdel-naim A, Khalifa A, El-Denshary ES. Protective effects of curcumin against ischaemia/reperfusion insult in rat forebrain. *Pharmacol Res* 2002;46:273-9.

13. Heim C, Zhang J, Lan J, Sieklucka M, Kurz T, Riederer P, *et al.* Cerebral oligoemia episode triggers free radical formation and late cognitive deficiencies. *Eur J Neurosci* 2000;12:715-25.

14. Benzi G, Moretti A. Age and peroxidative stress-related modifications of the cerebral enzymatic activities linked to mitochondria and the glutathione system. *Free Radic Biol Med* 1995;19:77-101.

15. Adibhatla RM, Hatcher J. Phospholipase A2, reactive oxygen species, and lipid peroxidation in cerebral ischemia. *Free Radic Biol Med* 2006;40:376-87.

16. Danielisova V, Nemethova M, Gottlieb M, Burda J. Changes of endogenous antioxidant enzymes during ischemic tolerance acquisition. *Neurochem Res* 2005;30:559-65.

17. Galvao RI, Diógenes JP, Maia GC, Filho AS, Vasconcelos SM, Menezes DB, *et al.* Tenoxicam Exerts a Neuroprotective Action after Cerebral Ischemia in Rats. *Neurochem Res* 2005;30:39-46.

18. Canadian Council on Animal Care (CCAC). Guidelines for the use of animals in Psychology. In: Olfert ED, Cross BM, McWilliam DVM, McWilliam AA (Eds.) Ottawa: Bradda Printing Services Inc. 1997; 155-62.

substrate. It was performed by adding, 650 μL of buffer, 340 μL of the substrate and 35 μL of the homogenate, in a thermostatic cuvette at 37 °C, mixing, and measuring the OD at 240 nm every 5 s for 20 s. The measurements were performed by duplicate, and a unit of enzyme activity was defined as the amount required to transform 1 mmol of H_2O_2 in 1 min at 37 °C.

Determination of the concentration of MDA

One-hundred microliters of the homogenate were mixed with 1.5 mL of 0.08 N sulfuric acid and 250 μL of 10% phosphotungstic acid and centrifuged at 3000 rpm for 10 min, discarding the supernatant. The precipitate was resuspended in 1 mL of 0.08 M sulfuric acid and 250 μL of 10% phosphotungstic acid, repeating the centrifugation and discarding the supernatant. The pellet was mixed with 1.5 mL of distilled water and 1 mL of 0.67% thiobarbituric acid (TBA) and incubated at 100 °C for 1 h. Then, the samples were mixed with 2 mL of n-butanol and centrifuged at 3000 rpm for 20 min, reading the OD of the supernatant at a wavelength of 532 nm. The MDA concentration assays were performed by triplicate, using an MDA standard at a known concentration to obtain the results. The concentrations were expressed in nM.

Assay for total protein concentration

Total protein concentration was determined according to Bradford [22]. One-hundred microliters of the sample were mixed with 2 mL of 0.1 mg/mL Coomassie brilliant blue in 8.5%/0.05% H_3PO_4 /ethanol, reading the OD at 595 nm. A standard curve was obtained using bovine serum albumin solutions at concentrations of 0.045; 0.089; 0.134 and 0.178 mg/mL, as determined using an extinction coefficient at 280 nm (k) of 0.68 mL/mg.

Study of behavioral disorders

Learning and spatial memory

The Morris water maze test consists of measuring the time taken by the experimental animals to locate a transparent platform with an 11 cm diameter. The platform was placed 1 cm above the water surface (visible platform) or 1 cm under the surface (hidden platform) on a circular water reservoir with a diameter of 1.5 m and a depth of 40 cm. The rats should find the platform using as a reference the visual cues that surround the tank. The measured variable was the escape latency (defined as the time taken by the subjects to find the platform and escape from the water) with a maximum search time allowed of 60 s. A camera was placed over the center of the tank to collect the data of either the control rats ($n = 14$) or those with brain injury ($n = 10$).

Evaluation of sensitive-motor and motivational deficits

This evaluation was performed at day 10 after the lesion. The platform was located at a visible place on the northeastern quadrant and 8 tests were performed per rat. The search for the platform began each time from a different position on the border of the tank, randomly selected from the eight possible positions (east, west, north, south, northeast, northwest,

southeast and southwest). This procedure was used for the evaluation of sensitive-motor and motivational alterations that could bias the results of the evaluation of memory and learning.

Evaluation of long-term or reference memory (Spatial memory)

This evaluation was performed at days 11 to 14 after the lesion and involved a search for the hidden platform, which remained at the same position throughout the tests. A total of 29 tests per rat were made during the 4 day evaluation period (8 during the first 3 days and 5 on the last day). The exact location at the tank from where the rats were released into the water was changed for every evaluation day. The assays of the fourth day evaluated retention. This assessment consisted of removing the escape platform and releasing the rat into the tank, measuring during a free swimming period of 60 s the number of times the subject passed through the spot where the platform had been located on the previous days (number of crosses). Escape latency and retention were measured again 30 days after the lesion, keeping the platform at the same position. In this case there were 16 tests performed, evaluating the number of crosses in the last assay.

Evaluation of short-term or working memory (learning)

Reference memory was evaluated after the oligoemia had had a one month evolution. In this experiment the animals were evaluated during 3 days, in which the position of the platform (that was hidden in all cases) was changed daily. A total of 12 tests were performed per rat (4 tests per day). The length of the period between consecutive tests was also changed daily, consisting of 20 s, 20 min. and 2 h for days 1, 2 and 3, respectively.

Statistical analyses

The adjustment of the quantitative variables to a normal distribution was evaluated by the Kolmogorov-Smirnov test. Values were expressed as the mean plus/minus its standard error. The activities of SOD and CAT, as well as the MDA concentrations of each brain area, were compared between the control and ischemic groups using a t-test for independent means; this test was also used for comparing the number of crosses in the Morris water maze test between the experimental groups. The Mann-Whitney U-test was used to compare the escape latency of the experimental groups, while using the Friedman test followed by a Wilcoxon test to compare the escape latency in the same experimental group on different days. The result was considered statistically significant when $p < 0.05$.

Results

Alterations of oxidative metabolism

Table 1 shows the activity of anti-oxidative enzymes and the concentration of MDA at the cortex, hippocampus and striatum of the control and oligoemic animals. According to these results, there were no alterations in oxidative metabolism for the hippocampus and striatum on day 15 after the lesion.

19. Paxinos G and Watson C. The rat brain in stereotaxic coordinates. 2nd. Academic Press; 1986.

20. Marklund J. Involvement of superoxide anion radical in autoxidation of pirogallol as a convenient assay for superoxide dismutase. *Eur J Biochem* 1992; 47:469-74.

21. Aebi H. Catalase *in vitro*. *Methods Enzimol* 1984;105:121-6.

Table 1. Mean \pm SE of the enzymatic activity of SOD (U/mg of protein) and CAT (KU/mg of protein) and of MDA concentration (nmole/L). The statistical analysis detected significant differences (t-test for independent means) between the control group (mock lesion) and the group of animals with bilateral CCA occlusion

Indicator	Control group			Oligoemic group		
	Cortex	Hippocampus	Striatum	Cortex	Hippocampus	Striatum
CAT	58.66 \pm 6.81	57.66 \pm 13.47	58.21 \pm 10.54	74.76 \pm 8.75	58.47 \pm 13.9	59.13 \pm 12.77
SOD	1.68 \pm 0.05	1.78 \pm 0.05	1.77 \pm 0.05	1.86 \pm 0.02*	1.75 \pm 0.03	1.71 \pm 0.08
MDA	9.16 \pm 3.38	5.22 \pm 2.23	10.37 \pm 2.63	18.72 \pm 4.60*	2.44 \pm 1.46	8.96 \pm 1.80

*Statistically significant differences, $p < 0.05$.

At the cortex, on the other hand, the ischemic event resulting from the reduction of cerebral blood flow induced a statistically significant ($p < 0.05$) change in SOD activity, which increased by 10.7% compared to the control group. Additionally, although no statistically significant differences were found between the control and ischemic animals in relation to CAT activity for any of the three brain areas, the value of this parameter did increase for the cortex, in contrast to the hippocampus and striatum where it remained constant. The concentration of MDA also showed a statistically significant ($p < 0.05$) increase of 104.4% in the cortex of the animals submitted to the ischemic treatment, compared to the control rats.

Behavioral disorders

The behavior of the oligoemic rats in the Morris water maze test with the visible platform was undistinguishable from that of the control rats (Figure 1), with no statistically significant differences between the escape latencies of both experimental groups.

When assessing long-term memory, there was a decrease in the escape latency with each passing day of the evaluation period in rats with the brain injury compared to the control ($p < 0.001$, Figure 2A). Although this decrease was significant for both experimental groups, the plateau of the escape latency curve for oligoemic animals was less pronounced than that of the control individuals. The escape latencies of oligoemic animals began to show a statistically significant difference compared to the controls on the third and fourth days of the evaluation period ($p < 0.01$), and this difference still remained one month after the lesion ($p < 0.01$).

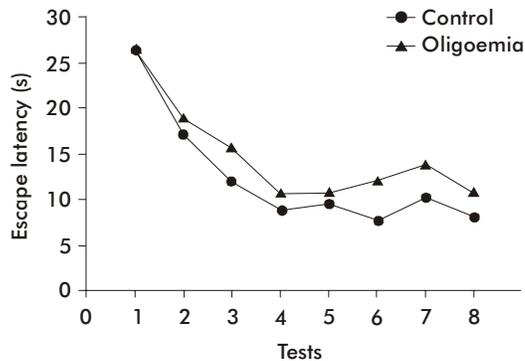


Figure 1. Evaluation of sensitive-motor and motivational deficits for oligoemic and control rats. A visible platform was used for these tests. The eight tests were performed on day 10 after the lesion.

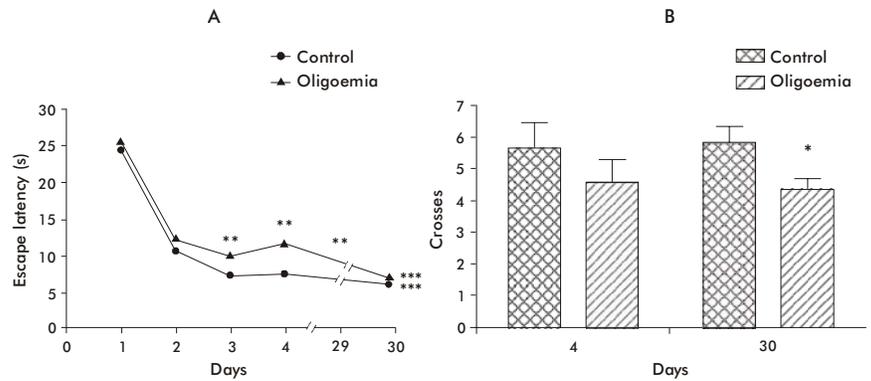


Figure 2. Evaluation of long-term (reference) memory. A hidden platform was used for these tests, which remained at the same location throughout the assay (A) Escape latency of both experimental groups. (B) Performance of the animals during the retention test on day 4 and day 30 of the evaluation. Statistically significant (*, $p < 0.05$) and highly significant (**, $p < 0.01$) differences between both experimental groups are shown, and also differences in latency within the same group (***, $p < 0.001$).

There were no statistically significant differences between both groups of rats in the retention tests on the fourth day of evaluation, although the average number of crosses of the ischemic individuals was 2-fold lower than in the control group (Figure 2B). However, during the retention tests performed 30 days after the lesion, the number of crosses of the ischemic animals were significantly lower ($p < 0.05$) than those of the control (Figure 2B). The escape latency of the oligoemic rats was significantly higher ($p < 0.05$) on the second day of the evaluation (Figure 3).

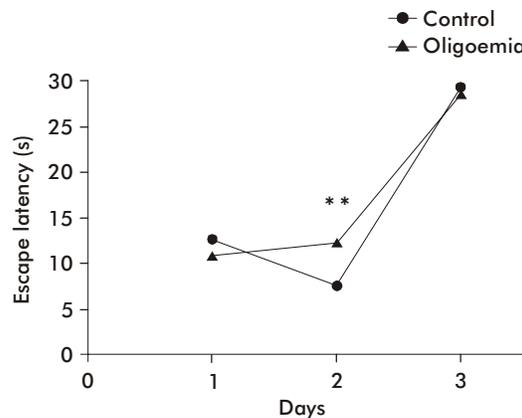


Figure 3. Evaluation of short-term (work) memory. This scheme used a hidden platform, whose location was changed daily. The inter-assay period was 20 s (day 1), 20 min (day 2) and 2 h (day 3). ** $p < 0.01$.

Discussion

In rats there is a connection between the anterior (carotidal) and posterior (vertebrobasilar) cerebral circulation, due to the existence of an anatomical structure similar to circle of Willis [2, 11]. The vertebrobasilar arteries, which were not ligated in this study, irrigate the hippocampus [23]. Among the brain areas examined in this study, the cortex was the only one showing alterations in oxidative metabolism due to the lesions; this is an expected outcome when the collateral irrigation from the vertebrobasilar circulation after the occlusion of the CCA is enough to maintain blood flow to the hippocampus and the striatum, but not to the cortex. A similar study in rats with permanent CCA occlusion also showed a marked effect on the cortex and a severe decrease in blood flow and glucose utilization in this area [23]. These alterations were maintained for up to 1 week after the occlusion of the CCA.

The increase in SOD activity in the cortex of ischemic animals can be considered a response to the generation of superoxide anions at high concentrations. SOD is known to be the main defense system against the excess of superoxide anions formed during reperfusion [16]. It has been demonstrated that the overexpression of this enzyme protects neurons against damage caused by ischemia-reperfusion [24], and similar results have been obtained elsewhere [3, 12]. The analysis of our results and those previously reported in the literature suggests that the behavior of SOD is an important indicator for an ischemic lesion in the model of bilateral CCA occlusion without arterial hypotensive manipulations.

The increase in CAT activity at the cortex of the animals with brain injury is coherent with the increase in SOD observed for this brain area. Hydrogen peroxide, one of the products of the reaction catalyzed by SOD, activates CAT at high concentrations [25]. Since an excess of H₂O₂ in the presence of metal ions can generate hydroxyl radicals -which can only be eliminated through the continuous cellular turnover of damaged molecules-, once this species is formed cell damage is unavoidable [26].

A similar study evaluating CAT activity after a 1 hour occlusion of the CCA followed by 1 hour of reperfusion [12], showed that the activity of this enzyme was not affected by oligoemia. While CAT activity at the cortex of ischemic animals did not significantly increase in our study, it was enhanced in 27.4%. This discrepancy may stem from the fact that we have evaluated a different period of the evolution of oligoemia, since there is data supporting a differential pattern of protein expression at different time points after ischemia [27] and, particularly, it has been shown that CAT activity does increase after an ischemic event, but peaks at 24 h post-lesion [16].

Furthermore, it is important to underline that the variations in SOD and CAT activity may be due to the regulatory activation of their expression or activity. If the increase in H₂O₂ concentration overwhelms the defensive action of CAT, the enzyme itself is affected, leading to variations in its specific activity. There is evidence supporting the theory that oxidative damage proceeds through self-perpetuating mechanisms that last much longer than the initial triggering event [1].

The brain, compared to other organs, has a higher concentration of poly-unsaturated fatty acids and phospholipids. This fact, together with its high energy requirements, oxygen consumption and tissue iron concentration, make this organ highly susceptible to lipid peroxidation [15]. The measurements of MDA concentration serve as a surrogate for lipid peroxidation, since this parameter correlates directly with ROS-mediated cell damage, especially for the case of the hydroxyl radical. The increased concentration of MDA obtained in this study agrees with previous results obtained by Ghoneim *et al.* [12], who observed increased levels of lipid peroxidation 1 hour after oligoemia as a consequence of the re-oxygenation of the brain tissue.

The generation of ROS at the cortex of the ischemic animals (which increases the activity of CAT and SOD, as well as the concentration of MDA) was still in course on day 15 after oligoemia. These findings demonstrate the existence of intracellular oxidative damage, and suggest that the oxidative stress parameters measured in this study can be used as indicators of the magnitude of oxidative damage to the cortex after an ischemic lesion.

The behavioral test of the Morris water maze is a useful tool to study the biological basis of learning and memory. This test evaluates the capacity of the animal for learning, remembering and moving towards a point in space which is defined only by its relative position to extra-labyrinth visual cues. Some of its advantages are that it is not painful, the learning phase is fast, the animal can be repeatedly evaluated before and after a defined treatment, and it does not provide intra-labyrinth olfactory cues to the experimental subject [28].

The behavioral test with a visible platform allows the dissociation of cognitive elements from sensorial, motor and motivational factors, since the platform is visible to the rats and therefore their escape does not depend on skills for learning and memorizing a predetermined position, but rather on their visual and natatory abilities [29]. The similarity between the behavior of animals with brain injury and the controls in the test with the visible platform, therefore demonstrates that the bilateral occlusion of the CCA did not significantly affect their capacity to spot the platform, swim towards it and escape from the water.

Both groups of animals were able to learn and remember the location of the platform as evidenced by a statistically significant decrease ($p < 0.001$) in escape latency with the progress of the evaluation period. However, the induced oligoemia led to an alteration of the reference memory in the animals with brain injury, since the plateau of their escape latency curves was less marked than those of the control animals (Figure 2A).

The fact that an effect on retention can be detected in the ischemic animals after 1 month of evolution but not after 14 days can be attributed to the greater memory demands of the assay after a longer period. The determination of the number of crosses on day 13 of the oligoemia took place after 28 consecutive tests with the platform in the same place; however, those performed after 1 month took place only after 15 tests.

These results agree with those of other authors who have worked with the model of bilateral CCA

22. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-54.

23. Otori T, Katsumata T, Muramatsu H, Kashiwagi F, Katayama Y, Terashi A. Long-term measurement of cerebral blood flow and metabolism in a rat chronic hypoperfusion model. *Clin Exp Pharmacol Physiol* 2003;30:266-72.

24. Saito A, Hayashi T, Okuno S, Ferrand-Drake M, Chan PH. Overexpression of Copper/Zinc superoxide dismutase in transgenic mice protects against neuronal cell death after transient focal ischemia by blocking activation of the bad cell death signaling pathway. *J Neurosci* 2003;23:1710-8.

25. Yu L, Wan F, Dutta S, Welsh S, Liu Z, Freundt E *et al.* Autophagic programmed cell death by selective catalase degradation. *Proc Natl Acad Sci USA* 2006;103:4952-7.

26. Blomgren K, Hagberg H. Free radicals, mitochondria, and hypoxia-ischemia in the developing brain. *Free Radic Biol Med* 2006;40:388-97.

27. Burda J, Hrehorovska M, Bonilla LG, Danielisova V, Cizkova D, Burda R *et al.* Role of protein synthesis in the ischemic tolerance acquisition induced by transient forebrain ischemia in the rat. *Neurochem Res* 2003;28:1213-9.

28. Giuliani A, Ottani A, Mioni C, Bazzani C, Galantucci M, Minutoli L *et al.* Neuroprotection in focal cerebral ischemia owing to delayed treatment with melancortins. *Eur J Pharmacol* 2007;570:57-65.

29. Monteiro SC, Matté C, Bavaresco CS, Netto CA, Wyse ATS. Vitamins E and C pre-treatment prevents ovariectomy-induced memory deficits in water maze. *Neurobiol Learn Mem* 2005;84:192-9.

occlusion, but including hypotensive manipulations [7]. During the evaluation of the reference memory, these authors used two schemes with different complexity levels, and although the low-complexity test did not detect differences in the long-term memory between ischemic and control animals, the assay with a high degree of complexity did detect differences in learning and retention capacity due to the induction of an ischemic event. Our results, together with those described above, indicate that the use of complex behavioral tests can detect the presence of behavioral alterations that go unnoticed when employing assays that place a lower demand on the capacity of the animals.

The oligoemic animals also showed alterations in short-term or working memory. However, these differences were significant only on the second day ($p < 0.05$) and became statistically undetectable by the third day. This result can be attributed to the different inter-assay periods employed for different days of the evaluation (20 min and 2 h for the second and third days, respectively; during the first day the inter-test rest was only 20 s long, and the animals could find the platform sooner).

The bilateral occlusion of the CCA reduces the blood flow to the cortex at the temporal-parietal region [30, 31]. It has been shown that 1 hour after the occlusion, the irrigation of this zone decreases by 50% [30]. On the other hand, it is known that cerebral hypoperfusion alters the ultrastructure of the capillary vessels of the brain [32]. The mechanisms involved in the formation and conservation of the memory include the activity of NMDA-type glutamatergic receptors, the stimulation of enzymes such as

calcium-dependent protein kinase II, the activation of transcriptional factors and the induction of the synthesis of neurotrophic factors. Since many of the molecules playing a role in this process are sensitive to changes in the redox state of the intracellular milieu [33], an oxidative unbalance in the cell can lead to behavioral alterations due to the infliction of damage to molecules involved in the process of memory and learning.

Although there are no reports dealing with alterations to memory and learning in the rat model of CCA occlusion without cerebral hypoperfusion after an oligoemia which has been allowed to evolve for an entire month, these alterations have been shown to occur in other models of cerebral hypoperfusion [7, 32], where the spatial learning skills of the animals showing brain injury are diminished compared to the controls. Other symptoms observed during these studies are an increase in escape latency and a decrease in the time spent by the subjects at the previous position occupied by the platform, once it was withdrawn.

Our results reveal that the model of oligoemia in rats subjected to a bilateral occlusion of the CCA disturbs the oxidative metabolism at the cortex, together with alterations of the reference and working memories. These behavioral disorders are evident at least after 10 days of evolution of the oligoemia, and can be evaluated with adequate schemes of the Morris water maze test. Therefore, the rat model of induced cerebral hypoperfusion without hypotension can be useful for the evaluation of the efficacy of therapeutic neuro-restorative, neuro-protective and anti-oxidative strategies aimed at the recovery of cognitive skills.

30. Butte M, Fortin T, Pappas BA. Pinealectomy: behavioral and neuropathological consequences in a chronic cerebral hypoperfusion model. *Neurobiol Aging* 2002;23:309-17.

31. Fadyukova O, Storozhevykh T, Vsevolod G, Pinelis V, Koshelev V. Ischemic and hemorrhagic disturbances in cerebral circulation alter contractile responses of the rat middle cerebral artery. *Brain Res* 2004; 995:145-9.

32. Wilde MC, Farkas E, Gerrits M, Kiliaan A, Luiten P. The effect of n-3 polyunsaturated fatty acid-rich diets on cognitive and cerebrovascular parameters in chronic cerebral hypoperfusion. *Brain Res* 2002; 947:166-73.

33. González ME, Fernández CI, Bauza JY. Indicadores de estrés oxidativo en cerebros de ratas viejas con déficit cognitivo. *Biotechnol Apl* 2007;24:145-50.

Received in May, 2008. Accepted for publication in January, 2009.