

BIOCHEMICAL EVALUATION OF FOCAL NON-REPERFUSION CEREBRAL ISCHEMIA BY MIDDLE CEREBRAL ARTERY OCCLUSION IN RATS

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Abstract – Cerebral ischemia is an important event in clinical and surgical neurological practice since it is one of the diseases that most compromise the human species. In the present study 40 adult rats were submitted to periods of focal ischemia of 30, 60 and 90 min without reperfusion and animals submitted to a sham procedure were used as controls. We analyzed the levels of ATP, malondialdehyde and caspase-3. No significant differences in the biochemical measurements were observed between the right and left brain hemispheres of the same animal in each experimental group. Reduced ATP levels were observed after the three periods of ischemia compared to the sham group. No significant increase in malondialdehyde or caspase-3 levels was observed. Despite significant changes in ATP levels, the results indicated cell viability in the ischemic region as shown by the low rates of lipid peroxidation and apoptosis, findings probably related to the lack of reperfusion.

KEY WORDS: cerebral ischemia, ATP, malondialdehyde, apoptosis.

Avaliação bioquímica da isquemia cerebral focal, não associada à reperfusão, por oclusão da artéria cerebral média em ratos

Resumo – Isquemia cerebral é um acontecimento importante na prática neurológica clínica e cirúrgica, uma vez que é uma das doenças que mais comprometem a espécie humana. No presente estudo 40 ratos adultos foram submetidos a períodos de isquemia focal de 30, 60 e 90 min e como controle foram utilizados animais do grupo *sham*. Foram analisados os níveis de ATP, malondialdeído e caspase-3. Nenhuma diferença significativa nas dosagens bioquímicas foram observadas entre os hemisférios cerebrais direito e esquerdo do mesmo animal em cada grupo experimental. Foi observada redução nos níveis de ATP após os três períodos de isquemia, em comparação com o grupo *sham*. Nenhum aumento significativo dos níveis de malondialdeído ou caspase-3 foi observado. Apesar das alterações significativas nos níveis ATP, os resultados indicaram viabilidade celular na região isquêmica como demonstrado pela baixa taxa de peroxidação lipídica e apoptose, achados que provavelmente estão relacionados com a falta de reperfusão.

PALAVRAS-CHAVE: isquemia cerebral, ATP, malondialdeído, apoptose.

Cerebral ischemia is an important event in clinical and surgical neurological practice since it is one of the diseases that most compromise the human species. In neurosurgical practice, arterial vasospasm after subarachnoid hemorrhage and arterial clamping represent two frequently occurring situations whose study is justified¹. The lack of energy substrates and of oxygen due to the reduced blood flow triggers a series of successive events that compromise cell integrity, possibly leading to necrosis in the central area and to apoptosis, more frequently occurring

in the area of penumbra surrounding the central area, or to both events^{2,3}. The pathophysiology of cell injury and death is poorly understood and therefore the therapeutic procedures commonly employed in ischemic events are essentially based on life support and on the treatment of the consequences of the injury, usually without intervening in the peri-ischemic phenomena which in most cases are the true cause of injury⁴.

The effects of cerebral ischemia can be evaluated by various methods ranging from clinical observation to sophis-

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ticated biochemical methods that provide data about cell metabolism and permit the quantization of normal metabolic reactions in tissues, such as the activity of different enzymes⁵⁻⁷, the determination and the study of the kinetics of neurotransmitter release from tissue⁸, the determination of lipid peroxidation by the measurement of malondialdehyde (MDA), the dosage of intracellular ATP⁹, and endothelial and mitochondrial function¹⁰. Our group has conducted several studies to evaluate the effects of cerebral ischemia using biochemical methods and the determination of mitochondrial function and of neurotransmitter release¹¹⁻¹⁵.

The objective of the present study was to determine the energy metabolism patterns in the early phase of ischemia, aiming to avoid possible influence of reperfusion using ATP dosage, the patterns of oxidative stress based on lipid peroxidation with MDA measurement, and the patterns of apoptosis by caspase-3 measurement.

METHOD

The experiment was carried out according to the Ethical Principles for Animal Experimentation adopted by COBEA (Brazilian College of Animal Experimentation).

Forty male Wistar rats weighing 250 to 350 g were divided into four experimental groups of 10 animals each. The animals of groups 1, 2 and 3 were submitted to 30, 60 and 90 min of ischemia, respectively, by occlusion of the left middle cerebral artery (MCA) with intraluminal mononylon 4.0 suture introduced through the internal cervical carotid artery from an initial access through the artery¹⁶. The animals were anesthetized with halothane inhalation, submitted to orotracheal intubation and ventilated with a respirator, with monitoring of physiological pa-

rameters. After ischemia the animals were killed and samples of the left (ischemic) and right (non-ischemic) brain hemisphere were collected for biochemical determinations and stored in liquid nitrogen at -196°C . Samples from the animals of group 4 (sham), considered to be the control of the surgical procedure, were similarly collected and stored.

Biochemical evaluation

Homogenates were obtained from the samples stored in liquid nitrogen using Tris-HCl 10 mM Ph 7.4 and centrifuged 3000g for 10 minutes at 4°C (in Eppendorf 5417R centrifuge, Hamburg, Schleswig-Holstein, Germany). 150 mM KCl in 10 mM Tris-HCl buffer, pH 7.4, and immediately used for all biochemical determinations. Protein dosage was performed in the supernatant using the method of biuret¹⁷, modified by addition of collate 1%¹⁸ and the aliquots of homogenate were stored at -70° for all biochemical dosages.

Determination of ATP

Aliquots of the homogenate (2 mg) were treated with perchloric acid 1N, neutralized with KOH 2M, centrifuged and analyzed using the commercial Kit Adenosine 5'-Triphosphate (ATP) Bioluminescent Assay Kit (Sigma, Saint Louis, Missouri, USA). ATP levels are reported as $\mu\text{M} \times 10^{-10}/\text{mg}$ of protein.

Determination of lipid peroxidation

Peroxidation was determined spectrophotometrically using a commercial kit (Lipid Peroxidation Assay Kit, Cat. n^o 437634, Calbiochem, San Diego, California, USA) for the detection of MDA together with 4-hydroxy-2(E)-nonenal (4-HNE). MDA levels are reported as μM .

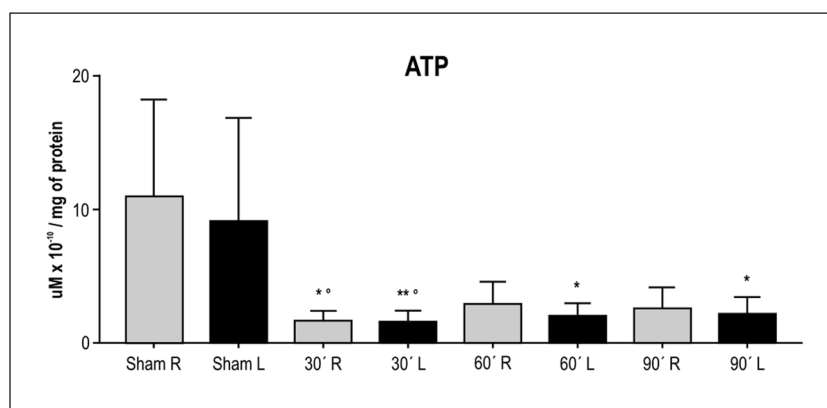


Fig 1. ATP levels in samples from the right (R) and left (L) brain hemispheres of sham animals and animals submitted to ischemia for 30, 60 and 90 min. Data are reported as means \pm SD. There was a significant difference between the samples from the right hemisphere ($p < 0.0001$, one-way ANOVA). The Bonferroni multiple comparisons post-test showed a significant difference between sham \times 30 min ($p < 0.001$), sham \times 60 min ($p < 0.001$) and sham \times 90 min ($p < 0.001$). There was a statistically significant difference between the samples from the left hemispheres ($p < 0.0003$, one-way ANOVA). The Bonferroni multiple comparisons post-test showed a significant difference between sham \times 30 min ($p < 0.001$), sham \times 60 min ($p < 0.01$) and sham \times 90 min ($p < 0.01$).

Determination of caspase-3 activity

Caspase-3 activity in the homogenate was determined using commercial kits (Caspase 3 Assay Kit, Colorimetric, Sigma, Saint Louis, Missouri, USA), Caspase-3 levels are reported as $\mu\text{mol pNA} \times 10^{-2}/\text{min/mL}$.

Statistical analysis

The results obtained for ATP, MDA and caspase-3, were compared between samples from the two hemispheres of the same animal using the nonparametric Wilcoxon test for paired samples, with the level of significance set at $p < 0.05$. Comparisons between groups were made using one-way ANOVA nonparametric test followed by the Bonferroni post-test for multiple comparisons using the GraphPad PRISM software, version 2.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Determination of ATP

The ATP levels (mean \pm SD) determined in samples of the right and left brain hemispheres of the animals in the four groups are presented in Figure 1. Application of the Wilcoxon test for paired samples revealed no significant differences in ATP concentrations between the left (ischemic) and right (non-ischemic) brain hemisphere of animals from the sham group ($p=0.1602$) or from the groups submitted to 30 min ($p=1.000$), 60 min ($p=0.1309$) and 90 min of ischemia ($p=0.5566$).

Despite a great dispersion in the results, there was a significant difference between the samples from the right ($p < 0.0001$, one-way ANOVA) and left hemispheres of the experimental groups ($p < 0.0003$, one-way ANOVA). The Bonferroni post-test for multiple comparisons showed a significant difference for the groups sham \times 30 min

($p < 0.001$), sham \times 60 min ($p < 0.001$) and sham \times 90 min of ischemia ($p < 0.001$) in the right hemispheres and for the groups sham \times 30 min ($p < 0.001$), sham \times 60 min ($p < 0.01$) and sham \times 90 min ($p < 0.01$) in the left hemispheres.

Determination of MDA

Figure 2 presents the MDA levels (mean \pm SD) determined in samples of the right and left brain hemispheres of the animals in the four groups studied.

The Wilcoxon test for paired samples revealed no significant difference between the levels of MDA in the samples of the left (ischemic) and right (non-ischemic) brain hemispheres of the sham group ($p=0.6953$) or of the animals submitted to 30 min ($p=1.000$), 60 min ($p=0.0840$) and 90 min of ischemia ($p=0.9219$).

There was no statistically significant difference between the samples obtained from the right brain hemispheres of the animals ($p=0.3728$, one-way ANOVA), but there was a significant difference between the left brain hemispheres ($p < 0.0404$, one-way ANOVA). The multiple comparisons Bonferroni post-test showed a difference between the groups 60 \times 90 min of ischemia ($p < 0.05$) in the samples from the left brain hemispheres.

Determination of caspase-3

The caspase-3 levels (mean \pm SD) determined in samples from the right and left brain hemispheres of the animals in the four groups are presented in Figure 3. The Wilcoxon test for paired samples showed no significant differences in caspase-3 levels in samples from the left (ischemic) and right (non-ischemic) brain hemispheres of the sham group ($p=0.6250$), or of the groups submitted to 30 min ($p=0.2324$), 60 min ($p=0.9219$) or 90 min ($p=0.6953$) of ischemia.

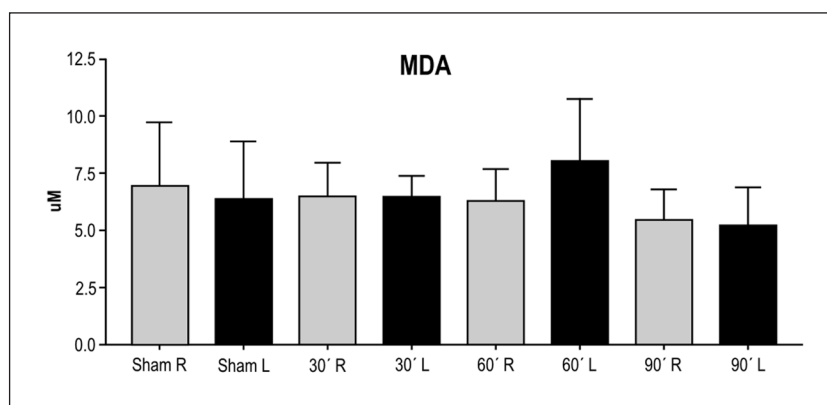


Fig 2. Malondialdehyde (MDA) levels in samples from the right (R) and left (L) brain hemisphere of sham animals and animals submitted to ischemia for 30, 60 and 90 min. Data are reported as means \pm SD. There was no significant difference between groups in the right hemisphere ($p=0.0638$, one-way ANOVA) but there was a significant difference between groups in the left hemisphere ($p=0.4694$, one-way ANOVA) and the multiple comparisons Bonferroni post-test showed a significant difference between the groups submitted to 60 and 90 min of ischemia ($p < 0.05$).

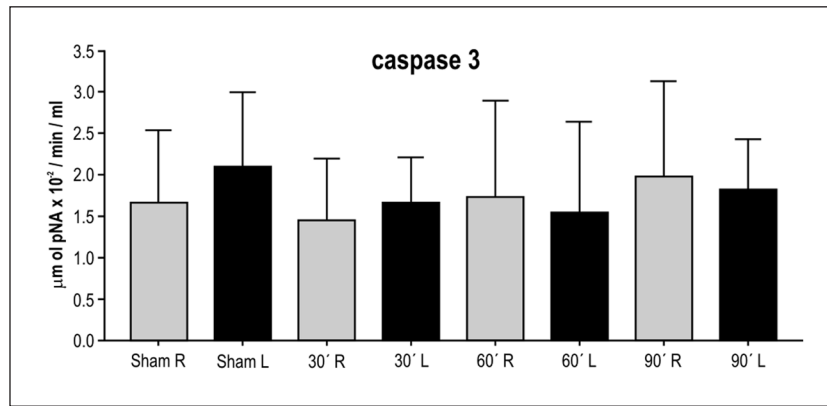


Fig 3. Caspase-3 levels in samples from the right and left brain hemispheres of sham animals and of animals submitted to 30, 60 and 90 min of ischemia. There was no statistically significant difference between samples from the right ($p=0.6739$) and left ($p=0.4694$) hemispheres of the animals of all four experimental groups.

There was no significant difference between the samples from the right ($p=0.6739$) and left ($p=0.4694$) brain hemispheres of the animals in the four experimental groups.

DISCUSSION

ATP is a critical source of energy for the maintenance of the $\text{Na}^+ \text{K}^+$ ATPase ion pump, which regulates the ion concentration gradient for the generation of action potentials by the neurons, and its reduction has been suggested to be a critical factor in the determination of cell death¹⁹.

Energy-rich components such as ATP are necessary to maintain cell structure and functions such as active transport, protein synthesis and phosphorylation, synaptic transmission and the $\text{Na}^+ \text{K}^+$ ATPase ion pump which regulates the ion concentration gradient necessary for the generation of action potentials by the neurons^{19,20}.

Energy status and metabolic changes can be demonstrated and evaluated earlier in temporary ischemia of short duration. As demonstrated in the literature, temporary ischemias (5 to 30 min) impair the energy status of the cell, with partial or total recovery after reperfusion. Morphologic and functional changes are observed in a more delayed manner^{5,11,14,21,22}.

Lee et al.²³ detected a small but significant reduction in the concentrations of ATP, ADP and phosphocreatine in the rat striate after 3 h of ischemia followed by 1 h of reperfusion, strongly indicating the presence of cell viability in this region. Hermann et al.²⁴ observed that intermittent unilateral occlusion of the MCA resulted in a delayed evolution of the focal infarct in the cerebral cortex, in the caudate nuclei, and in the putamen. This process, defined as a phenomenon of maturation of the ischemic lesion, is characterized by initial recovery of ATP metabolism in the brain, followed by slow and gradual failure secondary to

the energy status. Zhan and Yang²⁵ observed a significant decrease (49%) in $\text{Na}^+ \text{K}^+$ ATPase activity in the brain of rats submitted to 2 h of ischemia and 22 h of reperfusion compared to the sham group, demonstrating secondary failure of the energy status of this organ, as reported by Hermann et al.²⁴.

In the present study, in which we used focal ischemia without reperfusion, we observed a significant reduction in ATP in the animals of the ischemic groups compared to the sham group. However, this reduction occurred both in the ischemic and non-ischemic hemispheres, i.e., unilateral ischemia also caused a reduction of ATP production in both hemispheres. This was probably a global response to ischemic stress, as suggested by Harrison et al.²⁶, regarding to the transient decrease in the caspase-8 levels in both cerebral hemispheres of rats after 3 h of focal ischemia by occlusion of the left MCA.

Brain ischemia followed by reperfusion triggers a cascade of molecular events, among them lipid peroxidation²⁷. To assess the level of oxidative stress, Onem et al.²⁸ estimated the levels of MDA, one of the most sensitive indicators of lipid peroxidation, and observed an increase in its levels after 15 min of ischemia followed by 15 min of reperfusion. Damage to membrane permeability due to lipid peroxidation can cause a reduction of the enzymatic activity of $\text{Na}^+ \text{K}^+$ ATPase in the membrane, of the release of lysosomal proteolytic enzymes and of the mitochondrial matrix in the cytoplasm, initiating intracellular proteolysis and cell destruction. Bas et al.²⁹ observed that induction of ischemia for 45 min by occlusion of both carotid arteries followed by 30 min of reperfusion caused an accumulation of oxidation products, among them MDA and nitric oxide (NO), as well as induction of apoptosis in the hippocampal formation of rats.

Some studies^{28,29} have demonstrated significant increases in MDA levels in experiments with ischemia followed by different periods of reperfusion, with lipid peroxidation being highly influenced during the period of reperfusion²⁸. Serteser et al.³⁰, in a study on rats submitted to 60 min of ischemia by MCA occlusion, observed changes in lipid peroxidation, with significantly higher MDA values in the ipsilateral cortex compared to the contralateral one. The present results show that there was no significant increase in MDA levels in ischemic animals compared to sham animals, a fact possibly explained by the absence of reperfusion, nor any difference between the ischemic and non-ischemic hemispheres.

Neurons are among the cells most vulnerable to an ischemic event. While complex processes including both necrosis and apoptosis seem to be involved in neuronal cell death, the mitochondria are known to be involved in both necrotic and apoptotic pathways by releasing mitochondrial proteins such as cytochrome c and anti-apoptotic proteins³¹.

Apoptosis, which is controlled by cysteine-proteases, particularly caspases, is mediated by the mitochondrial release of apoptotic proteins, especially cytochrome c. The latter binds to the cytosolic protein Apaf-1 in the presence of ATP and facilitates the activation of caspase-9, which in turn activates caspase-3³². Several studies have indicated that cerebral ischemia and reperfusion can induce apoptosis in brain tissue^{31,33}. Hermann et al.³⁴, in a study of brain injury in mice after 30 min of focal ischemia followed by reperfusion, demonstrated that protein synthesis in the cerebral cortex was partially recovered after 24 and 72 h of reperfusion, but remained suppressed in the caudate nucleus and in the putamen. The mRNA levels for caspase-3 in the caudate nucleus and in the putamen increased after 24 h of reperfusion and remained unchanged for 3 days, when the rate of protein synthesis was still decreased. However, the mRNA level for caspase-3 did not increase in the cerebral cortex in which protein synthesis was recovered, demonstrating that the recovery of protein synthesis may be a factor that influences tissue survival after transitory focal ischemia.

After MCA occlusion for 1 h followed by reperfusion for 3 and 24 h, Li et al.³⁵ observed by immunohistochemistry an increase in the expression of caspase-3 in the ischemic cortex of rats at 3 and 24 h of reperfusion, which was not observed immediately after the period of ischemia. In the present study, apoptosis assessed by the determination of caspase-3 in ischemic animals not submitted to reperfusion did not show a significant difference as reported by Li et al.³⁵. These changes observed only after reperfusion indicate that "reperfusion injuries",

which lead to a cascade of molecular events, seem to be of great importance for the expression of this apoptotic enzyme and for the consequent worsening of previous tissue changes.

The present study, in which focal ischemia was induced without reperfusion, demonstrated no significant differences in ATP, MDA or caspase-3 levels between the right and left brain hemispheres of each animal in each experimental group. Despite significant changes in ATP levels, the results indicated cell viability in the ischemic cortical region after 90 min based on the low rates of lipid peroxidation (absence of an increase in MDA) and on the apoptosis process (lack of increase in caspase-3 levels), suggesting that they seem to depend on the reperfusion injury and not only on the ischemic process.

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