Memory deficit associated with increased brain proinflammatory cytokine levels and neurodegeneration in acute ischemic stroke

Déficit de memória associado ao aumento dos níveis cerebrais de citocinas pró-inflamatórias e neurodegeneração na isquemia cerebral aguda

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ABSTRACT
The present study aimed to investigate behavioral changes and neuroinflammatory process following left unilateral common carotid artery occlusion (UCCAO), a model of cerebral ischemia. Post-ischemic behavioral changes following 15 min UCCAO were recorded 24 hours after reperfusion. The novel object recognition task was used to assess learning and memory. After behavioral test, brains from sham and ischemic mice were removed and processed to evaluate central nervous system pathology by TTC and H&E techniques as well as inflammatory mediators by ELISA. UCCAO promoted long-term memory impairment after reperfusion. Infarct areas were observed in the cerebrum by TTC stain. Moreover, the histopathological analysis revealed cerebral necrotic cavities surrounded by ischemic neurons and hippocampal neurodegeneration. In parallel with memory dysfunction, brain levels of TNF-α, IL-1β and CXCL1 were increased post ischemia compared with sham-operated group. These findings suggest an involvement of central nervous system inflammatory mediators and brain damage in cognitive impairment following unilateral acute ischemia.

Keywords: brain, ischemia, memory, inflammation, cytokines.
cortex. Interestingly, reperfusion may paradoxically exacerbate brain injury, by involvement of neutrophils, glial reaction, cytokine and chemokine production. Cognitive deficits have often been reported following brain ischemia, being memory the most affected domain, followed by attention and executive function. To the best of our knowledge, no previous study investigated the role of central nervous system (CNS) inflammation in the development of cognitive impairment in acute brain stroke following experimental unilateral common carotid artery occlusion. Thus, the aim of the current study was to investigate the association between memory deficits, neuropathology and CNS inflammatory cytokines in adult C57BL/6 mice submitted to UCCAO.

**METHOD**

**Animals**

The Animal Ethics Committee of Federal University of Minas Gerais (UFMG) approved all experiments (protocol 4412/2012). Eight-to-ten-week-old male C57BL/6j mice were obtained from the Centro de Bioterismo of the UFMG, in Brazil, and kept in the animal facilities of the Immunopharmacology Laboratory, in Department of Biochemistry and Immunology at Biological Science Institute, UFMG. Mice were maintained with filtered water and food in a controlled environment (stable temperature and humidity).

**Unilateral common carotid artery occlusion (UCCAO)**

Mice were anesthetized by intraperitoneal injection of ketamine hydrochloride (150 mg/kg) and xylazine (10 mg/kg). Transient cerebral ischemia was induced by occlusion of the left common carotid artery (UCCAO). Briefly, a midline cervical incision was made and the left common carotid artery was exposed and occluded using microaneurysm clamps. The clamps were removed after 15 minutes of occlusion. In the sham group, arteries were visualized, but not occluded.

**Novel object recognition task**

The novel object recognition task is based on the innate tendency of rodents to differentially explore novel objects over familiar ones. The object recognition task was performed to assess memory 24 hours after ischemia as previously described. Briefly, animals had the opportunity to explore an open field for 5 min (habitation session). On the following day, a training session was conducted by placing individual mice for 5 min into the field in the center of the arena, in which two identical objects (object A1 and A2 were positioned in two adjacent corners at 10 cm from the walls). In the long-term memory (LTM) test (24 h after training), the mice explored the field for 5 min in the presence of a familiar (A) and a different novel (B) object. Objects (Lego toys) had only distinction in shape. The exploratory preference was defined as the percentage of total time (seconds) that the animal spent investigating the novel object and calculated for each animal by the ratio "TB/(TA + TB)*100 ([TA = time spent exploring the familiar object A; TB = time spent exploring the novel object B). The distance traveled in the apparatus arena was also recorded as a locomotor activity parameter. The Anymaze software (Stoelting Co., Wood Dale, IL, USA) was employed for behavioral analysis. All tests were performed by the same investigator who was blinded to the animal status (sham operated or I/R). A total of ten animals per group were used.

**Assessment of cerebral infarction**

At 24 h after UCCAO, mice were deeply anesthetized and brains were removed. The brain was carefully removed and placed in a mouse brain matrix slicer (Insight Ltda., Ribeirão Preto, SP, Brazil), and the entire brain was coronally sectioned at 2 mm intervals. Sections were incubated for 30 minutes in 2% TTC in saline at 37°C and fixed in formalin for 24 hours. Infarcted (unstained) and viable tissue (stained) areas from brain sections were imaged.

**Histopathological examination**

Brains from sham and UCCAO mice were preserved in 10% buffered formalin. Sections of 5 µm thickness were cut at intervals of 10 µm and mounted for hematoxylin and eosin staining.

**ELISA of proteins in cerebral tissue**

Brain tissue extracts were obtained from sham-operated and UCCAO mouse at 24 hours after the ischemia and stored at -20°C. Thereafter, the brain tissue was homogenized in an extraction solution (100 mg of tissue per 1 mL of extraction solution) containing 0.4 mol/L NaCl, 0.05% Tween® 20, 0.5% BSA, 0.1 mmol/L phenylmethyl sulfonil fluoride, 0.1 mmol/L benzethonium chloride, 10 mmol/L EDTA, and 20 KI aprotinin, using Ultra-Turrax (Fisher Scientific, Pittsburgh, PA, USA). The brain homogenate was centrifuged at 3000×g for 10 min at 4°C, and the supernatant was collected and stored at -20°C. Concentrations of the cytokines TNF-α and IL-1β and of the chemokine CXCL1 were determined using ELISA. The brain tissue supernatants were assayed in an ELISA setup using commercially available antibodies, according to the manufacturer's procedures (R&D Systems, Minneapolis, MN, USA).

**Statistical analysis**

Data are shown as mean ± SEM. The t student was used for comparisons between two groups. Statistical significance was set at p < 0.05.

**RESULTS**

The effect of unilateral common carotid artery occlusion and reperfusion in cognition was analyzed using novel object recognition task. UCCAO mice presented an impairment of...
long-term memory 24 hours after ischemia compared to controls, indicated by a significant reduction in the percentage of time exploring the novel object (Figure 1A; p < 0.05). There was no significant difference observed in the distance travelled between UCCA₀ and sham groups, indicating no difference in motor activity (Figure 1B) (n = 10 per group).

Representative coronal brain sections from UCCAO group are shown in Figure 2. TTC staining shows deep red staining of normal brain tissue and white nonstaining of the infarct areas in the cerebrum (asterisks). No morphological changes were observed in brain tissue from sham-operated mice (Figure 3A: hippocampus and D: cerebral cortex). H&E sections from UCCAO mice showed several shrunken neurons with triangulated pyknotic nuclei in all hippocampal subfields (CA1-CA4) (Figure 3B) and hemorrhagic foci (Figure 3C). Ischemic neurons were also observed in the cerebral cortex (Figure 3E). Infarcted areas characterized by formation of cavities surrounded by ischemic neurons were visualized in the cerebrum (Figure 3F).

The inflammatory mediators were measured in the brain from sham and UCCA₀ animals. The concentrations of TNF-α and IL-1β in the CNS of UCCA₀ mice were significantly increased in comparison with sham animals (p < 0.05). Higher levels of CXCL1 were also detected in UCCA₀ animals compared with sham. (p < 0.005) (Figure 4: n = 6 per group).

**DISCUSSION**

Cerebrovascular diseases can directly or indirectly damage brain structures associated with cognitive functions. There is evidence of cognitive decline in patients with large volume of brain ischemia or multiple cortical microinfarcts\(^1\). Clinically asymptomatic vascular brain injury might also cause cognitive impairment\(^6\). In the current study, we demonstrated that object recognition memory impairment was associated with histopathological lesions in cerebrum and hippocampus of UCCA₀ mice. Moreover, an increase in CNS inflammatory cytokines was also found following acute brain ischemia and reperfusion. In the evaluated period, UCCA₀ did not induced locomotor alterations, which supports this model as a valuable tool to study memory impairment following ischemia. Cerebral ischemia can cause severe neuronal injury and death, which can further lead to learning and memory impairment in patients\(^1\). Hippocampus plays a vital role in information processing, memory formation, and subsequent regulation of behavior\(^16\). In our study, cognitive deficit was accompanied by neuronal death in hippocampal CA region 24 hours after reperfusion. The acute brain ischemia induced in this work could be a suitable model to study cognitive changes and neurodegeneration characterized by large cortical infarcts and hippocampal neuronal loss.

![Figure 1](image1.png)  **Figure 1.** Novel object recognition memory impairment without locomotor changes following brain ischemia and reperfusion. C57BL/6 mice (n = 10 per group) were submitted to 15 minutes of ischemia and 24 hours of reperfusion (I/R group). Sham-operated animals were anesthetized and arteries were only exposed (n = 10 per group). All animals were submitted to object recognition task training and test session, respectively. (A) Long-term memory and (B) distance travelled were recorded 24 hours after ischemia. Results are expressed as mean ± SEM and are representative of at least two independent experiments. Asterisk indicates statistical difference (*p < 0.05).

![Figure 2](image2.png)  **Figure 2.** Representative TTC stained brain sections were shown where mice were subjected to 15 minutes of ischemia followed by 24 h reperfusion (I/R). White is infarct area and red is normal area. Infarcted areas were visualized in the cerebrum and hippocampus (asterisks).
We also observed marked cytokine and chemokine up-regulation (IL-1β, TNF-α and CXCL1) after stroke. Astroglial and microglial reaction to the ischemic brain induces the production of inflammatory cytokines and chemokines6,17. We measured the expression of the chemokine CXCL1, the rodent homolog of human IL-8, which is chemoattractant for neutrophils. Neutrophils are the first inflammatory cells in the ischemic tissue and during reperfusion contribute to expansion of brain injury by production of radical oxygen species, proteinases and cytokines7,10. We previously demonstrated that blockade of CXCR1/2 receptors by reparixin promoted neuroprotective effects by reducing the levels of neutrophil infiltration and tissue damage in the brain after cerebral occlusion and reperfusion in mice7. We also detected increased levels of pro-inflammatory cytokines TNF-α and IL-1β, which can contribute to the expansion of brain damage and death of neurons18. Under physiological conditions, IL-1β and TNF-α play an important role in neurogenesis, synaptic plasticity, long-term potentiation (LTP) and memory formation and consolidation19,20. On the other hand, the over-expression of these cytokines in the CNS has been associated with behavioral and cognitive impairment in several pathological conditions21,22, 23, 24.

There are some limitations in the present study. The study is largely descriptive and does not show proof of causality.

Several studies have examined the pathophysiological mechanisms underlying the ischemic process, with the objective of discovering potential targets in the treatment of stroke. Therefore, efforts need to be done not only to preserve the cerebral blood flow, but also to prevent the mechanisms that trigger brain damage after ischemia.

In conclusion, we found that memory deficits after acute brain ischemia are associated with increased levels of brain pro-inflammatory cytokines and neurodegeneration. These findings suggest a role for CNS inflammatory mediators and brain damage in cognitive impairment following ischemia.
References


