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USE OF CONFOCAL MICROSCOPY IN THE STUDY OF ISCHEMIA-INDUCED HIPPOCAMPAL NEURONAL DAMAGE

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Abstract — The present study was undertaken to reveal by means of confocal laser microscopy the cytoarchitecture of hippocampal CA3 neurons in Mongolian gerbils before and after cerebral ischemia of different duration. The common carotid arteries of gerbils were occluded for 5, 10, or 15 min. On the 4th, 14th and 28th day after reperfusion, neuronal damage was examined by laser scanning confocal microscopy in the CA3 region of hippocampus (30 μm slices). Slices were stained with fluorescent Nissl staining and fluorescent membrane tracer DiI. Increased duration of cerebral ischemia resulted in a progressive loss of hippocampal CA3 neurons. Four days after the ischemic insult, neuronal damage in the hippocampal CA3 region was mild but visible. On the 28th day after reperfusion, neuronal damage in the observed brain structure was most severe. These results demonstrate the temporal profile of neuronal damage after an ischemic insult as observed using confocal microscopy.

Key words: Cerebral ischemia, hippocampal cell loss, confocal microscopy, gerbils

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INTRODUCTION

Global cerebral ischemia leads to a cascade of pathophysiological processes that contribute to ischemic cell damage (Love et al., 2000). During the ischemic period and early reperfusion a massive release of excitatory amino acids, an intracellular overload with Ca^{2+}, and an increase in free radicals are the hallmarks of a phase called excitotoxicity (Sengpiel et al., 1998; Sattler and Tymianski 2000; Bailey et al., 2001; Ciriolo et al., 2001). Identification of these events and the underlying cellular mechanism, has led investigators to attempt to selectively intervene with drugs to reduce or prevent the neuronal damage that occurs in a delayed fashion as a consequence of an ischemic episode.

Recently, animal models of transient global cerebral ischemia have been extensively used in investigations of the cellular mechanisms of ischemic cell death and the pharmacology of cerebral ischemia (Liu et al., 1998; Schmidt et al., 2002). In gerbils, due to the lack of posterior communicating arteries, which normally connect the posterior circulation of the brain from the vertebral arteries with the anterior circulation from the carotid arteries in the circle of Willis (Olsson et al., 2003), global cerebral ischemia is easily induced by simple bilateral common carotid artery occlusion.

Cerebral ischemia is a leading cause of death and permanent disability, one for which there is currently no effective treatment. In both humans and animals, ischemia kills neurons in vulnerable regions of the brain, including the hippocampus, which plays a very important role in learning and memory (Liu et al., 1998; Schmidt and Reymann, 2002). In animal models of ischemia, neuronal damage is typically determined by quantifying the degree of cell loss or reductions in infarct volume shortly after the ischemic insult. However, the employed histological methods were unable to reliably detect more subtle forms of neuronal death and dysfunction arising

from injury to non-homogenous cell populations (hilar and striatal neurons) or to dendrites (loss of structural proteins or decreased synaptic transmission).

The present study was undertaken to reveal the cytoarchitecture of hippocampal CA3 neurons before and after cerebral ischemia of different duration by means of confocal laser microscopy.

MATERIALS AND METHODS

Animals

Adult male Mongolian gerbils (*Meriones unguiculatus,* 60 - 75 g) were submitted to different duration of cerebral ischemia. Groups of four gerbils per cage (Erath, FRG), were housed in standard conditions [23 ± 2 °C, 55 ± 10 % humidity, lights for 12 h/day (07.00 - 19.00)], and commercial food and tap water *ad libitum*). Animals used for procedures were treated in strict accordance with the NIH Guide for Care and Use of Laboratory Animals (1985) and with approval of the Ethical Committee of the Serbian Laboratory Animal Science Association (SLASA, reg. No. 210-1342/2-2005-06).

Occlusion of common carotid arteries

Because mature gerbils lack collateral communications between carotid and vertebrobasilar circulations, occlusion of both common carotid arteries results, reproducibly, in global forebrain ischemia. The Mongolian gerbils were anesthetized with diethyl ether and placed in the dorsal position. The neck area was shaved, and both common carotid arteries were exposed carefully by blunt dissection and then clamped for 5, 10, or 15 min with microaneurysm clips. After the clips were removed, reperfusion was confirmed visually, and the skin was sutured by 3 - 4 loose silk stitches. For sham-operated animals, both common carotid arteries were exposed but not occluded. Rectal temperature was controlled carefully and maintained close to 37 °C during ischemia using a heating lamp and a heating pad. Gerbils were allowed to recover in their home cages for 2 h and then returned to the animal quarters.

Confocal microscopy

The gerbils were decapitated on the 4^{th}, 14^{th} and 28^{th} day after reperfusion. Brains were removed from the skull, fixed in 4% paraformaldehyde for at least 24 h before dehydration through a range of alcohol dilutions, and embedded in paraffin wax. Hippocampal 30-μm thick sections were cut on a microtome and thaw-mounted onto gelatin-coated glass slides. To reveal the cytoarchitecture of the hippocampal CA3 region, these sections were stained with the Neurotrace fluorescent Nissl (Molecular Probes) and fluorescent membrane tracer DiI (Molecular Probes) and viewed using a confocal laser scanning microscope (Zeiss LSM 510). An argon laser (488 nm) and helium-neon laser (543 nm) were utilized for the excitation of Nissl staining and DiI, respectively. Following acquisition, images were processed using the Zeiss LSM 510 Basic software package ver. 3.2.

The extend of cell damage to the CA3 hippocampal region was quantified under a Zeiss Axioscope 2 as the mean number of persisted, intact neurons on coronal sections. At least three defined 300 μm^2 fields of the CA3 region (medial, intermediate, and lateral) were saved by a camera of the MC 10095 type (Carl Zeiss Jena), and remaining neurons were counted in a computer-assisted image analysis system (KS 300, Carl Zeiss Jena).

RESULTS AND DISCUSSION

Morphological comparison of neuron appearance within gerbil coronal sections in intact and sham-operated of animals should check for the effect of mechanical stress caused by the surgical intervention. Our results revealed no significant difference between the morphological appearance of neurons in these two groups (not shown). This means that the surgical intervention by itself is not sufficient to trigger oxidative stress and/or excitotoxicity and cause neuronal damage. Thus, sham-operated animals were considered as controls.

Upon examination on a confocal microscope (Fig. 1) ischemia-induced cell damage was observed in three portions of the CA3 region (medial, intermediate, and lateral). In control conditions, pyramidal neurons of the hippocampal CA3 region are morphologically characterized by being in thick layers. The pyramidal neurons have a round shape, with a

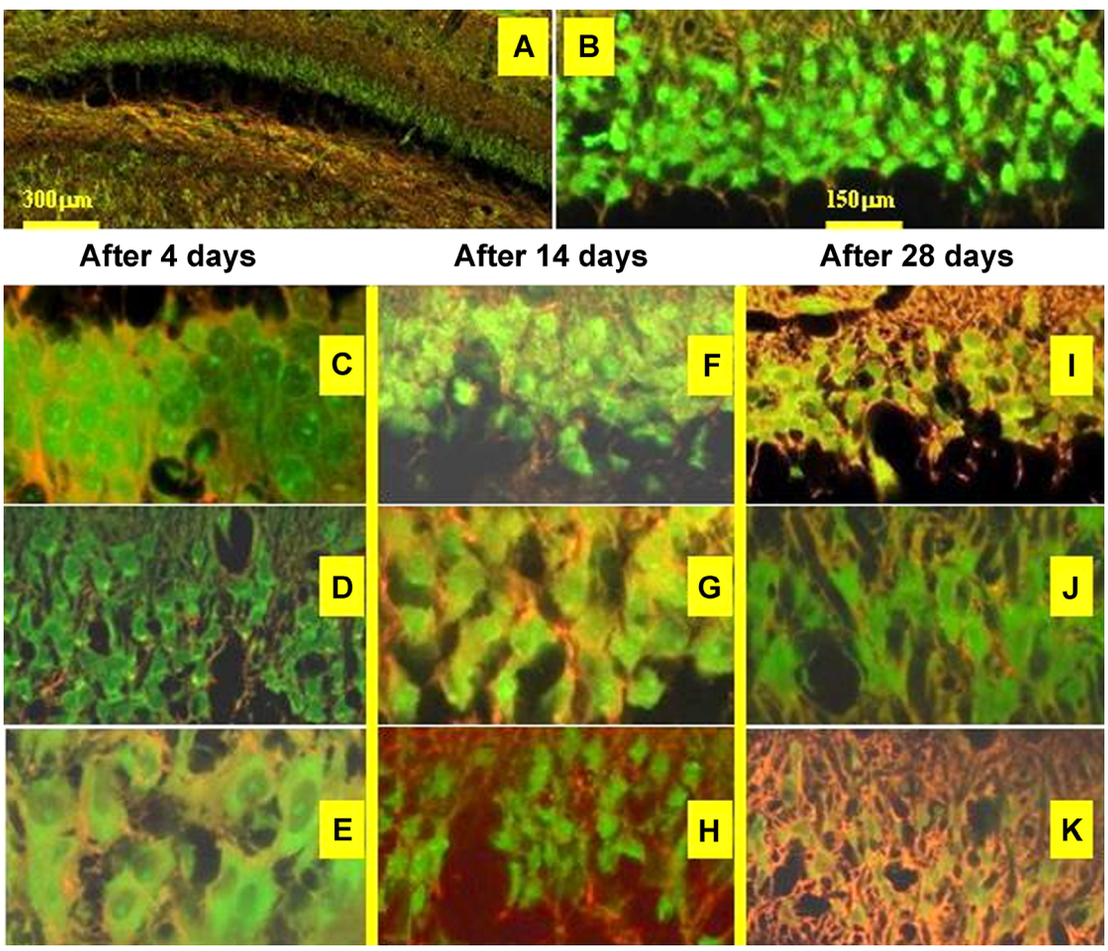

Fig. 1. Morphological appearance of neurons within the CA3 region of the gerbil hippocampus 4, 14, and 28 days after global cerebral ischemia as revealed by staining with fluorescent Nissl stain (green) and fluorescent membrane tracer DiI (red) viewed using a confocal laser scanning microscope. A, B – control; C, D, E – 5, 10, or 15 min of ischemia, respectively, 4 days after reperfusion; F, G, H – 5, 10, or 15 min of ischemia, respectively, 14 days after reperfusion; I, J, K – 5, 10, or 15 min of ischemia, respectively 28 days after reperfusion.

well-defined nucleus and intact cell membrane. The cells are very close to each other and well connected. Morphological indications of ischemic cell damage were seen as neuronal loss, with abnormal shape of remaining cells. Neurons became stretched, spindle-shaped, separated from each other. Their connections were disturbed. The cytoplasm appeared granular, while the nuclei lost definition and became disintegrated. Also, neuronal decay was morphologically characterized by moderate cytoplasmic and whole cell shrinkage or indentation, associated with perineuronal and, at later stages, more generalized vacuolation of the neuropil.

In order to quantify our results, neurons with a well-defined nucleus and intact cell membrane were counted in three portions of the CA3 region (medial, intermediate, and lateral). The total cell counts were averaged from at least three sections per gerbil (Fig. 2). Neuronal damage parameters were the degree of cell loss and morphology of damaged cells. Neurons with intact neurites of uniform diameter and a soma with smooth round appearance were considered as viable, whereas neurons with fragmented neurites and vacuolated soma were considered as non-viable. Our results showed that the degree of hippocampal neuronal loss was closely associated with duration of the ischemic insult.

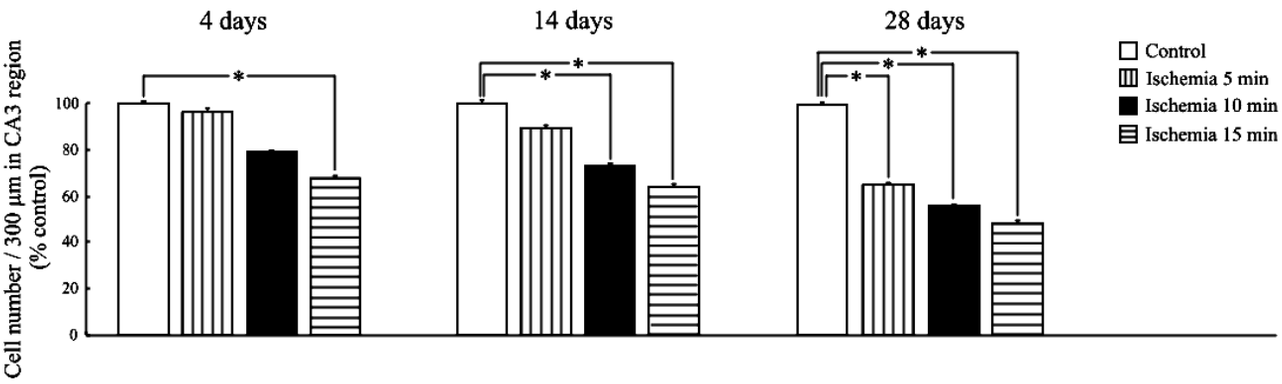

Fig. 2. Number of hippocampal CA3 neurons remaining in gerbils submitted to various durations of carotid occlusion. Values represent means ± SEM. * $p < 0.5$ - vs. control.

Global cerebral ischemia leads to a cascade of pathophysiological processes that contribute to ischemic cell damage. Transient global cerebral ischemia in rodents induces selective degeneration affecting hippocampal CA1 and CA4 neurons with relative sparing of CA3 pyramidal neurons and dentate granule cells. Although degeneration of CA4 neurons begins within hours after the ischemic insult, CA1 neurons remain intact for up to 2 days and then degenerate (Y o k o t a et al., 1999). In our study, we shed light on the temporal profile of ischemia-induced degeneration of the CA3 region, the hippocampal region with no delayed neuronal damage. Our histological results showed that the degree of hippocampal CA3 neuronal loss was closely associated with the duration of the ischemic insult. Ischemia caused extensive degeneration of CA3 pyramidal neurons. After morphological assessment by confocal scanning microscopy, we demonstrated that already four days after the ischemic insult neuronal damage in the CA3 hippocampal region was mild but visible. On the 28th day after reperfusion, the neuronal damage in the observed brain structure was most severe. These features of ischemic cell death agree with previous reports (O l s s o n et al., 2003). However, the signal transduction and intracellular biochemical pathways from external stimuli to cell death during reperfusion are still uncertain.

CONCLUSION

Our results revealed the temporal profile of neuronal damage after an ischemic insult. This may contribute to a better understanding of pathophysiological processes causing ischemic neuronal damage and provide a potential therapeutic time window for neuroprotective intervention after the insult.

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ПРИМЕНА КОНФОКАЛНЕ МИКРОСКОПИЈЕ КОД ИСХЕМИЈОМ ИЗАЗВАНОГ ОШТЕЋЕЊА ХИПОКАМПУСА ПУСТИЊСКИХ МИШЕВА

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Експериментални модел за изучавање исхемије били су џербили - пустињски мишеви, који су били изложени 5, 10 или 15-минутној исхемији. Четвртог, 14 и 28 дана после реперфузије степен оштећења неурона у CA3 региону хипокампуса је процењиван применом конфокалне микроскопије (двоструко бојење) на 30 μm пресецима. Наши резултати показују да је степен оштећења неурона зависан од дужине исхемије и повећава се временом после реперфузије. Највећи степен оштећења неурона CA3 региона хипокампуса био је 28. дана после 15-минутне исхемије.