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Hypoxic damage of the cerebellum in 7-day-old rats Ultrastructural and histochemical study

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Abstract. The damaging influence of hypoxia on the cerebellum in immature rats, which is still discussed, was investigated. Using material obtained in a modified Levine model for combined hypoxic-ischemic damage in 7-day-old rats, we examined changes in cerebellum submitted to hypoxia only. The results demonstrated classic features of hypoxic nervous tissue damage and calcium accumulation in mitochondria and endoplasmic reticulum. This was investigated using electron microscopy combined with the oxalate-pyroantimonate method. We propose that Ca^{2+} increases in endoplasmic reticulum and mitochondrial Ca^{2+} pools may be involved in damage-mediated mechanisms. These results support a role of calcium as a mechanism of cerebellar cell loss after this form of injury.

Key words: hypoxia, cerebellar damage, newborn rats

The damaging influence of hypoxia on the maturing nervous system has been a subject of extensive research. Chronic hypoxia is considered to be cause of delayed myelination (Sarnat 1992, Dąmbska and Wiśniewski 1999). On the other hand, Mujsce et al. (1990) emphasised that perinatal brain hypoxia alone produces no damage in a hypoxic-ischaemic model influencing some brain structures. Their observation was based on investigations of the evolution of brain oedema in 7-day-old rats in a modified Levine model of unilateral permanent carotid artery ligation in newborn rats, followed by generalised 90 min hypoxia. This bears many similarities to birth asphyxia (Hagberg et al. 1997). Using this model, generalised tissue oedema and neuronal and glial cell lesions in the hemisphere submitted to hypoxic-ischaemic damage were observed (Rice et al. 1981). In addition, ultrastructural histochemical investigations revealed the accumulation of calcium deposits intra- and extracellularly in damaged nervous tissue (Gajkowska et al. 1992).Using the material obtained in this model we decided to examine the ultrastructural changes in the cerebellum after severe hypoxia only.

Four 7-day-old rats anaesthetised with halothane were submitted to a hypoxic-ischaemic model of brain damage in the course of which a 90-min hypoxia was produced by placing the animals in an atmosphere of 8% oxygen and 92% nitrogen.

Animal experiments were approved by the Local Ethical Committee of the Medical Research Centre in Warsaw. For morphological and histochemical ultrastructural studies the rats were sacrificed 30 min, 3 and 24 h after hypoxia. Untreated littermates were used as controls.

Animals were anaesthetised with ether and perfused through the heart: 5 min with washing buffer (90 mM potassium acetate and 1.9% sucrose, pH 7.4-KOH-adjusted) followed by 60 min with fixation buffer (90 mM potassiumoxalate and 1.9% sucrose, 3% glutaraldehyde, 0.5% paraformaldehyde, pH 7.4-KOH-adjusted) according to the oxalate pyroantimonate method described by Mata et al. (1987). Pieces of 1 x 1 x 1 mm were cut from the cerebellum. The samples were immersed in perfusion buffer for 2 h at 4°C in potassium oxalate (90 nM in 1.9% sucrose, pH 7.4), post-fixed 2 h at room temperature in 1% O_sO_4 and 2% potassium pyro- antimonate and subsequently washed 10 min in 2.5% glutaraldehyde, pH 10 (adjusted with KOH). After fixation, the samples were dehydrated in alcohol and embedded in Epon 812. Ultrathin sections (500-900 Å, approximately 90 sections from each area) were cut on an LKB Nova Ultratome, stained with uranyl acetate and lead citrate, and examined and photographed with a JEOL 1200 EX electron microscope. The specificity of the oxalate-pyroantimonate reaction in our hands was previously tested by immersion of sections in a solution containing 10 mM of the calcium chelator ethylene glycol-bis-(β-aminoethyl)-N,N'-tetra-acetic acid (EGTA) for 1 h at 60°C. EGTA-treatment, in turn, was controlled by incubation of the sections in distilled water for 1 h at 60°C (Mata et al. 1987).

The ultramicroscopic observation of cerebellum revealed lesions in the tissue only 3 h after hypoxia. Several damaged mitochondria and flaky calcium precipitates within nerve and glial cells were also observed in these cells (Fig. 1). The neuropil presented a more loose structure with enlarged canals of smooth endoplasmic reticulum due to oedematous changes. In many canals calcium precipitates were seen (Fig. 2). 24 hours after hypoxia the tissue lesions were more advanced. Apoptosis of single cells appeared within

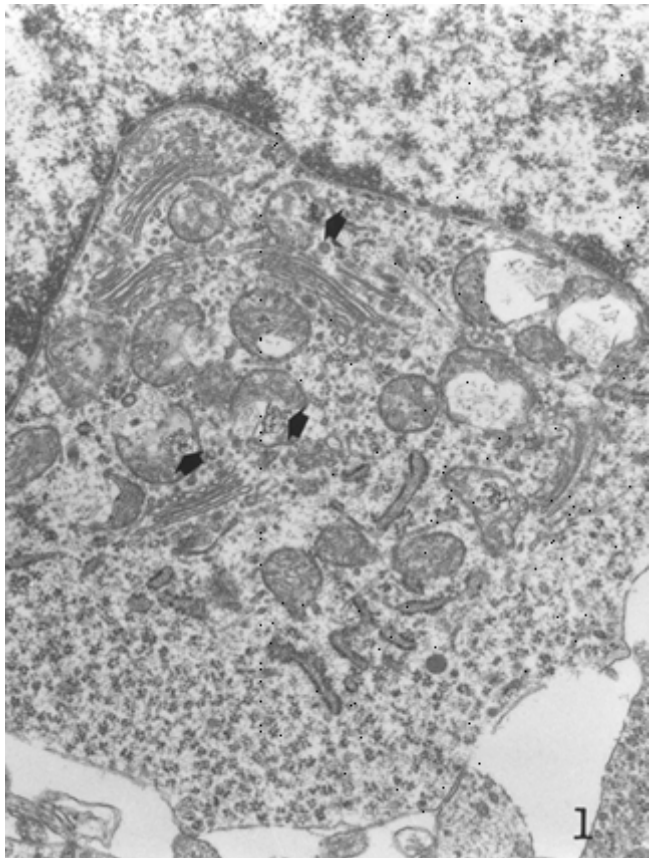

Fig. 1. Electron micrograph of the cerebellum 3 h after hypoxia. Numerous damaged mitochondria with calcium in the matrix (arrows). Magnification, x 30,000.

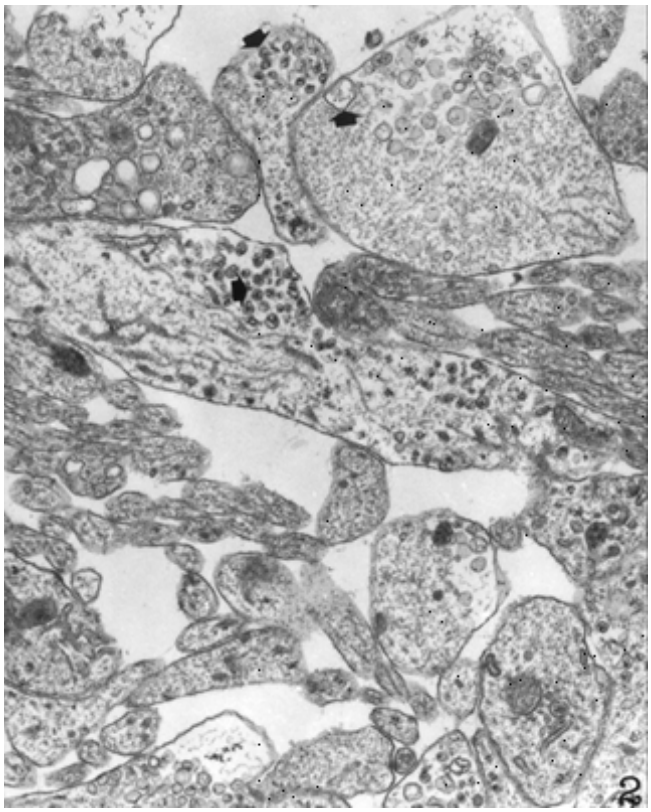

Fig. 2. Electron micrograph of the cerebellum 3 h after hypoxia. Note accumulation of calcium (arrows) in canals of smooth endoplasmic reticulum and in small vacuoles of axons and astrocytic processes. In neuropil, note wide extracellular spaces. Magnification, x 30,000.

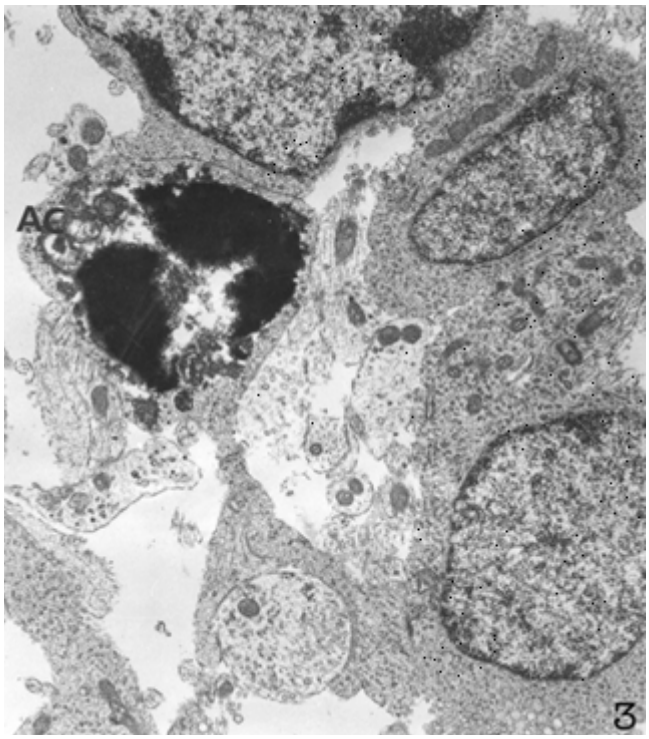

Fig. 3. Electron micrograph of the cerebellum 24 h after hypoxia. Core of injury in immature cerebellum. Note wide extracellular space with apoptotic cell (AC). Magnification, x 18,000.

perivascular oedematous regions (Fig. 3). Cellular lesions with damaged mitochondria demonstrated some calcium deposits in the mitochondria and in endoplasmic reticulum. Such deposits were more abundant in neuropil with evident oedematous changes (Fig. 4).

Control cases showed cerebellar nerve tissue elements normal for the developmental age of the examined animals. The majority of synapses were still immature. Within the loose structure of the neuropil, myelin sheets around nerve fibers were not seen. No evident precipitation of calcium was observed (Fig. 5).

Ultrastructural changes in cerebellum of rats that survived 90 min of severe oxygen deprivation demonstrated the features of nervous tissue damage expressed by generalised oedema and cellular damage only 3 h after the experiment. The observed changes suggested imminent cell death by necrosis or apoptosis. This seems to be in agreement with observations of Rice et al. (1988), that the evolution of changes after hypoxia-ischaemia in the Levine model was more rapid in 7-day-old rats than

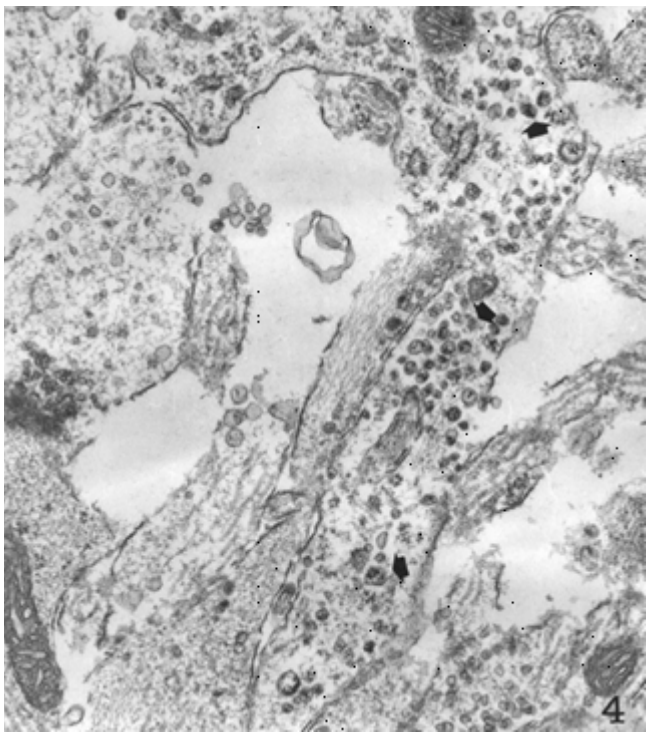

Fig. 4. Numerous vesicles containing calcium deposits (arrows) are present in damaged cells. Magnification, x 45,000.

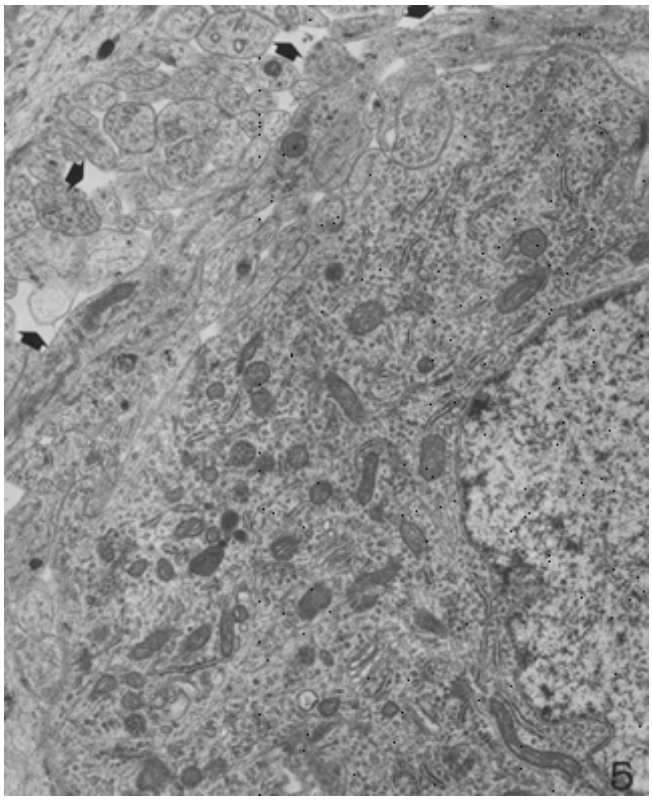

Fig. 5. Electron micrograph of the cerebellum of a control rat at postnatal day 7. Fragment of ultrastructurally normal nerve cell and neuropil with elements of immaturity – wide extracellular space (arrows). Magnification, x 18,000.

in adults. Our observations demonstrated that the changes were very rapid also in hypoxia alone, inducing in experimental animals moderate changes.

The picture of all post-hypoxic lesions in the examined material was dominated by rather abundant calcium deposits within nerve and glial cells. Mitochondria were evidently damaged reflecting their role as a central organelle mediating cellular death (Kroemer et al. 1998). Intra-mitochondrial calcium accumulation may trigger mitochondrial permeability transition (MPT) characterised by opening of pores leading to the disruption of the electrochemical gradient across the inner mitochondrial membrane and mitochondrial swelling (Zoratti and Szabo 1995). Even though swelling of a few mitochondria does not lead to profound depletion of energy production, it may lead to the release of proapoptotic proteins leading to the activation of caspase-3 and DNA fragmentation (Kroemer et al. 1998, Gajkowska et al. 2000, Motyl et al. 2000). The phenomenon of calcium deposition seems to be important for the evaluation of hypoxic lesions in our animals. According to the statement of Berridge et al. (1998), calcium is one of the major signalling compounds in the human body. It influences the beginning and consecutive steps of brain development but may change the signal of life into that of death.

The excessive accumulation of Ca^{2+} in neuronal and other tissues may represent the "final common pathway" for cell death arising from hypoxia-ischaemia because of the disruption of intracellular calcium homeostasis (Stein and Vanucci 1988). When the pathologic influence results in an increased permeability of the cellular membrane and the Ca^{2+} level in cytosol rises, these ions can enter the cells, playing a particular role in their damage by activating enzymes such as lipases, proteases, and endonucleases (Sjesjo and Bengston 1989). This may lead to necrotic cell death, but also in the model of hypoxia-ischaemia was found to lead to apoptosis (Pulera et al. 1998).

Our results, demonstrating an excessive calcium accumulation in cerebellum of rats submitted to severe hypoxia, confirm that this damaging factor leads to tissue damage expressed by some classic features of such changes and also by accumulation of abundant calcium deposits.

CONCLUSION

90 min hypoxia in 7-days-old rats induced classic features of hypoxic lesions in cerebellar nervous tissue and an abundant accumulation of calcium deposits in nerve and glial cells.

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