BRIEF COMMUNICATION

VISUALIZATION OF PROTEIN AGGREGATION IN NERVE CELLS AFTER ISCHEMIA/REPERFUSION BY UBIQUITIN IMMUNOHISTOCHEMISTRY AND IMPREGNATIVE NAUTA METHOD

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Summary: Using ubiquitin immunohistochemistry and impregnative Nauta method we demonstrated that ubiquitin positivity and Nauta positivity in the neurons affected with ischemic injury in the lumbosacral spinal cord of rabbits and dogs may be of the same origin. Increased number of ubiquitin-positive aggregates was found in the cytoplasm of neurons in the intermediate zone and lamina IX of ventral horns of spinal cord in rabbits after 30 min of ischemia followed by 24 h lasting reperfusion. Nauta-positive, flocculent, intracytoplasmic, dark clusters appeared in the same localization in the canine lumbosacral spinal cord neurons after 30 min of ischemia and 24 h of reperfusion. Ubiquitin aggregates and Nauta-positive dark clusters in the injured spinal cord neurons could be the first light microscopic signs of slow neuronal death following spinal cord ischemia and reperfusion.

Key words: *Spinal cord; Rabbit; Dog; Ischemia; Ubiquitin; Immunoreactivity; Nauta method*

Introduction

Ubiquitin is a low molecular weight stress protein found in all eukaryotic cells that is covalently ligated to short lived and denatured proteins produced by various forms of injury. Once ubiquitinated proteins are degraded by 26S proteasome (8,1) in an ATP-dependent nonlysosomal way. It has been proposed that loss of protein ubiquitination causes the accumulation of affected proteins forming aggregates and leads to severe cellular dysfunction and eventually cell death (3).

Neuropathological verification of spinal cord ischemic injury is based on currently used cellular stainings, histochemic methods (10), immunohistochemic methods or silver impregnation procedures. The results of our laboratory suggest that neurons after ischemic injury and reperfusion are stained, in early stage after ischemia, by suppressive Nauta method (7). Only degenerating neurons are stained in dark brown color by this method. Undamaged, normal nerve cells are seen as very light yellow shadows.

The aim of our study was to compare the light microscopic picture of sections from lumbosacral spinal cord of rabbits and dogs after 30 min of abdominal aorta ligation and 24 h of reperfusion after having used ubiquitin immunohistochemistry and impregnative Nauta method.

Material and methods

The experiments were carried out on 6 male rabbits weighing 2.5–3 kg and 8 adult mongreal dogs of both sexes weighing 18–25 kg.

The control group (n=3) and experimental group (n=3) of rabbits were evaluated.

The directions of the Council of European Communities (86/609/EEC) on animal care have been maintained. Handling of experimental animals was performed under the supervision of the Ethical Committee of Medical Faculty of P.J. Šafárik University. The rabbits were anesthetized with thiopental (40 mg/kg, i. v.) and 30 min ischemia of spinal cord was induced by occlusion of abdominal aorta below the left renal artery. After 24 h of reperfusion, the animals were transcardially perfused with 0.9% saline and 4% paraformaldehyde in phosphate buffered saline (pH 7.4). Spinal cords were carefully dissected out and post-fixed in 4% paraformaldehyde for 2 hours. Cryostat sections, 40 μm thick, of the L_5, L_6 spinal cord segments were treated immunohistochemically using rabbit polyclonal antibody to ubiquitin (U-5379, Sigma, 1:100). The reaction product was visualized by the avidin-biotin-peroxidase complex method using DAB (3, 3'-diaminobenzidine, Fluka) as the chromogen. The sections were counterstained with hematoxylin.

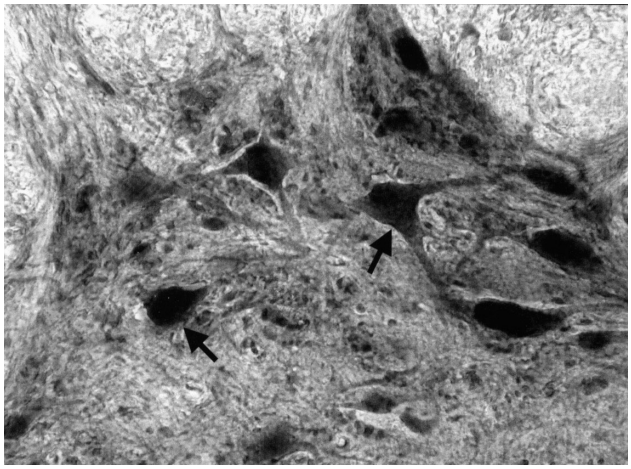

Fig. 1: Spinal cord L_5 segment after 30 min of ischemia and 24 h of reperfusion. Ubiquitin aggregates fill in the cytoplasm of neurons in lamina IX of the ventral horn (arrows). x 400.

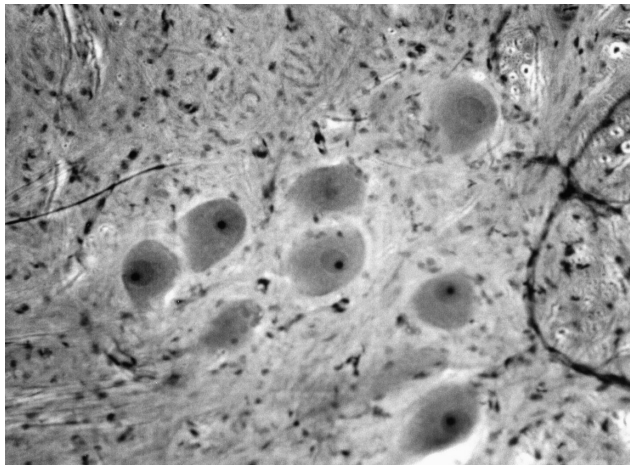

Fig. 2: Spinal cord L_5 segment-control section. Undamaged nerve cells contain round nucleus and darkly stained nucleolus. Nauta staining. x 400.

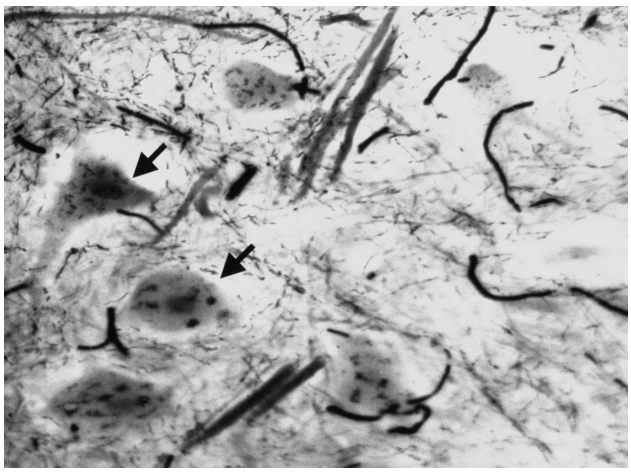

Fig. 3: Spinal cord L_5 segment after 30 min of ischemia and 24 h of reperfusion. Dark Nauta positive argyrophillic material is present in the cytoplasm of the neurons in lamina IX of the ventral horn (arrows). x 400.

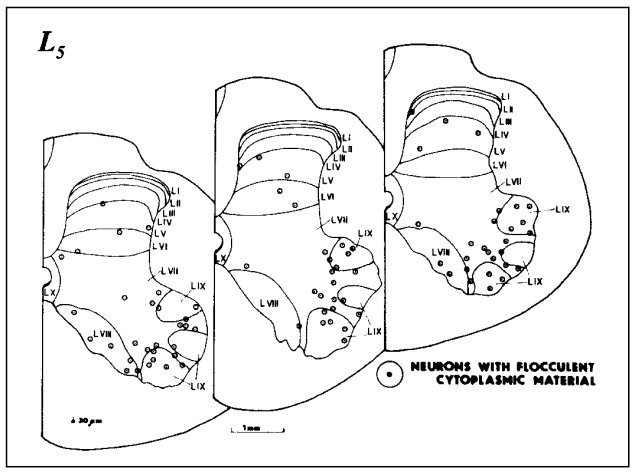

Fig. 4: The distribution of Nauta positive neurons with dark argyrophillic material in the L_5 spinal cord segment.

The control group (n=3) and experimental group (n=3) of dogs were evaluated.

All animals were anesthetized (pentobarbital, 30 mg/kg, i. v.), intubated and placed on a volume-cycled ventilator (Anemat N8, Chirana) using room air. Spinal cord ischemia was induced by aortic cross-clamp in the duration of 30 min, below the origin of left subclavian artery. After 24 h of reperfusion perfusion fixation was made with 0.9% saline and 4% paraformaldehyde in phosphate buffered saline (pH 7.4). Transverse, 30 μm thick cryostat sections were impregnated by Nauta method (7). The slides were analyzed on Olympus light microscope using Olympus DP-SOFT program.

Results

Localization of ubiquitin in the lumbar spinal cord segments was analysed immunohistochemically in rabbits after 30 min of ischemia and 24 h of reperfusion.

In control sections, a weak ubiquitin positivity of the neurons were seen throughout the gray matter of L_5 spinal cord segment. Both, the cytoplasm and the nuclei of the neurons were weakly ubiquitin positive.

Clinically, after 30 min of ischemia and 24 h of reperfusion the animals were paraplegic. In the cytoplasm of neurons in the intermediate zone and in lamina IX of ventral

horns (Fig. 1) the increased amount of ubiquitin positive aggregates was found.

The analysis of control Nauta stained L_5 dog's spinal cord segments did not reveal any signs of Nauta positivity. In all parts of the gray matter pale-stained perikarya of neurons were visible (Fig. 2) without signs of argyrophilia.

After 30 min of aorta ligation and 24 h of reperfusion in L_5 spinal cord segments of the dog argyrophilia occurred in affected neurons. In relation to laminar distribution, the large neurons containing dark, flocculent, argyrophilic material were present in lamina IX (Fig.3). This phenomenon is only occasionally seen in small neurons of the dorsal horn and in the intermediate zone. The number of ischemia/reperfusion damaged argyrophilic neurons was counted with respect to their laminar distribution using 10 serial sections taken from L_5 segment in each animal (Fig. 4). The drawing shows that the majority of Nauta positive neurons with dark argyrophilic material was localized in the ventral and lateral parts of the ventral horn.

Discussion

Protein aggregates that contain ubiquitinated proteins are commonly present in nerve cells in neurodegenerative disorders and after metabolic stress such as ischemia and reperfusion (4, 3). Ubiquitin, as one of the stress proteins, undoubtedly is required under stressful conditions for cell survival and is essential for rapid degradation of altered proteins by 26S proteasomes (1). Using immunohistochemic method we demonstrated that the ubiquitin positivity in the neurons of rabbit lumbosacral spinal cord after 30 min of aorta ligation and 24 h of reperfusion may have the same origin as Nauta positivity in affected neurons in the dog. The protein aggregates observed under electron microscope after 15 min of ischemia and 4 h of reperfusion were described by Hu (3). The protein aggregates present on the nuclear envelope, in the Golgi apparatus and on the mitochondrial membrane cause the impairment of nuclear envelope, damage of post-translational protein modification and transportation, overproduction of reactive oxygen species and severe secondary energy failure that initiates ischemic neuronal death (2,6,9).

Dark clusters of intracytoplasmic and intradendritic bodies were visible in the canine spinal cord neurons after 30 min of ischemia and 24 h of reperfusion. These dark Nauta positive structures in general resemble the cytoplasmic distribution of Nissl bodies. Presumably the membranes of the endoplasmic reticulum might be the organelles to become argyrophilic due to the ischemic damage (5).

The increased protein ubiquitination is an early change resulting from ischemia and is a protective response (11). On the other hand, massive aggregation of ubiquitinated abnormal proteins in cells damaged by ischemia is the result of broken proteolytic ubiquitin pathway which leads to ischemic cell death (3).

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