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Review Article

Changes In Bcl-2 Levels in the Hippocampus of Rats with Different Degrees of Cerebral Ischemia

Bon E.I *, Maximovich N.E, Karnyushko O.A, Zimatkin S.M, Novak A.A, Sorokina T. S, Sidzineuskaya A. I, Misik V.A, Kipen K.M

Grodno State Medical University, Republic of Belarus.

***Corresponding Author:** Bon EI, Grodno State Medical University, Republic of Belarus.

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Abstract

Objective – to study and analyze the dynamics of changes in the BCL-2 protein content in several groups of rats with varying degrees of ischemia. We modeled cerebral ischemia (CI) of varying degrees of severity (partial, subtotal, and stepwise subtotal) under intravenous thiopental anesthesia (40-50 mg/kg). Using the immunohistochemical method with monoclonal antibodies, we measured the Bcl-2 protein content in the cytoplasm of pyramidal neurons in the CA1 field of the hippocampus.

Keywords: Bcl-2 protein; ischemia; rats; immunohistochemical method

Introduction

The pathogenesis of ischemic brain damage includes a biochemical cascade, part of which is the activation of apoptosis [4,7]. In addition to its pathogenic role, apoptosis plays an important role in the process of embryogenesis and organogenesis, as well as in the involution of organs and tissues in an aging organism, immune response, and other processes.

The manifestations of neuronal apoptosis include perikaryon swelling, neuropile destruction, chromatin condensation, and DNA fragmentation followed by karyorrhexis.

The external (receptor-mediated) mechanism of apoptosis is induced by extracellular stress signals that are perceived by specific transmembrane receptors.

Caspase-dependent cell death can be inhibited by broad-spectrum inhibitors, such as N-benzyl-oxycarbonyl-Val-Ala-Asp-fluoromethylketone.

There is a mitochondrial mechanism for enhancing the function of effector caspases. In this process, caspase-3 in its active state cleaves the Bid protein in the cytoplasm, which then moves to the mitochondria and becomes embedded in the membrane, leading to the release of cytochrome c from the mitochondria into the cytosol. Cytochrome c interacts with Apoptoticproteaseactivatingfactor 1(APAF-1) and procaspase-9, activating the latter and activating procaspase-3, which in turn activates caspases-6 and 7 [1].

The causes of apoptosis through changes in mitochondrial function include a lack of growth factors in the cell's environment, increased production of reactive oxygen species, DNA damage, and others [2].

Under the influence of these stimuli, the function of proteins belonging to the Bcl (B-CellLeukemia/Lymphoma). Proteins of this family are divided into two classes.

The first class of Bcl family proteins inhibits apoptosis. They are embedded in the outer mitochondrial membrane and regulate membrane permeability, as well as reduce the formation of reactive oxygen species. The representatives of this class of proteins are Bcl-2 and Bcl-xL.

The Bcl-2 protein is localized on the outer mitochondrial membrane, inhibiting the actions of pro-apoptotic proteins that normally act on the mitochondrial membrane, increasing its permeability and triggering apoptosis [10].

The second class of Bcl family proteins stimulates the development of apoptosis. These proteins are located in the cytosol, but then move to the mitochondrial membrane, where they interact with the first class of proteins, Bcl-2 and Bcl-xL, inhibiting them. The members of the second class of Bcl family proteins include Bid, Bad, Bax, and others.

Activation of the second class of Bcl causes an increase in the permeability of the inner mitochondrial membrane, which leads to swelling of the mitochondrial matrix and rupture of the outer membrane [9]. When the function of the Bcl-2 family members that inhibit apoptosis is suppressed, a "non-selective mega-channel" may form in the mitochondrial membrane, increasing the membrane's permeability and leading to the release of cytochrome c and the DIABLO protein from the mitochondria into the cytosol.

Cytocrome c, which enters the cytosol, interacts with APAF-1, attracting caspase-9, which leads to the formation of a protein complex called the apoptosome. In the presence of ATP, caspase-9 is activated. The

DIABLO protein inactivates protein inhibitors of apoptosis, leading to the activation of caspase-3 [3].

The initiating factors of the mechanism involving the internal pathway of apoptosis are intracellular changes, such as DNA damage, oxidative stress, cytosolic calcium overload, excitotoxicity (overstimulation of glutamate receptors), accumulation of denatured proteins in the endoplasmic reticulum, and others [5]. The main mechanism of the internal pathway of apoptosis is an increase in the permeability of mitochondrial membranes [6].

The caspase-independent pathway of cell death is associated with chromatin lysis and is accompanied by depletion of NAD+ and ATP.

The study of changes in the Bcl-2 protein content in cerebral ischemia of varying severity is of interest [8].

Materials and Methods of the Study

The experiments were performed on 42 outbred male white rats weighing 260±20 g, in compliance with the requirements of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Cerebral ischemia (CI) of varying severity (partial, subtotal, and staged subtotal) was modeled under intravenous thiopental anesthesia (40-50 mg/kg).

Partial cerebral ischemia (PCI) was modeled by ligation of the right common carotid artery (CCA).

Subtotal cerebral ischemia (SCI) was modeled by simultaneous ligation of both CCAs.

Staged subtotal CI (SSCI) was performed by sequential ligation of both CCAs with intervals of 7 days (subgroup 1), 3 days (subgroup 2), and 1 day (subgroup 3).

Tissue sampling (hippocampal regions) was performed 1 hour after the surgery.

The control group consisted of sham-operated rats of similar sex and weight.

The determination of Bcl-2 content in the cytoplasm of pyramidal neurons in the CA1 field of the rat hippocampus was carried out using an immunohistochemical method with monoclonal antibodies. For this purpose, after decapitation, the brains were quickly removed from the rats, fixed in zinc-ethanol-formaldehyde at +4°C (overnight), and then embedded in paraffin.

Paraffin sections, 5 μm thick, were prepared using a microtome and mounted on slides. The specimens were processed according to the immunocytochemical reaction protocol for light microscopy, omitting the heat-induced antigen retrieval procedure. To determine the immunoreactivity of the molecular marker Bcl-2, primary monoclonal mouse antibodies (Anti-Bcl-2 rabbit pAb antibody, Finetest, China, Catalog No. FNab00839) were used at a dilution of 1:1600 at +4°C, with an exposure of 20 hours in a humid chamber. The content of the Bcl-2 protein in the neuron cytoplasm was studied in immunohistochemical preparations based on the optical density value of the chromogen precipitate using an Axioscop 2 plus microscope (Zeiss, Germany), a Leica DFC 320 digital video camera (Germany), and ImageWarp image analysis software (Bitflow, USA).

To prevent systematic measurement errors, brain samples from the compared control and experimental groups of animals were studied under identical conditions.

The studies yielded quantitative continuous data. As the experiment involved small samples with a non-normal distribution, the analysis was performed using nonparametric statistical methods with the licensed computer program Statistica 10.0 for Windows (StatSoft, Inc., USA). The data are presented as Me (LQ; UQ), where Me is the median, LQ is the lower quartile value, and UQ is the upper quartile value. Differences between groups were considered significant at $p < 0.05$ (Kruskal-Wallis test with Bonferroni correction).

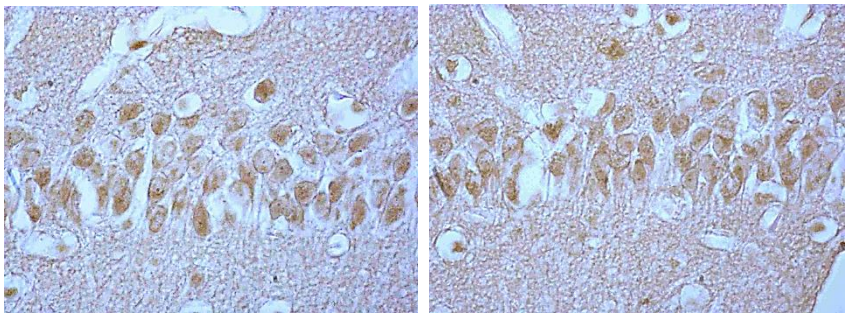
Results and Discussion

In rats with PCI, no changes in Bcl-2 content relative to the level in the control group ($p > 0.05$) were detected (Table 1, Figure 1), indicating the preservation of normal apoptosis regulation in this type of CI.

Control	PCI	SCI	SSCI		
			1 pg	2 pg	3 pg
0,22 (0,20; 0,23)	0,23 (0,22; 0,24)	0,16* (0,15; 0,16)	0,24 (0,23; 0,25)	0,16* (0,15; 0,16)	0,16* (0,16; 0,17)

Table 1: Bcl-2 content in the cytoplasm of pyramidal neurons in the CA1 field of the hippocampus in rats with cerebral ischemia, Me (LQ; UQ)

Notes: * – $p < 0.05$ compared to the control group; PCI – partial cerebral ischemia; SCI – subtotal cerebral ischemia; SSCI – staged subtotal cerebral ischemia.



Control **PCI**

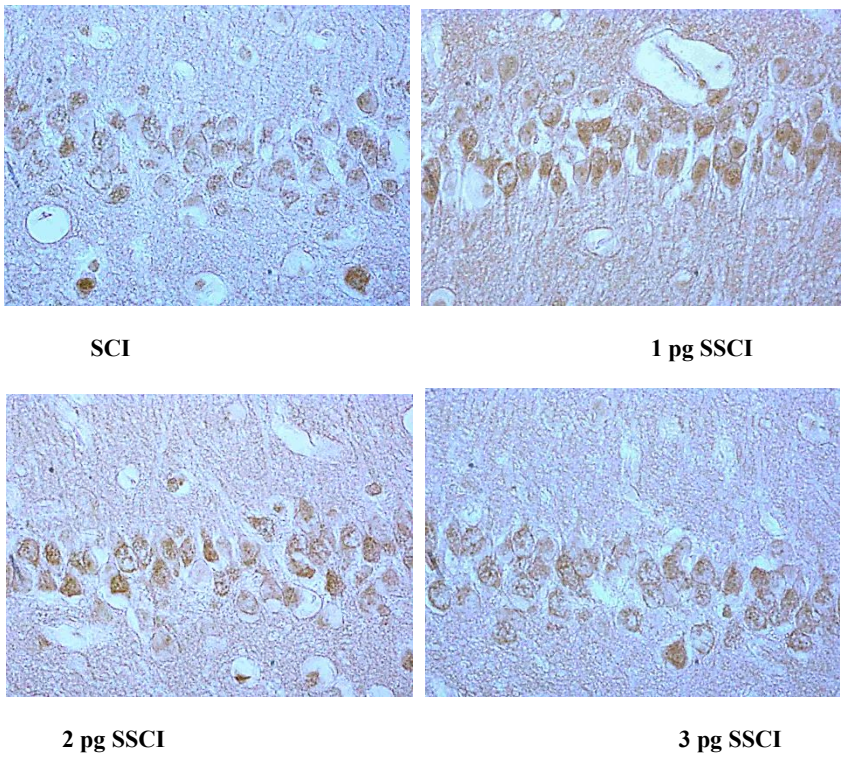


Figure 1: Bcl-2 content (in optical density units) in the cytoplasm of pyramidal neurons in the CA1 field of the rat hippocampus in partial (PCI), staged subtotal (SSCI), subtotal (SCI), and total (TCI) cerebral ischemia. Digital micrograph. Magnification x40.

Note: PCI – partial cerebral ischemia; SSCI – staged subtotal cerebral ischemia; SCI – subtotal cerebral ischemia.

In SCI, compared to the control group, the Bcl-2 content in the hippocampus decreased by 27% ($p < 0.05$), which may be a sign of impaired apoptosis blockade in a more severe type of CI.

Compared to PCI, the Bcl-2 content in SCI was lower by 30% ($p < 0.05$).

Compared to the control group, in the 1st subgroup of SSCI with a 7-day interval between CCA ligations, the Bcl-2 content did not change ($p > 0.05$), while in the 2nd and 3rd subgroups of SSCI (with intervals between ligations of 3 days and 1 day, respectively), it decreased by 27% ($p < 0.05$).

There were no differences in the Bcl-2 content in the cytoplasm of hippocampal neurons between the 1st subgroup of SSCI and the PCI group ($p > 0.05$), whereas compared to the SCI group, it was higher by 33% ($p < 0.05$).

Thus, the changes in Bcl-2 content in the 1st, 2nd, and 3rd subgroups of SSCI were multidirectional: in the 1st subgroup with the maximum interval between CCA ligations (7 days), it did not change, indicating normal regulation of apoptosis processes, while in the 2nd and 3rd subgroups, the Bcl-2 content decreased, which may indicate a lack of blockade of neuronal death processes in more severe forms of cerebral ischemia.

Conclusion

Thus, the most significant changes in Bcl-2 content occurred in subtotal cerebral ischemia lasting 1 day, whereas in partial and staged subtotal cerebral ischemia with a 7-day interval between ligations of both common carotid arteries, the Bcl-2 content did not change compared to the control group. In staged subtotal cerebral ischemia with intervals between ligations of both common carotid arteries of 1 and 3 days, the Bcl-2 content was similar to that observed with simultaneous ligation of both common carotid arteries.

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