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Apoptotic death of cortical neurons following surgical brain injury

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Abstract

Unilateral surgical brain injury of the rat is a model of surgery-related brain damage of humans. Our preliminary experiments showed two phenomena within the damaged cortical region of rat brain. Those were: degeneration and death of neurons and massive gliosis. In the present study we aimed to investigate the mechanisms of neuronal death following brain injury and to characterize responses of glial cells to the damage. We analyzed the morphological changes and alterations of immunochemical profile of cells localized in the brain areas adjacent to the lesion. Our data show the massive neuronal death following the lesion. Neurons undergo necrosis and apoptosis, but on the 4th day following the operation apoptosis prevails. Apoptotic cells showed heavy immunostaining for proapoptotic Bax and caspase 3. This result suggests the involvement of these proteins in neuronal apoptosis in our experimental model. Neuronal death is accompanied by the induction of astrogliosis in the perilesional cortical area. Astrocytes became hypertrophic. We did not detect any dying astrocytes at the investigated time point, but there is a possibility that apoptosis may occur in astroglia during another time period following the damage. This question requires further studies in our experimental model.

Key words: *neuron, apoptosis, Bax, caspase 3, brain injury.*

Introduction

A number of mechanisms have been implicated following brain injury, including energy failure and inflammation. Central to all of these mechanisms are cell death pathways that resemble apoptosis and necrosis. At least in the adult nervous system, mechanisms of ischaemic cell death often involve elements of both or aponecrosis. Dynamic mechanical deformation of neuronal tissue produces a microstructural response at the cellular level, triggering a complex array of signalling events. These events, such as activation of second messengers, phosphorylation of proteins, and changes in the synthesis of transcriptional factors [5,10,13,20], lead to damage and cell death in the initially affected cells as well as neighbouring cells within hours or days post-injury [12].

In particular, neuronal cell death, especially via apoptosis, has recently become an area of intense investigation [16], because of its potential reversibility. The major issue is to find proteins which regulated and executed the apoptotic process in each individual experimental model. One of these proteins is Bax. It is a member of the Bcl-2 family of proteins. Bax can promote apoptosis [for example: 9,11,15,23]. The main protein involved in the

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execution of apoptosis in mammals is caspase 3 [for example: 17,18], which can cleave and activate other caspases (e.g. caspases 6, 7 and 9) and cut many cellular key proteins, such as nuclear enzyme poly (ADP-ribose) polymerase – PARP [3]. Caspase 3 belongs to the family of cysteine proteases, and is the most extensively studied apoptotic protein among caspase family members.

Our experimental model of surgical injury of rat cerebral cortex imitates surgery-related brain damage of humans. This injury initiates disruption of brain parenchyma and glial scar formation. In our preliminary studies we detected two different phenomena within the damaged cortical region of rat brain. Those were: rapid neuronal degeneration and death, and massive gliosis. Both these phenomena are very intense on the fourth day post-lesion, so we decided to perform our present studies at this time point.

In the present study we aimed to investigate mechanisms of neuronal death following brain injury. We also aimed to characterize responses of non-neuronal cells within the injured cortical region.

Material and Methods

All surgical procedures and treatments were approved by the local animal Ethical Committee.

Eighteen adult, male Wistar rats (200-250 g) were used in this experiment. Twelve animals were subjected to surgical brain injury. Then rats were utilized 4 days after the operation. Six intact animals served as a control group.

Animals were anaesthetized with 20 mg/kg ketamine hydrochloride. The head skin was incised and removed. Traumatic injury was induced in the fronto-temporal region of the cerebral cortex [see 4]. The operated region was hemisected with a small scalpel and the wound was closed. The wound was sutured and dressed under aseptic conditions. The rats remained under standard laboratory conditions for 4 days.

Four days after the operation six animals (four operated and two control) were anaesthetized with Nembutal (80 mg/kg b.w.) and perfused through the ascending aorta with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 20°C. Material for ultrastructural morphological studies was sampled from the cerebral cortex, contacting with the operated region. The tissue was fixed in the same solution for 20 h and placed in a mixture of 1% OsO_4 and 0.8% $K_4[Fe(CN)_6]$. Then, material for microscopic studies was processed for transmission electron microscopy [4] and analyzed in a JEM-1200EX.

Twelve animals (eight operated and four control) designed for immunohistochemical (IHC) and histological studies were anaesthetized with Nembutal (80 mg/kg b.w.) and perfused (see above) with 4% paraformaldehyde in 0.1 M phosphate buffer. Then brains were removed from the skull and postfixed with the same fixative for 2 h. Tissue was subsequently infiltrated in 10% sucrose in phosphate buffer, followed by 20% and 30% sucrose solutions infiltration for the following days, and cut on a cryostat (Leica). IHC reactions and histological staining were carried out on 40-µm-thick free-floating or slide-mounted brain sections. Primary antibodies (Abs) against NeuN (1:1000, Chemicon), GFAP (1:1000, Chemicon), Bax (1:400, Santa-Cruz) and active caspase 3 (1:200, Cell Signaling) and secondary antibodies conjugated to HRP (1:1000, Bio-Rad) were used. The HRP reaction product was visualized with an SG kit (Vector, Vector Labs). For the analysis of cell morphology several dyes were used. They were cresyl violet (Sigma), haematoxylin & eosin (Sigma), toluidine blue (Sigma) and bisbenzimide (Hoechst; Sigma).

To control the immunostaining specificity we performed an experiment in which the primary antibodies were omitted in the incubation mixture. In this experiment we did not detect immunolabelling within the investigated brain sections.

Results

Unilateral surgical brain injury caused massive cell degeneration and subsequently death within the injured cortical area. On the fourth day following the injury we observed cortical atrophy and the loss of tissue within the operated region (Fig. 1).

In our histological investigations we found neurons that had undergone necrosis and apoptosis, as well, in the sections stained with cresyl violet, haematoxylin & eosin and toluidine blue (data not shown). On the 4th day following the operation we observed only a few necrotic swollen neurons and numerous neurons with apoptotic appearance (for example, cell shrinkage and chromatin condensation) and apoptotic bodies. This result was confirmed by the bisbenzimide (Hoechst) staining technique (Fig. 2). At

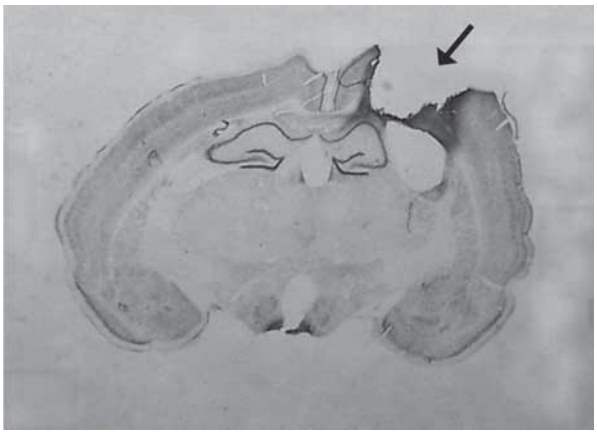

Fig. 1. The area of cortical degeneration on the 4th day following the operation

the same time point the activation of astroglia was detected in the proximity of the lesion (Fig. 3). Active astrocytes became hypertrophic. We did not find any apoptotic astroglial cells (data not shown).

Our data obtained from electron microscopy studies confirm these results. Within the area immediately adjacent to the lesion we found numerous places rich in neurons showing ultrastructural features of cellular death. Some of them were characterized by electron-dense organelles and nucleus. These were so-called dark neurons (Fig. 4B). In addition to dark neurons (and neurons undergoing autophagy; data not shown), the analyzed specimens

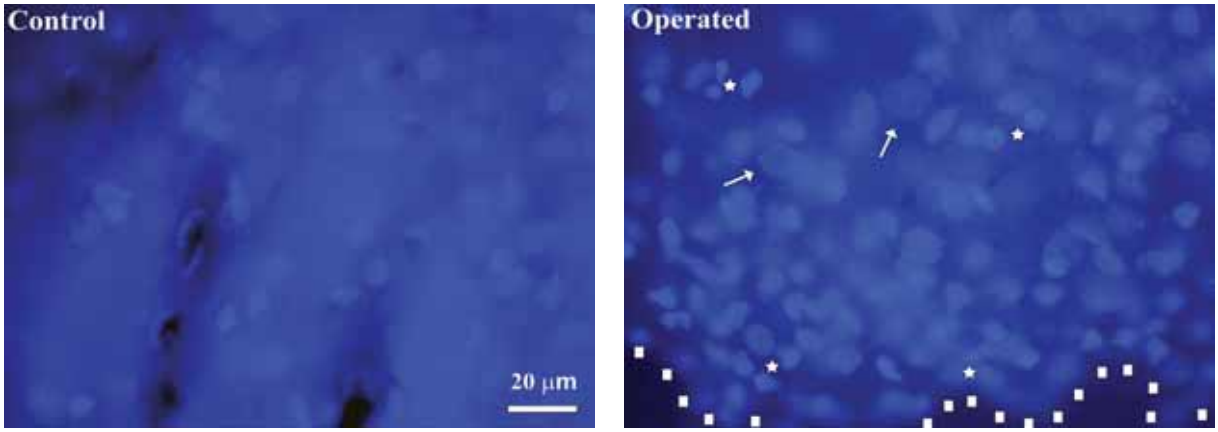

Fig. 2. Rat cerebral cortex stained by bisbenzimide (Hoechst). Left microphotograph shows section derived from the control animal. Note the regular morphology of the cell nuclei. Right microphotograph shows section derived from the operated animal four days following the lesion. Note the presence of necrotic swollen nuclei (arrows) and apoptotic bodies (asterisks). White squares demarcate the border of the lesion

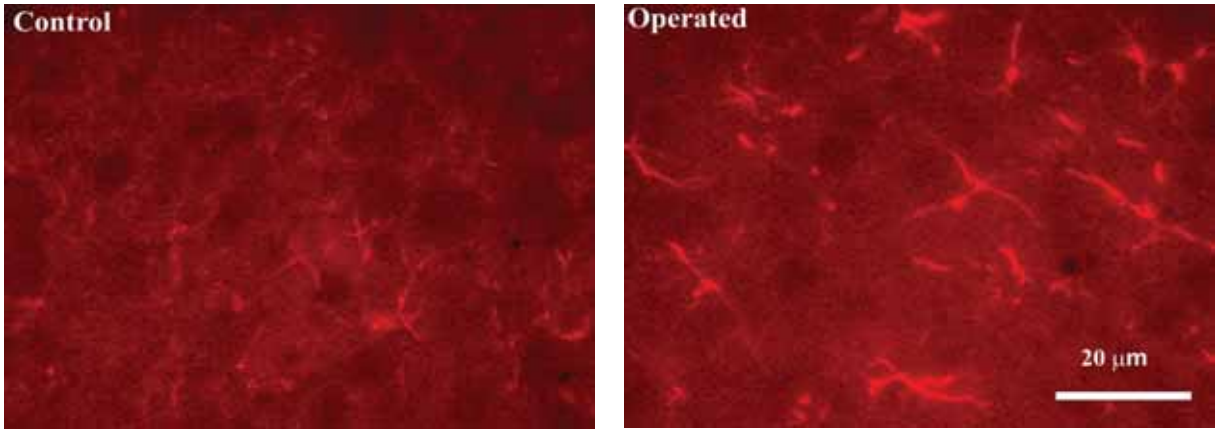

Fig. 3. Cerebral cortex stained for GFAP (marker of astrocytes). Within the control cortex (left microphotograph) astrocytes with unchanged morphology were detected. In the operated cortex (right microphotograph) numerous astrocytes were detected. They became hypertrophic

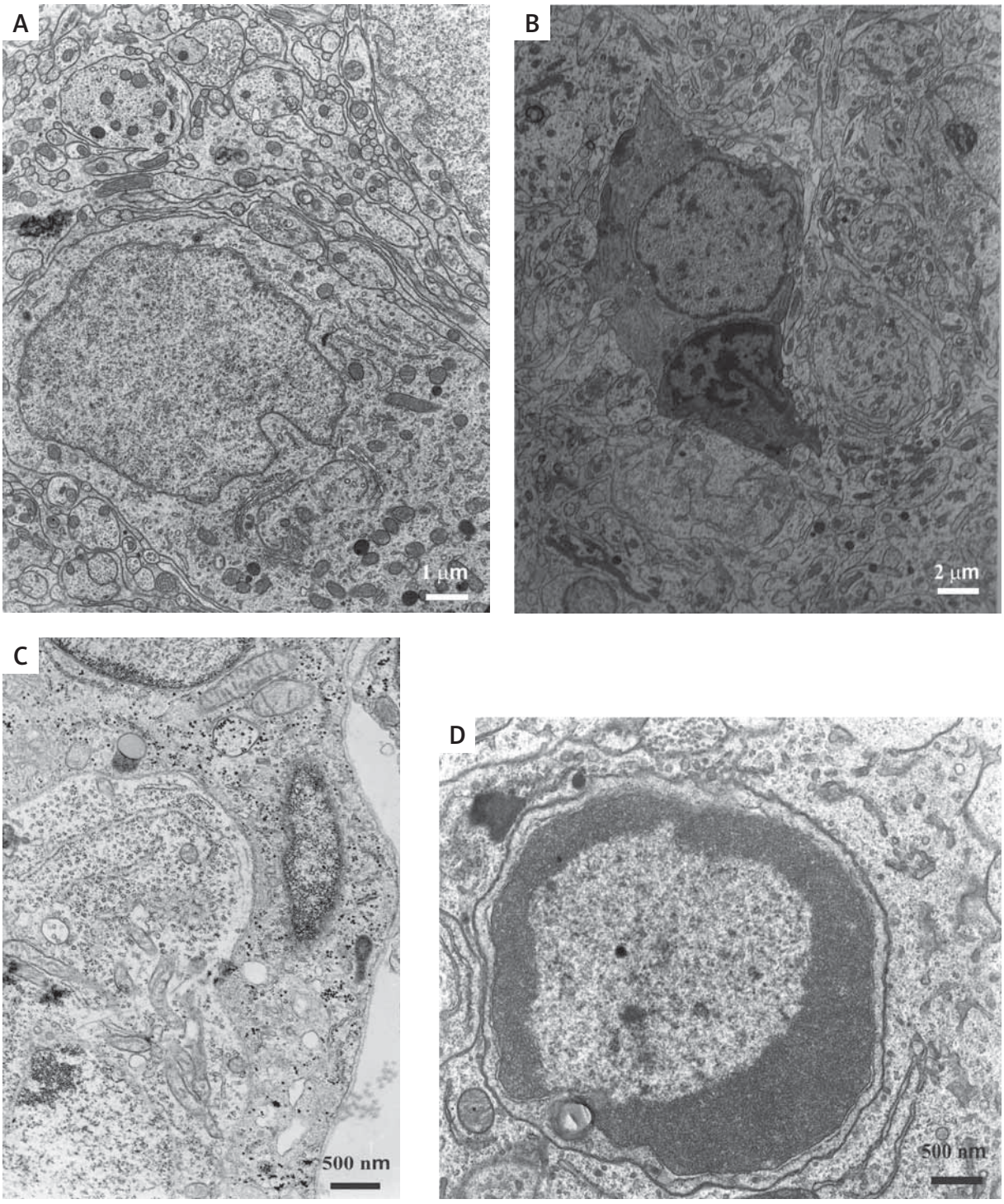

Fig. 4. Ultrastructure of control and dying neurons. A. Neuron with regular ultrastructure of the nucleus with one invagination. Cytoplasm with normal organelles contains a few lipofuscin grains. B. Dark degenerating neurons with shrunken cell bodies. Note dark, electron-dense cytoplasm. C. Necrotic neuron with electron-lucent cytoplasm contains the remnants of organelles and dilated ER. Note strongly swollen mitochondria and dispersion of polyribosomes. D. Apoptotic neuron with characteristic nuclear changes. Note aggregation and marginalization of chromatin

contained neurons with necrotic features (Fig. 4C), characterized by electron-lucent cytoplasm with a distinct nucleus containing loose, electron-lucent chromatin. Among ultrastructurally altered neurons we observed numerous cells containing electron-dense cytoplasm with nuclei separated by a lucid rim from the remainder of the cell. It was the most interesting group of apoptotic neurons. The apoptotic cells undergo shrinkage and grow denser and their cytoplasmic organelles are condensed. Nuclear chromatin is condensed and undergoes marginalization close to the nuclear membrane (Fig. 4D). The endoplasmic reticulum of these cells is dilated, but the mitochondria do not change morphologically. In some observed cells the nucleolus is disintegrated and the nucleus is fragmented and packed into vesicles, called apoptotic bodies, which may be phagocytosed.

We tried to explain the mechanism of neuronal apoptosis. The results obtained from the IHC studies showed an increase of immunoreactivity (IR) for proapoptotic Bax protein in neurons localized in the area where the cells are destined for death (Fig. 5). Neurons from the control intact rats showed only a slight constitutive immunoreactive signal.

The sections derived from control animals did not show any staining for active caspase 3 (Fig. 6). Following the surgical injury numerous neurons with a strong IR signal for active caspase 3 were detected in the perilesional cortical area.

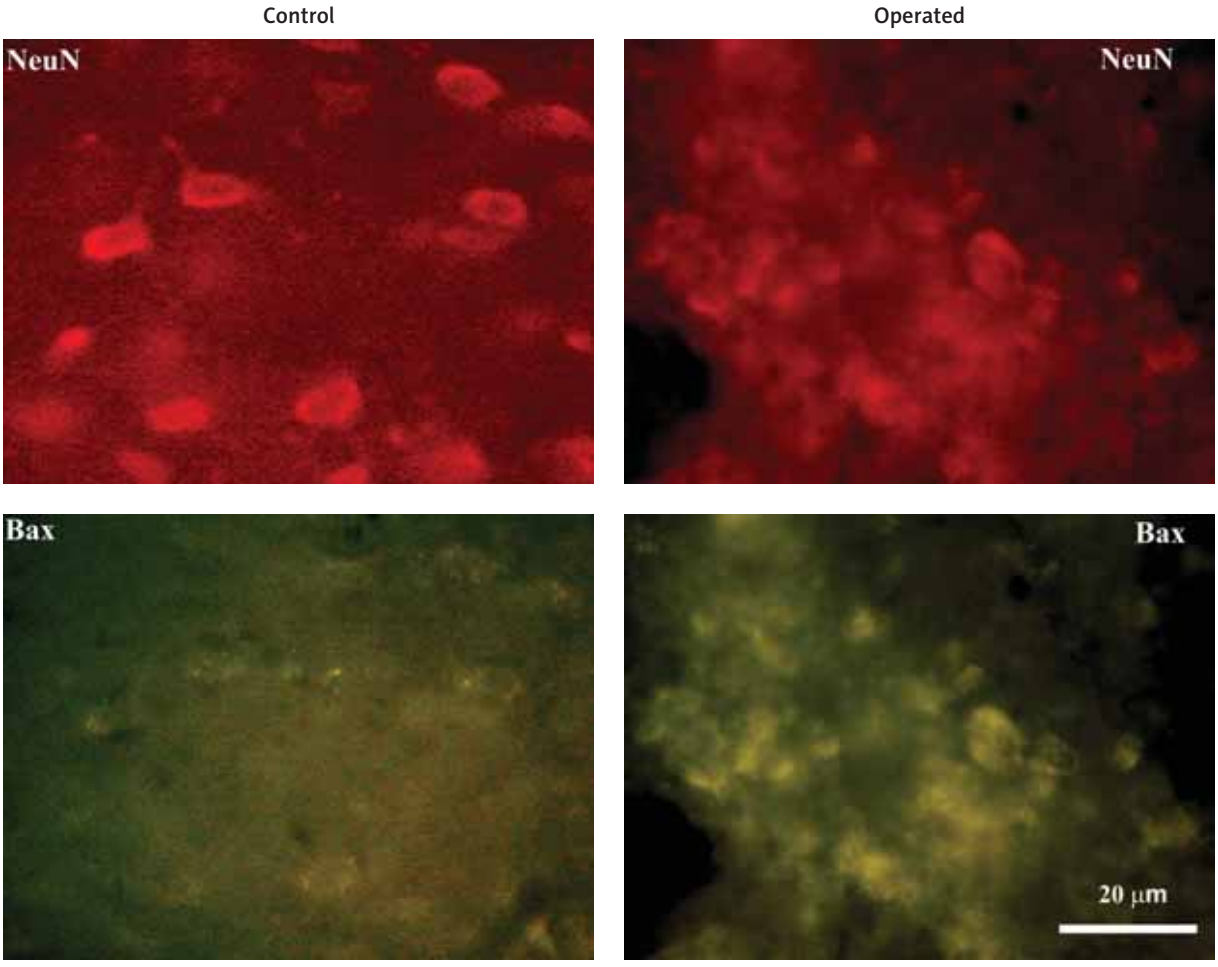

Fig. 5. Immunofluorescent double-labelling of NeuN (neuronal marker) and Bax. Microphotographs show the part of cerebral cortex derived from control and operated animals. In control rats Bax basal expression was detected in neurons. Following the operation an increase of Bax immunoreactivity was observed in numerous neurons located in the proximity of the injury

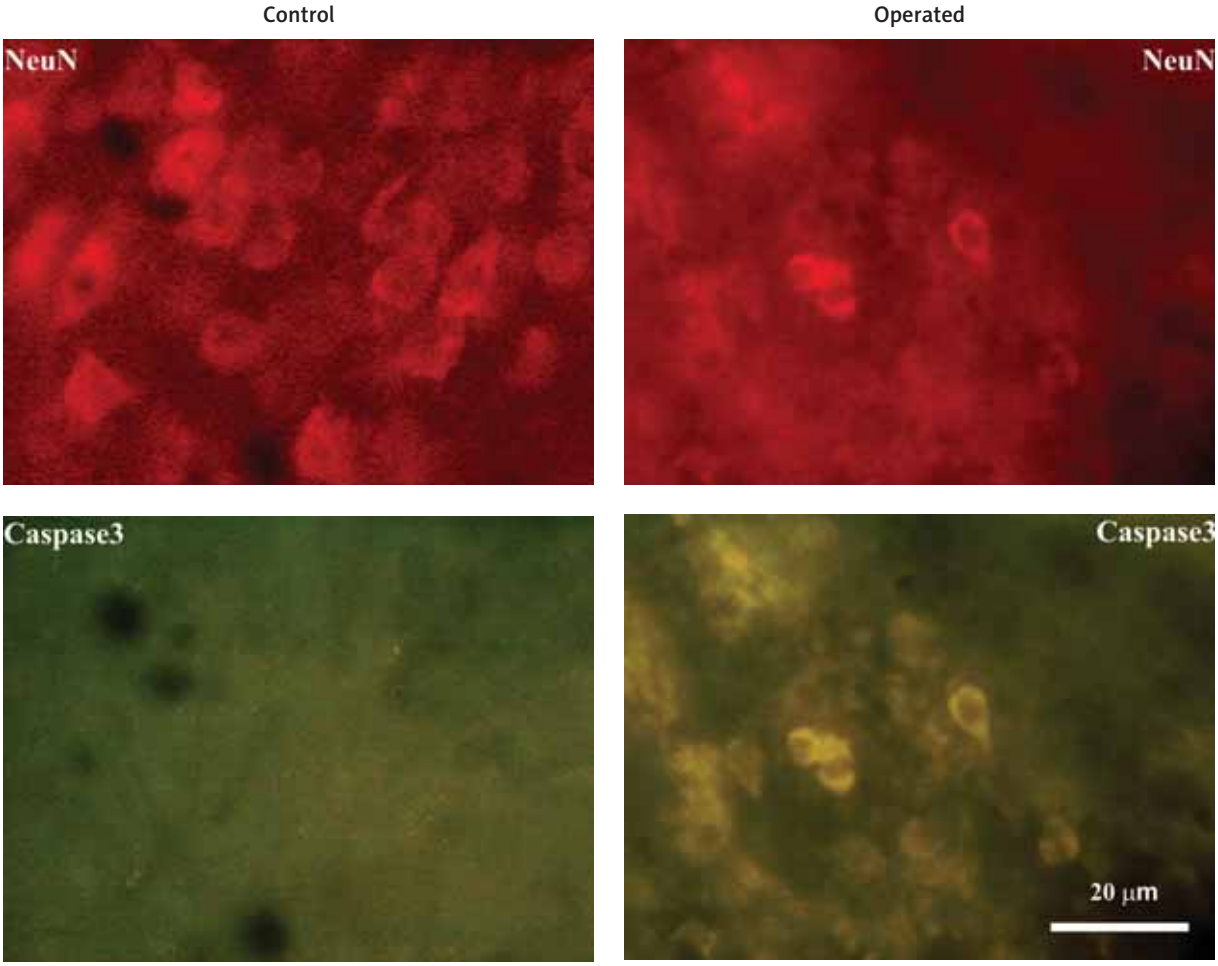

Fig. 6. Immunofluorescent double-labelling of NeuN (neuronal marker) and caspase 3. Microphotographs show the part of cerebral cortex derived from control and operated rats. In the control animal the expression of caspase 3 was not detected. Following the operation a strong induction of caspase 3 IR was observed in many neurons located in the proximity of the lesion

Neurons immunopositive for Bax and caspase 3 often revealed morphological changes characteristic of apoptosis, such as shrinkage of the pericaryon as well as chromatin condensation.

Discussion

We studied the phenomenon of cell death following surgical brain injury. Our investigations compare morphological changes detected in dying cells with the expression of proteins characteristic for apoptosis (Bax and caspase 3).

We found that surgical brain injury causes massive neuronal degeneration and death within the affected cortical region. Neurons die via necrosis or apoptosis but apoptosis is the major type of cell death in our experimental model. This result is in agreement with numerous data collected from several experimental models [6,19].

To explore the mechanism of neuronal apoptosis we investigated the changes of the level of proapoptotic protein Bax and active caspase 3 in the cortical neurons following the injury and correlated it with apoptotic signs. Apoptotic cells showed strong immunostaining for Bax and for active caspase 3, as compared to neurons from intact controls. This result suggests the involvement of both these proteins in the neuronal apoptosis in our experimental model.

Recent literature data confirm our results and show the involvement of Bax and caspase 3 in neuronal death following several traumatic brain injuries [1,2].

Neuronal degeneration and death caused by surgical injury is accompanied by the induction of astroglial cells in the perilesional cortical area. These cells revealed a hypertrophic appearance. Many authors have shown that the phenomenon of astrogliosis takes place in the cellular response to several brain injuries [7,8,22]. Our results are in agreement with these data.

We did not find any dying astroglial cells at the investigated time point, but there is a possibility that apoptosis may occur in astroglia during another period following the damage. The results collected by other authors indicate that trauma may cause apoptotic cell death in astrocytes or may not induce astroglial apoptosis [14,21]. This question is very interesting but in our experimental model requires further studies.

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