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Source / Izvornik: Collegium antropologicum, 2012, 36, 1319 - 1323

Journal article, Published version Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

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# Calpain Expression in the Brain Cortex after Traumatic Brain Injury

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#### ABSTRACT

Traumatic brain injury (TBI) is the leading cause of death and disability worldwide. Calpains, a family of cysteine proteases have been implicated in cells death following TBI. Using immunohistochemistry calpain expression was analyzed in post mortem brain tissue obtained from patients who died after TBI, and findings were compared with the brain tissue from patients who died from sudden cardiac arrest. In the injured cortex an increase in calpain expression was observed in all resident brain cells: neurons, glial and endothelial cells in comparison to the control group (all p < 0.001). Calpain expression was analyzed in different post-traumatic intervals, from day 0 until 10 days post-injury, in order to establish a time course of expression in the brain cortex after TBI. Expression was detected in the cortex 5 hours after the accident, peaked at 72 hours, and substantially reduced by 10 days after TBI. Calpain expression in the cortex significantly changed during the time from TBI to death (p < 0.001), and the most prominent expression was detected in the cortex 3 days after TBI. Our results indicate that prolonged calpain expression in resident brain cells (neurons, glial and endothelial cells) plays an important role in neuronal degeneration following TBI.

**Key words**: calpain, neuronal damage, traumatic brain injury, post-mortem study

#### Introduction

Traumatic brain injury (TBI) is a major cause of death and disability throughout the world. Solely in the United States, approximately 52,000 people die and 80,000 people suffer permanent disability as the consequence of TBI¹. The Centers for Disease Control and Prevention (CDC) estimate that at least 5.3 million Americans currently have a long-term requirement for assistance with daily living activities as a result of TBI².

The primary injury to the brain initiates a secondary injury process that spreads through multiple molecular mechanisms in the pathogenesis of TBI.

Calpains are implicated in neuropathologic events following TBI<sup>3</sup>. Neuronal calpain activation has been detected after experimental TBI in animals, suggesting that calpains are an early mediator of neuronal damage<sup>4</sup>.

Calpains are nonlysosomal, neutral cysteine proteases activated by calcium. The best characterized, and the predominant calpains in the CNS, are the ubiquitous mand  $\mu$ -calpains<sup>3</sup>. These are heterodimers consisting of a unique 80 kDa large subunit and a common 28 kDa small

subunit<sup>4</sup>. Cytoskeletal proteins, membrane proteins, and transcription factors are known to be preferred calpain substrates<sup>3</sup>. Upon activation, calpains cleave a variety of biologically important proteins and serve, therefore, as key regulators of many cellular functions<sup>3,4</sup>. Whereas transient calpain activation triggers numerous cell signaling and remodeling events involved in normal physiological processes, the sustained calpain activation produced by trauma is associated with neuronal death in multiple models of TBI<sup>5</sup>.

In this study we have examined calpain expression in *post mortem* brain tissue obtained from patients who died from TBI and compared the findings with patients who died from sudden cardiac arrest.

#### **Patients and Methods**

The study was performed on cases of fatal TBI subjected to medicolegal autopsy at the Department of Forensic Medicine and Criminalistics, School of Medicine,

University of Rijeka. In 50 autopsy cases with TBI and known survival times, a sample of brain tissue was taken from the macroscopically visible contusion zone in the cerebral cortex. The survival time in the TBI group ranged from 5 h to 10 days. For the control group, brain tissues were taken from 34 autopsy cases without trauma in which death had resulted from sudden cardiac arrest. Collection and use of human tissue material was acquired according to guidelines and study was approved by the Institutional Ethics Committee. The ages of the individuals in the TBI group ranged from 40 to 70 years (the mean age was  $56.3\pm7.9$  years). There were 33 males and 17 females.

The control group included 22 males and 12 females. There were no significant differences between TBI patients and control patients for age or gender distribution: 42-69 years; the mean age  $56.3\pm7.3$  years.

#### Tissue preparation and histologic assessment

The resected brain tissue was immediately fixed in 10% buffered formalin. After fixation in formalin, the paraffin-embedded tissue was sectioned into 7  $\mu$ m slices and then mounted onto polylysine-coated slides. Sections of each specimen were processed for hematoxylin-eosin (HE) staining. Control samples were prepared in the same way. HE slides from the control and TBI group were submitted for neuropathological evaluation.

#### Immunohistochemical analysis

Sections of paraffin-embedded samples were processed for immunohistochemical analysis in a DAKO Autostainer Plus (DakoCytomation Colorado, Fort Collins, CO, USA) according to the manufacturer's protocol using the EnVision peroxidase procedure (ChemMate EnVision HRP detection kit K5007, DakoCytomation, Glostrup, Denmark). Microwave antigen retrieval was done in the presence of 1 mmol/L EDTA (pH 8.0) buffer.

The calpain antibody (Abcam, Cambridge, UK) was visualized by incubation with biotinylated goat anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark). Negative staining controls comprised sections immunostained as above apart from omission of the primary antibody.

#### Semiquantitative scoring

Calpain expression was assessed by immunohistochemistry. All the images were acquired and analyzed on a light microscope Olympus BX51 (Olympus, Tokyo, Japan) with camera attached Olympus DP72 (Olympus, Tokyo, Japan) by two different researchers blinded to the experimental conditions. For immunohistochemistry, the intensity of cytoplasmic chromogen staining was graded semiquantitatively on a scale of 0–3 arbitrary units with 0= no detectable staining, 1= weak, 2= moderate and 3= strong staining<sup>6</sup>.

#### Statistical analysis

Statistical analysis was carried out using the Statistica 9.0 (StatSoft Inc., Tulsa, OK, USA) program. Data were analyzed using Mann–Whitney test and Spearman rank correlation test. Results are given as  $\overline{X}\pm SD$ . Only p<0.05 values were regarded as significant.

#### Results

#### Histopathological analysis

HE sections from the cortex of the TBI group revealed destruction of the brain parenchyma. At the site of cortical contusion large brain edema was visible, along with loss of tissue cells, primary neurons. Also, blood extravasation and infiltration of inflammatory cells was detected. The necrotic neurons showed disappear of Nissl's body in cytoplasm, chromatolysis, nuclear pyknosis and eosinophilic cytoplasm (Figure 1). In brain parenchyma of head injured patients lack of cellular structure was observed in comparison to the control group where brain parenchyma showed intact morphology.

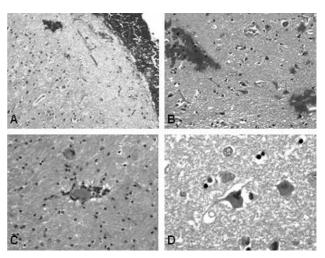


Fig. 1. HE staining of the cortex from TBI group. Leptomeningeal hematoma and diffuse brain edema (A; magnification: 100x), blood extravasation in the brain parenchyma (B; magnification: 200x), perivascular infiltration of inflammatory cells (C; magnification: 200x) and necrotic eosinophilic neurons (D; magnification: 400x) were observed in the injured cortex.

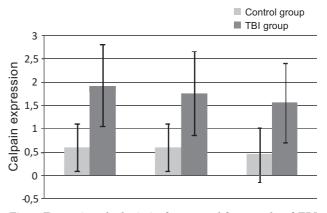


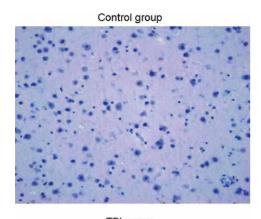
Fig. 2. Expression of calpain in the cortex of the control and TBI group. Diagrammatic presentation of semi-quantitative data for calpain expression analyzed by immunohistochemistry. In the injured cortex stronger calpain immunoreactivity was observed (p<0.001) compared to control group.

#### Immunohistochemical study of calpain expression

Immunohistochemical analysis of calpain expression was performed on the cortex from the control and TBI group. In the TBI group immunoreactivity to the calpain was detected in neurons, glial cells and endothelial cells, whereas the control group showed very weak calpain staining. Semi-quantitative analysis showed significantly elevated calpain expression in neurons, glial and endothelial cells in the traumatic brain cortex compared to control brain (all p<0.001) (Figure 2).

#### Neurons

In the cortex of TBI group immunohistochemical analysis showed intense calpain staining in a number of neurons, including pyramidal neurons. Significantly higher calpain expression in the neurons of TBI group was observed in comparison to the control group (Figure 3).



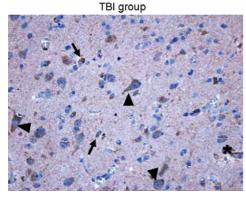


Fig. 3. Immunoreactivity for calpain in the cortex following TBI. Significantly stronger calpain staining was observed in both neurons (♠) and glial cells (→) in the TBI group. Also, calpain staining was observed in endothelial cells (★). No calpain staining was observed in the control brain (Scale bar, 200 μm).

#### Glial cells

We have observed a strong calpain staining in the cytoplasm of glial cells in the TBI group. Moreover, in the TBI group expression of calpain in glial cells was almost equal to one of neurons. A significant increase of calpain expression in the TBI group was determined in comparison to the control group (Figure 3).

#### Vessels

Endothelial cells of vessels were labeled for calpain in the TBI group, in contrast to the control group where seldom positive cells were found. Significantly increased calpain expression in endothelial cells was detected in the TBI group in comparison to the control brain (Figure 3).

## Calpain expression in the cortex following TBI in different post-traumatic intervals

Calpain expression was analyzed in different post-traumatic intervals, from day 0 until 10 days post-injury, in order to establish a time course of expression in the brain cortex after TBI. Calpain expression was detected in the cortex 5 hours after the accident, peaked at 72 hours, and substantially reduced by 10 days after TBI. Calpain expression in the cortex significantly changed during the time from TBI to death (p<0.001), and the most prominent expression was detected in the cortex 3 days after TBI (Figure 4).

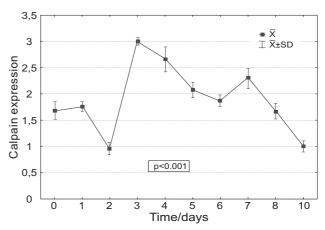


Fig. 4. Calpain expression in the cortex following TBI in different post-traumatic intervals. Calpain expression in the cortex significantly changed during the time from TBI to death (p<0.001), and the most prominent expression was detected in the cortex 3 days after TBI.

Correlation between calpain expression in the cortex and the period of time elapsed from trauma to the brain death in the TBI group was negative (Spearman rank coefficient R) and statistically significant in all resident brain cells (Table 1).

#### **Discussion and Conclusion**

TBI is a major cause of death and one of the most frequent neurological disorders. It is known that TBI results in a variety of pathological changes following injury<sup>7–9</sup>. We have confirmed that TBI results in loss of the basic cytoarchitecture of the brain causing significant damage to the brain parenchyma.

The first reports of calpain activation following experimental TBI were published in 1996. Saatman et al.

TABLE 1
CORRELATION BETWEEN CALPAIN EXPRESSION IN THE
CORTEX AND THE PERIOD OF TIME ELAPSED FROM TRAUMA
TO THE BRAIN DEATH IN THE TBI GROUP

	Spearman rank coefficient R	p
Cortex		
Neurons	-0.365	0.024
Glial cells	-0.373	0.020
Endothelial cells	-0.364	0.025

showed that lateral fluid percussion brain injury in the rat initiates calpain activation in the cortex and hippocampus<sup>10</sup>. Neuronal calpain activation has been observed within minutes to hours following either contusive or diffuse brain trauma in animals, suggesting that calpains are an early mediator of neuronal damage<sup>5</sup>.

In our study calpain expression in the cortex following TBI was evaluated by immunohistochemistry. We have confirmed calpain expression in all resident brain cells (i.e. neurons, glia and endothelial cells) of the injured brain. Moreover, in the cortex of TBI group significantly increased calpain immunoeactivity was detected in all cell populations in comparison to the control group. This observation suggests that neuronal survival may be compromised due to increased expression of calpain in the injured cortex.

Further, temporal changes in calpain expression following TBI were investigated. In the cortex calpain expression was recorded at different time points after the brain injury: expression was detected 5 hours after the accident, peaked at 72 hours, and substantially reduced by 10 days after TBI. Calpain expression in the injured cortex showed clear temporal variations' leading to assumption that its expression in a temporally restricted pattern is consistent with a pathological role this protease has in post-traumatic brain pathology.

Similar results were obtained by the authors who have studied the temporal expression of calpain in laboratory animals. Pike and colleagues in the rat model of TBI confirmed that calpain activation occurs during the first 3–4 hours after TBI and that gradually decreases up to 7 days after TBI<sup>11</sup>.

Also, several prospective case control studies of patients with severe TBI have reported increased levels of calpain- and caspase-mediated spectrin BDPs in ventricular cerebrospinal fluid from 6 h to 3–4 days after trauma<sup>12,13</sup>.

According to our study, prolonged calpain activation plays an important role in neuronal degeneration following TBI. Taken together with the previous demonstration of calpain involvement in the pathogenesis of many neurological diseases, including cerebral ischemia<sup>14</sup>, Alzheimer's disease<sup>15</sup>, Parkinson's disease<sup>16</sup>, and amyotrophic sclerosis<sup>17</sup> these data support the hypothesis that prolonged calpain activation contributes to post-traumatic cell death.

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### IZRAŽAJ KALPAINA U KORTEKSU OSOBA PREMINULIH NAKON TRAUMATSKE OZLJEDE MOZGA

#### SAŽETAK

Traumatska ozljeda mozga (TBI) vodeći je uzrok smrti i invalidnosti u svijetu. Poznato je da kalpaini, stanične neutralne cisteinske proteaze, sudjeluju u post-traumatskom oštećenju moždanog parenhima. Izražaj kalpaina u korteksu osoba preminulih nakon TBI i osoba kontrolne skupine koje su umrle uslijed naglog zatajenja u radu srca analiziran je imunohistokemijski. U korteksu osoba preminulih nakon TBI zabilježen je pojačan izražaj kalpaina u svim rezidentnim moždanim stanicama (neuronima, glija stanicama i endotelnim stanicama) u odnosu na kontrolnu skupinu (svi p<0,001). U različitim vremenskim razdobljima proteklim od traume mozga do smrti analiziran je izražaj kalpaina kako bi se ispitao vremenski obrazac ekspresije kalpaina u mozgu nakon TBI. Izražaj kalpaina zabilježen je u kori mozga 5 sati nakon ozljede, a najveća vrijednost je zabilježena 3 dana nakon TBI. Statističke analize pokazale su da se izražaj kalpaina u korteksu značajno mijenja s vremenom proteklim od traume mozga do smrti (p<0,001). Naši rezultati ukazuju da pojačan izražaj kalpaina u stanicama moždane kore igra važnu ulogu u post-traumatskoj neurodegeneraciji.