The Role of Cathepsin B Inhibition in Mitigating Traumatic Brain Injury: Effects of Calcium Levels on Cellular Viability

Nithila Madhan, Sriram Selvakumaran, Veronica Gomez-Godinez & Linda Shi

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Traumatic brain injury (TBI) is a significant public health concern due to its complex nature and the enduring impact it has on the physical, cognitive, and psychological well-being of patients. This study delves into the role of Cathepsin B, a type of cysteine protease, in TBI, and explores the potential of Cathepsin B inhibition in offering neuroprotection. To simulate TBI, PtK2 cells were utilized, and laser shockwaves were generated to examine the effects of inhibiting Cathepsin B on cell viability, particularly under varying calcium concentrations in the media. It was hypothesized that inhibiting Cathepsin B could reduce cell death following the laser shockwave that simulated TBI, with the protective effect expected to depend on the calcium levels in the media. Calcium plays a crucial role in various cellular processes, including signaling pathways that impact cell survival, proliferation, and apoptosis. Understanding the interaction between calcium levels and the inhibitor could help us uncover the mechanisms that influence cell viability. This understanding might indicate that adjusting calcium levels could improve the effectiveness of the inhibitor or reduce potential side effects. To test this hypothesis, cells were exposed to a laser shockwave to simulate blast-induced TBI and used Ethidium Dimer III dye to assess cell viability. The experimental groups that were subjected to the shockwave included cells in normal (+Ca) and low (-Ca) calcium media, with and without the Cathepsin B inhibitor (CA-074 methyl ester). The results showed that cells cultured in a +Ca media with a Cathepsin B inhibitor exhibited a significantly lower cell death rate than cells cultured in a -Ca media with a Cathepsin B inhibitor. In contrast to the hypothesis, calcium concentration did affect the effectiveness of Cathepsin B inhibition, indicating that optimal neuroprotection requires a specific calcium environment. These results highlight the interaction between calcium levels and Cathepsin B activity, suggesting more research is needed to understand their roles in TBI pathology and potential treatments. In conclusion, inhibiting Cathepsin B shows promise for reducing secondary brain injury after TBI. The study included only a 25-minute imaging period, which is insufficient to fully assess the effects of Cathepsin B inhibition on cell viability following TBI. Future research should explore the long-term effects of Cathepsin B inhibition through longer imaging periods in different cell and animal models to confirm these findings and develop targeted TBI therapies.

Keywords: Traumatic Brain Injury, Cathepsin B, Laser-Induced Shockwave, Apoptosis

Introduction

Traumatic brain injury (TBI) is a dysfunction in the brain that occurs when the brain is subjected to immense forces or when an external object penetrates the brain tissue[1]. There has been a steady increase in the number of military-related TBIs, as well as injuries observed in war zones and terrorist incidents, where the head is violently shaken due to exposure to radiation or explosions[2]. The extent of damage caused by TBI can vary and have a significant impact on life expectancy. TBI can lead to lasting physical, cognitive, and psychological disorders, which can be a burden on family members[3]. As a result, the number of friendships patients maintain and their social interactions substantially decrease. Additionally, families often report a decrease in economic resources as they allocate more resources to care for the patient[4].

Immediate emergency care for TBI involves maintaining internal blood pressure and blood supply while also focusing on minimizing secondary damage caused by inflammation, bleeding, or reduced oxygen supply to the brain[5]. Immediately following injury, there exists an acute inflammatory response sent by the immune system, which is necessary to clear damaged tissue and initiate the repair process. However, excessive or prolonged inflammation can exacerbate brain damage and contribute to a greater degree of injury[6].

Cathepsins are a group of enzymes categorized as cysteine proteases, containing cysteine residues in the enzymatic domain[7]. These enzymes play a crucial role in various cellular functions, including intracellular protein degradation, energy metabolism, and immune responses. Of particular interest is Cathepsin B, which has shown functional activity outside the lysosome, making it a potential drug target for TBI. Lysosomes

are membrane-bound organelles with an acidic interior and contain various hydrolytic enzymes such as lipases, proteases, and glycosidases involved in cellular catabolism[8]. Furthermore, despite Cathepsin D's optimal activity at pH 4, it has been observed to remain active even at a pH of 7.4, indicating its broad range of proteolytic activity beyond the lysosome[8]. Thus, Cathepsins have the unique ability to exit the lysosome and enter the cytoplasm or the extracellular matrix. Upon release, they can degrade proteins in these areas, leading to cell destruction through necrotic, apoptotic, autophagic, and activated glia-induced cell death[9].

In a previous experiment based on the correlation between TBI triggers and the Dysregulation of Cathepsin B Protein Levels in Brain and Cerebral Spinal Fluid, it has been observed that levels of Cathepsin B were notably elevated in cerebrospinal fluid collected within 3 days following TBI in comparison to non-TBI controls[10]. These findings collectively suggest that Cathepsin B and its cysteine protease activity could potentially function as comprehensive molecular indicators of TBI progression, exhibiting varying levels in both proximal and distal brain regions[10]. In certain pathological conditions, the upregulation of Cathepsin B and its protease activity is linked to an imbalance between lysosomal and autophagic processes, leading to either apoptotic or necrotic cell death[10].

The experimental study conducted on TBI uses PtK2 cells, which are derived from male long-nosed potoroo (Potorous tridactylus) epithelial kidney cells. To study TBI, PtK2 cells were utilized along with a laser-induced shockwave TBI. The laser shockwave compresses cells which leads to their lysis and thus, the release of harmful molecules from internal compartments, simulating the impact of TBI. A particular enzyme, Cathepsin B, can damage cellular components if leaked out of the lysosome. Therefore, Cathepsin B release is known to be one of the leading causes of cell death, it occurs only under the event of cell stress and activates as a trigger for many transcription factors that cause the production of proteins responsible for cell death. TBI is followed by primary and secondary brain damage, where primary damage occurs at impact, and secondary brain damage is triggered by the first. It is hypothesized that inhibiting the activity of released Cathepsin B from the lysosome reduces cell death following TBI, with the degree of neuroprotection depending on the calcium concentration in the media of PtK2 cells. Calcium is essential for various cellular processes, including signaling pathways that affect cell survival, proliferation[11], and apoptosis. By exploring the relationship between calcium levels and the inhibitor, mechanisms that influence cell viability can be discovered. This understanding could suggest that modulating calcium levels might enhance the inhibitor's effectiveness or minimize potential side effects.

Methods

The experimental study subjected cells to conditions similar to those during a blast-induced TBI by utilizing a Coherent 1030 nm 2000 Hz repetition rate system with a 1.5 ns pulse width (Spectra-Physics, Mountain View, CA) to induce a shockwave. A shockwave was administered once at the start of each 25-minute imaging session for each trial. A rotating optical polarizer mounted on a stepper-motor-controlled rotating mount attenuated the power (Newport, Irvine, CA). One pulse was allowed to enter the microscope using a mechanical shutter (Vincent Associates, Rochester, NY) with a 10–15 ms duty cycle. The laser beam diameter was adjusted to fill the back aperture of a 40x NA 1.3 Zeiss objective on a 200 M Zeiss microscope (Figure 1). The laser was focused 10 μm above the substrate. Rat kangaroo cells (PtK2) from the Potorous tridactylus were utilized due to their ability to remain flat and well adhered to the substrate. These cells were grown in Advanced DMEM/F12 with 1% Glutamax and 10% Fetal Bovine All cells were maintained in a humidified 5% CO2 incubator. Cells were plated onto glass-bottom gridded dishes from MatTek to a confluency of 80% and used 1–2 days post-subculture.

Prior to the shockwave Ethidium Dimer III (Biotium), a dye impermeable to intact cell membranes but able to penetrate damaged ones, was added to the cells at a concentration of 2uM to identify damaged or deceased cells. Cells were placed in media containing either low or normal concentrations of calcium to measure the influence of calcium on cell death. ImageJ software was employed to quantify pre - and post-shockwave dead or damaged cells using the Ethidium III dye. Additionally, Matlab was utilized to assess cell distance from the impact and determine cell death intensity. To inhibit Cathepsin B, CA-074 methyl ester was applied at a final concentration of 50μM. CA-074 has been employed in numerous studies to elucidate the role of this protease in cellular and physiological functions[12]. The experiment entailed quantifying cells before and after the laser shockwave while simultaneously measuring the intensity of cell death and its correlation with the distance from the shockwave, while also studying calcium's role in apoptosis. The imaging period was 25 minutes long with 600 images taken throughout the time interval.

In the study, cells were exposed to either a normal concentration of calcium, denoted as +Ca, or a lower concentration of calcium, denoted as -Ca, which served as the control groups for the experiment. The cathepsin B inhibitor was administered to cells in both calcium media to assess the impact of the media on the efficacy of the inhibitor. Phase images were captured prior to subjecting the cells to the laser-induced shockwave in both low and normal calcium media. This was done to compare the effect and confirm the accuracy of the laser-induced shockwave model.

Data was aggregated and structured using Google Spread-

sheets. A T-test, a statistical method for comparing sample means, was employed to analyze the results of the four trials. Prism software was utilized to generate visual representations of the T-test outcomes.

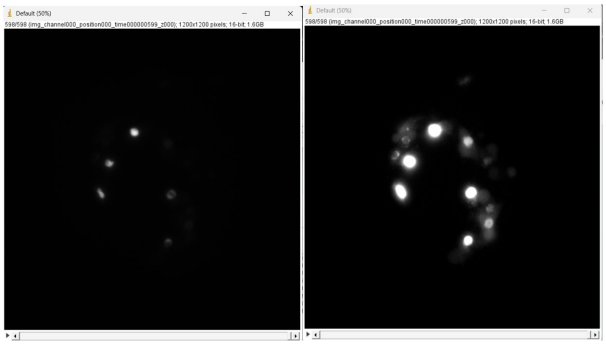

Fig. 1 Quantification of Cell Death Using Ethidium Dimer III Dye

This figure illustrates the quantification of cell death using Ethidium Dimer III dye. Ethidium Dimer III is impermeable to intact cell membranes but penetrates damaged ones, allowing detection of dead or dying cells. The dye stains the nucleus of apoptotic cells, providing a visual and quantitative method to assess cell death.

Overview of Image Analysis

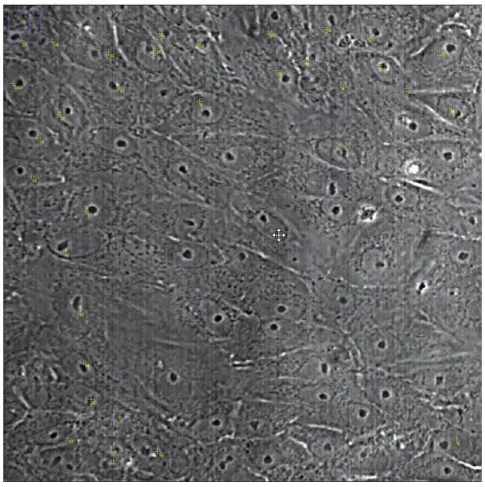

Fig. 2 Phase Images

Cell death is quantified as the percentage of cells that died from the shockwave at various time points, compared to the initial number of cells present before the shockwave (as seen in phase images). These phase images serve as a control for the shockwave model, validating that cell death is indeed a result of the shockwave. At any given moment, the percentage of cell death is calculated by dividing the number of dead cells by the total number of cells, then multiplying by one hundred. Dead cells are identified by their expression of a red fluorescent dye, indicating intense fluorescence.

To quantify cell death, ImageJ software was used, while MATLAB measured the intensity of cell death in relation to proximity to the shockwave, further validating its role. Once the number of dead cells and the initial cell count are obtained, an Excel sheet is created to compute the percentages of cell death in each field of view based on the time elapsed since exposure to the shockwave. From these percentages, graphs were generated to compare the effectiveness of different environmental conditions and treatments in reducing cell death.

Results

The validity of the laser-induced shockwave was demonstrated by the absence of cell death in the PTK2 cell model under all four conditions. Through analyzing graphical representations of cell death progression over the measured time interval, it is evident that cells cultured in a -Ca media without a Cathepsin B inhibitor demonstrated significantly lower cell death rates compared to cells cultured in a -Ca media with a Cathepsin-B inhibitor (Figure 3). Additionally, cells cultured in a +Ca media with a Cathepsin B inhibitor exhibited a lower cell death rate than cells cultured in a -Ca media with a Cathepsin B inhibitor (Figure 3). In absolute, cell death increased during the 25-minute imaging period across all four trials (Figure 4). However, the interaction between differing calcium levels in the media with either the presence or absence of a Cathepsin-B inhibitor resulted in varying percentages of cell death. These findings validate the hypothesis as varying calcium levels in the media impacted cell death with and without the Cathepsin B inhibitor.

Previous research on the impact of calcium levels in cell cultures has revealed that certain cell types may necessitate exposure to media with reduced calcium concentrations [13]. This is due to the susceptibility of cells grown in high-calcium environments to the toxic influx of calcium through cell membranes that cause damage by oxidative processes [13]. Heightened calcium levels trigger the activation of hydrolytic enzymes, leading to increased energy expenditure, impaired energy production, initiation of cytoskeletal degradation, and ultimately resulting in cell death [14].

The two distinct groups with varying levels of Calcium exhibited differing percentages of cell death. Notably, at 5 minutes (Figure 3), the group with a higher Calcium concentration demonstrated a significantly greater percentage of cell death. Thus, it can be concluded that the efficacy of a Cathepsin B Inhibitor in providing neuroprotection is contingent upon the concentration of Calcium in the media. This is evidenced by Figure 3, where the impact of the inhibitor varied significantly based on the level of Calcium in the media, with higher levels of Calcium resulting in a more effective interaction with

the inhibitor. Results suggest calcium modulates response to Cathepsin B inhibition.

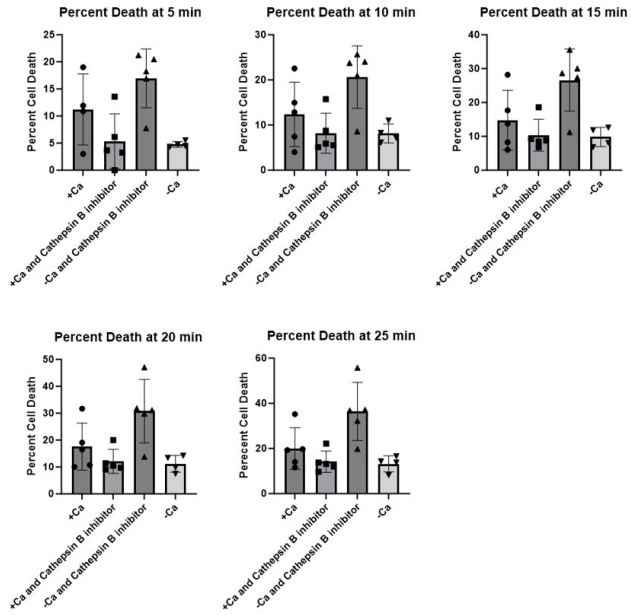

Fig. 3 Percentage of Cell Death Over 25 Minutes for PtK2 Cells Under Different Conditions

Figure 3 demonstrates the percentage of cell death throughout the 25-minute time interval. The experiment consisted of four groups: cells in low calcium medium with and without Cathepsin B inhibitor, and cells in normal calcium medium with and without Cathepsin B inhibitor. The data indicates an overall increase in cell death over time, with variations depending on calcium concentration and inhibitor presence.

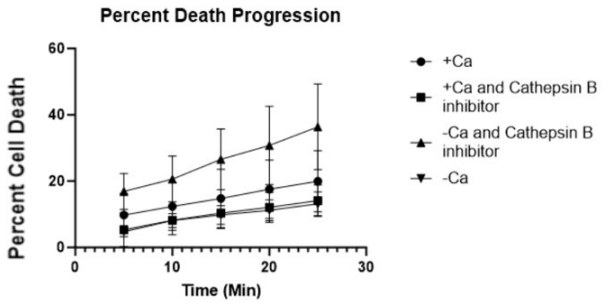

Fig. 4 Bar Graph of Feature Analysis on Gradient Boosting Model

Figure 4 compiles the percentage of cell death across four different experimental conditions over a 25-minute period. The groups consist of cells treated with low and normal calcium medium, both with and without the Cathepsin B inhibitor. The general trendline that all groups followed was an increase in cell death as time progressed, highlighting the impact of calcium levels and Cathepsin B inhibition on cell viability.

Discussion

TBI is followed by primary and secondary phases of cell death, also known as primary and secondary brain injury. The initial phase, triggered by the shockwave, causes sudden and profound damage to the brain [15]. This immediate damage to tissues, cells, and components is referred to as primary brain injury [16]. An increase in cell death can act as a trigger for the secondary phase. The secondary phase of cell death is also influenced by the debris of dead cells after the shockwave, which has damaging effects on nearby cells, including astrocytes or neurons. It involves a series of cellular, chemical, blood vessel, or tissue changes in the brain that contribute to further destruction of brain tissue 24-48 hours after impact [15].

In a previous study, it was discovered that neuroinflammation plays a significant role in causing secondary brain injury after TBI [16]. As a result, reducing the inflammatory response has been considered a potential therapeutic target [16]. While neuroinflammation is beneficial for clearing debris and promoting regeneration, it can also lead to collateral damage when it becomes dysregulated and excessive, ultimately causing secondary brain injury [16]. Clinical significance for patients through treatment lies in the possibility that chronic inflammation following TBI could persist for years and increase the risk of developing neurodegenerative disorders like dementia [17]. Therefore, it is crucial to focus on treatments that aim to inhibit secondary brain injury after TBI. It is believed that, with further investigation, Cathepsin B has the potential to mitigate this process. Cathepsin B, a cysteine protease implicated in protein degradation and cellular apoptosis, demonstrates elevated levels following TBI, thereby signifying its potential role as a molecular indicator and target for therapeutic intervention.

The experimental setup has demonstrated its validity in simulating blast-induced TBI in PtK2 cells through laser shockwaves. The observed significant cell lysis and stress following the shockwave confirms the relationship between shockwave intensity and cell death. The analysis of cell death intensity in relation to the distance from the shockwave further strengthens the correlation, indicating that cell death intensity is influenced by proximity to the shockwave induced by the laser. As cell death significantly increased post-shockwave, there has been a marked increase in Cathepsin B levels post-shockwave and a significant decrease through inhibition using CA-074 methyl ester under normal calcium levels of 2uM.

The relationship between Calcium and a Cathepsin B Inhibitor has not been extensively explored in past research. However, findings are consistent with previous research indicating the adverse effects of heightened Cathepsin B activity. Elevated levels of Cathepsin B have been observed in patients with Alzheimer's disease and TBI, and have been shown to be correlated with behavioral and injury outcomes [18]. In animal models of Alzheimer's disease and TBI, inhibiting Cathepsin B has led

to improvements in cognitive and behavioral deficiencies and a reduction in neuropathology [18]. Studies propose that lysosomal leakage occurs in these conditions, contributing to neurodegeneration, and posit that the redistribution of cathepsin B from the lysosome to the cytosol may trigger cell death and inflammation processes associated with neurodegeneration [18].

The presence of calcium plays a critical role in the effectiveness of the Cathepsin B inhibitor, as demonstrated by significant differences in cell death rates when cells were exposed to media with varying calcium levels. The inhibitor works best when it is applied in media with normal calcium concentrations of 2 microliters. From the research performed, it has been demonstrated that higher calcium levels may exacerbate cellular stress and enhance Cathepsin B activity, however, further studies are imperative to elucidate these molecular mechanisms in detail. This discovery suggests that the interplay between calcium levels and Cathepsin B inhibition presents a promising new area for research.

While PtK2 cells show a favorable response to the Cathepsin B inhibitor under optimal calcium conditions, it is important to note that these cells may not completely mimic the complexities of human TBI. Consequently, the response of neurons to the Cathepsin B inhibitor may differ from that of the experimental cells. Despite extensive research on cell cycle analysis utilizing Rat Kangaroo Cells (PtK2), there is a limited amount of research employing these cells for the analysis of brain function and response to brain injury. Additionally, the experiment recorded cell death progression over an interval of only 25 minutes, generating 600 images per field of view analyzed. The relatively short observation period leaves the long-term effects of Cathepsin B inhibition unexplored.

Nonetheless, inhibiting Cathepsin B shows promise in reducing secondary brain injury, as demonstrated in the research. However, further investigation is needed to optimize environmental cellular conditions, especially with regard to calcium levels, before considering clinical applications.

Observing the effects of Cathepsin B inhibition over extended periods of time in normal calcium media, along with experimenting with different cell types and animal models, allows for the validation of findings for new therapeutic targets through long-term studies. If the inhibitor proves to be significantly effective in models comparable to the human brain and in response to TBI, it could potentially be developed into a drug capable of inhibiting the secondary phase of cell death and ultimately providing some degree of neuroprotection.

Conclusion

The conducted experiment aimed to investigate the neuroprotective effects of a Cathepsin B Inhibitor on individuals with TBI. The results not only confirmed the neuroprotective properties of the inhibitor but also shed light on other influential factors. The findings suggest that the effectiveness of the inhibitor in reducing cell death and post-TBI is closely linked to the concentration of calcium in the surrounding media. Specifically, the data revealed a significantly lower percentage of cell death in Ptk2 cells treated with the inhibitor in a normal calcium media compared to those treated in a lower calcium concentration media. This indicates that the interaction between calcium and the inhibitor plays a crucial role in mitigating cell death. The study was limited to a 25-minute imaging period, which is not adequate to thoroughly evaluate the effects of Cathepsin B inhibition on cell viability after TBI. Future research should investigate the long-term impacts of Cathepsin B inhibition by using extended imaging periods across various cell and animal models to validate these findings and inform the development of targeted therapies for TBI. Still, these insights pave the way for further exploration and potential development of drugs based on Cathepsin B inhibition, offering the promise of alleviating the detrimental effects of TBI and hope for improved patient recovery and quality of life.

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