The Effects of Oleuropein Isolate on Apoptosis Inducing Factor and High Mobility Group Box-1 Levels In Traumatic Brain Injury Model Rats

Abdurrahman Mousa Arsyad[1]

[1]Department of Neurosurgery, Faculty of Medicine, Universitas Sumatera Utara
Email : fahmirasyid1995@gmail.com

Abstract

Background: Traumatic brain injury is a major public problem with an incidence of 10 million people annually, causing the most significant deaths and disabilities worldwide. Head injuries can be classified into primary and secondary head injuries. Secondary head injuries can be caused by ischemia, cerebral oedema, and neuroinflammation. AIF and HMGB-1 are two parameters that can be indicators in measuring the effect of Oleuropein on traumatic brain injury in rats. Oleuropein has many activities, such as antioxidant, anti-apoptotic, antimicrobial, anti-inflammatory, and neuroprotective.
Method: This laboratory study used a simple random sampling technique to select experimental animals and homogenous experimental locations. The study was conducted with a pre-test and post-test control group design to compare treatment effects in the experimental and controlgroups. Observations were made from the first 24 hours up to 72 hours after the onset of head injury, with the administration of Oleuropein intraperitoneally.
Result: The results showed that Oleuropein reduced AIF and HMGB-1 levels in rats with traumatic brain injury. This indicates that Oleuropein has a neuroprotective effect by reducinginflammation and apoptosis.
Conclusion: Oleuropein has a potential neuroprotective effect in traumatic brain injury byreducing inflammation and apoptosis. Therefore, Oleuropein can be considered as a potential therapeutic agent for traumatic brain injury in the future.

Keywords: Oleuropein Isolate, Apoptosis-Inducing Factor, High Mobility Group Box-1

M. M. Amin et al. (eds.), *Proceedings of the 6th International Conference on Neuroscience, Neurology and Psychiatry (ICONAP 2023)*, Advances in Health Sciences Research 71,
https://doi.org/10.2991/978-94-6463-310-8_2

Introduction

Head injuries caused by external force are a significant public health issue, affecting millions globally yearly. In the U.S., 2.5 million head injuries occurred in 2014, significantly impacting children. Head injury leads to the highest disability rate worldwide, with 13 million people living with related disabilities in Europe and the U.S. Severe head injury patients are typically treated in intensive care units [1] – [5].

Indonesia experiences a head injury incidence of 6-12%, with a mortality rate of 23-37%, andit is among the top ten diseases treated in hospitals. In 2020, there were 343 head injury patients with mild, moderate, and severe head injuries at the HAM Medan. Primary head injury occurs directly during trauma with acceleration, deceleration, and rotational mechanisms, causing inertiaforces on brain cells and tissue [1] – [5].

Secondary head injury is caused by biochemical, cellular, and molecular cascades activatedafter primary head injury, often due to systemic hypotension and hypoxemia. Various factors, such as cerebral oedema, ischemia, excitotoxicity, diffuse axonalinjury, neuroinflammation, energy failure, and apoptosis, can cause this type of injury. Current therapy aims to restrict the expansion of a secondary head injury from a primary head injury [1] – [5].

In head injury, necrosis and apoptosis can occur, with necrosis happening in response to severemechanical injury and brain tissue undergoing hypoxia marked by the release of excitatory neurotransmitters and metabolic failure. On the other hand, neuronal apoptosis requires an energy supply and an imbalance between pro-apoptotic and anti-apoptotic proteins. Head injury can induce apoptosis through several pathways, such as the B-cell lymphoma-2 pathway, which has pro-apoptotic and anti-apoptotic components, and the caspase-independent pathway, which is influenced by the Apoptosis Inducing Factor (AIF) [1] – [5].

AIF and HMGB-1 are molecules involved in cell death and inflammation, respectively. Mitochondria release AIF after cerebral ischemia, while HMGB-1 is expressed in the brain andcan cause an inflammatory response and blood-brain barrier disruption. High levels of HMGB-1 have been linked to poor outcomes after head injury, and it can contribute to secondary head injuryby causing neuronal hyperexcitation [1] – [5].

Oleuropein, a compound in olives, has antioxidation, antiapoptosis, antimicrobial, anti-inflammatory, and neuroprotection properties. It can help cells survive unfavourable conditions like ischemia, reduce apoptosis, and improve outcomes in vivo. It also reduces inflammation by lowering the expression of TNF-α and IL-1β. Previous research showed that an oleuropein extractfrom the Fraxinus rhynchophylla tree trunk could reduce AIF expression and glutamate-induced cell death [6] – [11]. Based on this background, the researchers are interested in investigating the effect of Oleuropein administration on rats with traumatic brain injury models using AIF and HMGB-1parameters.

Methods

This laboratory study uses a simple random sampling technique, so the research materials,such as test animals and experimental locations, can be considered homogeneous. All samples wereprocessed simultaneously using a pre-test and post-test control group design[12] for observation. The research design uses pre-test and post-test control groups. With this design, researchers can measure the impact of the treatment (intervention) on the experimental group by comparing the experimental group and the control group. This design allows researchers to determine the extent or magnitude of the changes because the test is performed at the beginning and end of the treatment. The experimental animal was treated for one day, and then brain tissue preparations were examined on day 3 in the form of the number of cells expressing AIF with a 1000x lightmicroscope. Positive cells were counted in 20 fields of view [12].

Traumatic brain injury was given to 5 experimental groups (Group B, C, D, E, and F) for one treatment, and after that the research subject of group C were given Oleuropein and D were given Oleuropein after treatment and groups E and F were given Oleuropein before and after treatment. Dose of Oleuropein According to the literature with the best neuroprotective effect is 30 mg/Kg. This study will use a dose ratio of 50-100 mg/KgBB in rats.

Observations were made within the first 24 hours and continued for the next 72 hours, as AIFand HMGB-1 increased within the first 24 hours of head injury onset [13] – [15]. The study used two measurements in the pre-and post-tests, as the effect of Oleuropein with pre-injury administrationwill be observed. This study has been approved by the Universitas Sumatera Utara research ethics committee.

Results

1. Histology of Brain Tissue Due to TBI Exposure

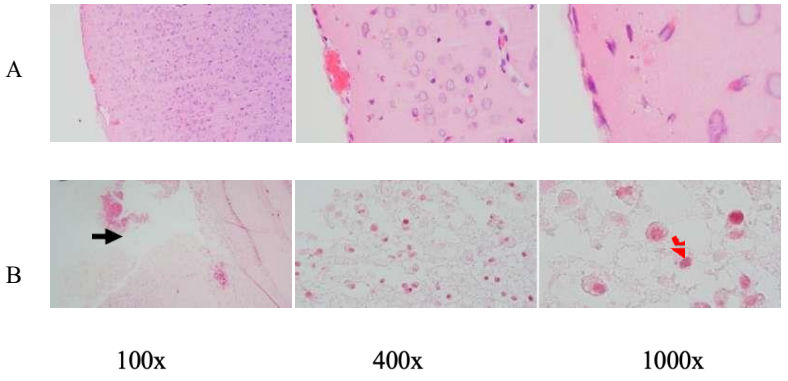


Figure 1. Photomicrograph of HE staining results, brain tissue of TBI rat model (Image A&B). The black arrow indicates the impact injury area in the TBI process. The red arrow indicates the presence of neutrophil cells in the impact area.

Cellular infiltration and glial activation occur in the injured brain, with neutrophil accumulation mainly occurring within the first 24 hours of injury. Clinical studies have found an increase in the number of neutrophils after TBI. Experimentally, an increase in neutrophil accumulation is associated with increased lesion volume and changes in the brain cytokine profile. However, there is no reduction in BBB dysfunction and motor impairment.

2. The TBI Treatment in Rats Increases The Number of Neurons Undergoing Apoptosis ThroughThe Aif Pathway, Which is Associated with An Increase in Hmgb1.

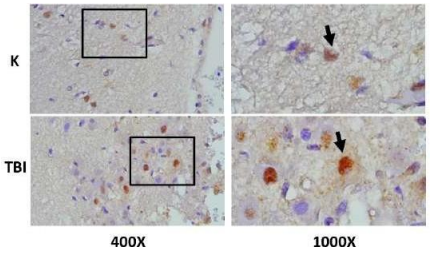


Figure 2. Shows the photomicrograph of TUNEL ASSAY staining results in the brain tissue of the TBImouse model (TBI). The black box indicates the observed area, and the arrow indicates the neuroncells undergoing apoptosis. It can be seen that there is a difference in the distribution of neuron cells undergoing apoptosis, with a significant increase in the TBI group.

3. The Administration of Oleuropein and the Duration of Administration Decrease the Expression of HMGB1 in Microglial Cells of the Rat Brain TBI Model

Microglia have diverse functions in the brain, and their morphology changes depending on their role. Recent research has shown that microglial surface antigens can change, resulting in different functions. Oleuropein has been found to inhibit Aβ aggregation and microglial activation.

This study observed the role of OLE in the expression of HMGB1 in microglia of the rat brain tissue due to TBI induction and its association with neuronal cell apoptosis in the surrounding area. Moreover, as mentioned before, the method used was the same - immunohistochemical techniques (HMGB1). The results showed that HMGB1 expression significantly decreased in the group with TBI treated with OLE compared to the TBI treatment group ($p < 0.05$).

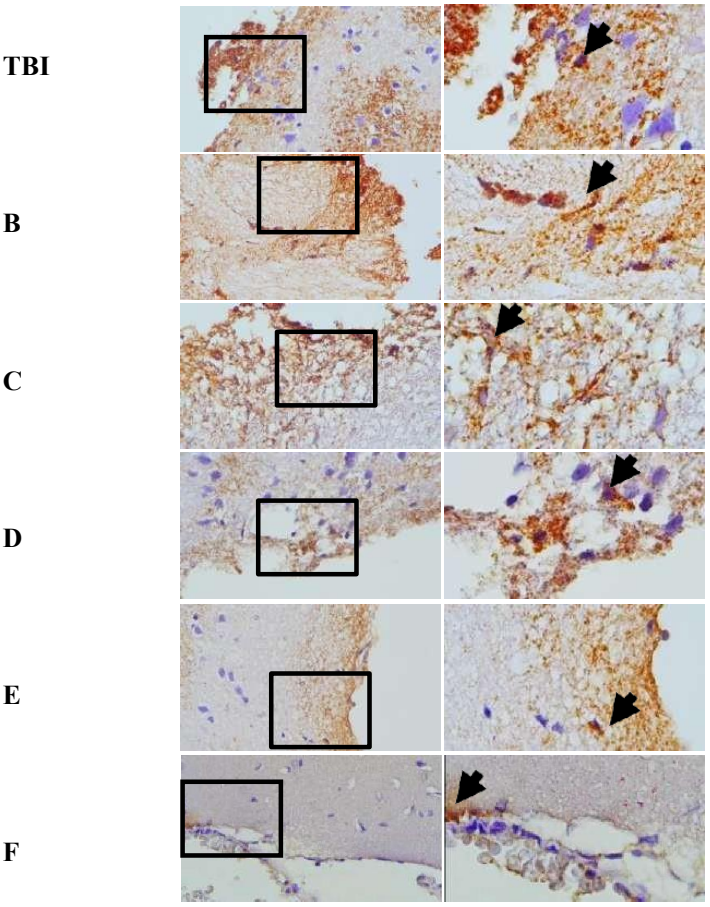


Figure 3. Represents the immunohistochemical staining results of mouse brain tissue samples treated with TBI and OLE treatment, using the immunoperoxidase technique with anti-gfap, photographed at 400x and 1000x magnifications. Glial cells expressing gfap are marked with brown colour in the cytoplasm (arrow). A represents the TBI group; B, C, D, E, and F represent differentdoses of the OLE treatment group.

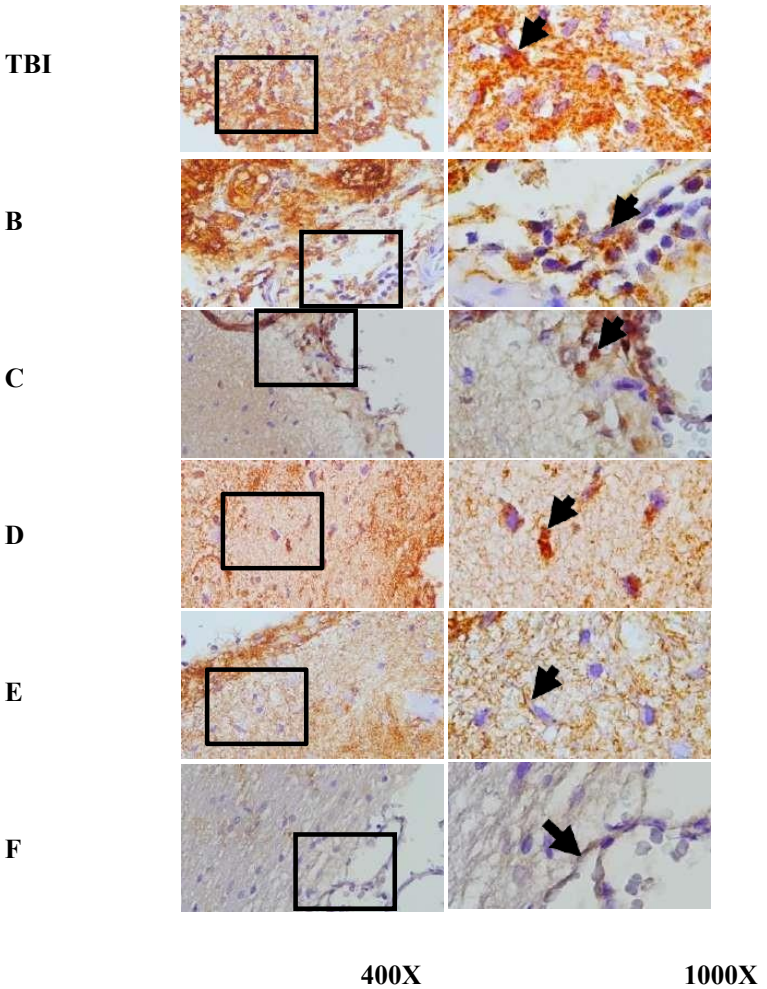


Figure 4. Shows the immunocytochemistry staining results of brain tissue sectionsfrom rats subjected to TBI with and without OLE treatment, using immunoperoxidase technique with anti-gfap, photomicrographs at 400x and 1000x. Glia cells expressing gfap are marked with brown colour in the cell cytoplasm (arrow). Group A represents the TBI rats, and groups B, C, D, E, and F represent the OLE treatment groups with various doses.

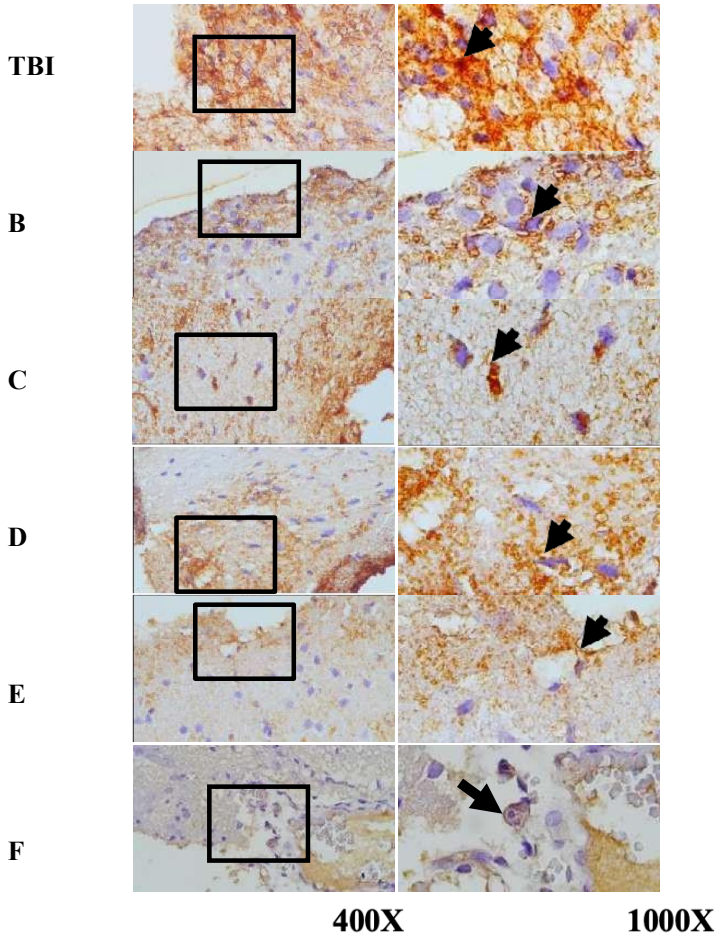


Figure 5. represents the immunohistochemical staining results of brain tissue sections from TBI-treated mice, stained with anti-HMGB1 antibodies and visualized with immunoperoxidase technique at 400x and 1000× magnification. Neurons expressing HMGB1 are marked with a browncolour in the cell's cytoplasm (arrow). TBI-treated mice without treatment are marked as groupTBI; B, C, D, E, and F represent different doses of OLE treatment. It is known that HMGB1 is typically localized in the nucleus (arrow in group K) and becomes inflammatory when translocatedto the cytoplasm or extracellular space.

Table 1. The results of the comparative test of the control group were negative

Observational group	Mean ± standard deviation
	hmgb1
B (24)	13,20±1,483
C (24)	7,60±1,140
D (24)	6,40±1,140
E (24)	6,00±1,000
F (24)	3,40+1,140
p-value	**0,000**

Note: The ANOVA test results are presented in the column as mean±SD. If they contain differentletters, it indicates a significant difference (p-value < 0.05), and if they contain the same letters, itindicates no significant difference (p-value > 0.05).

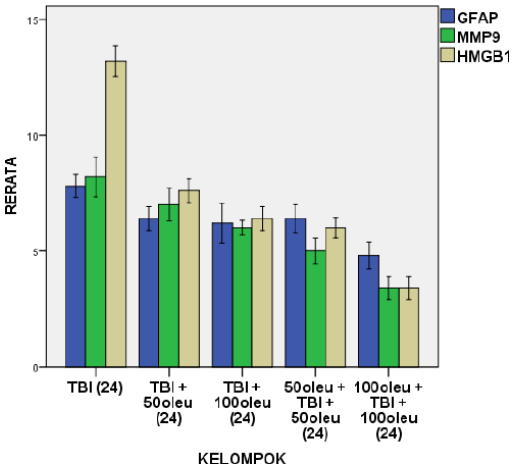


Figure 6. shows the mean histogram and analysis results of GFAP, MMP9, and HMGB1 expression in mouse brain tissue with a TBI model with OLE administration 24 hours post-treatment.

Table 2. The results of the comparative test of the control group were negative

Observational group	Mean ± standard deviation
	hmgb1
B (72)	13,60±1,341
C (72)	8,60±1,140
D (72)	6,20±1,095
E (72)	5,80±1,483
F (72)	2,20+1,303
p-value	**0,000**

Note: The results of the ANOVA test are shown in the column of mean±SD; if it contains differentletters it means there is a significant difference (p-value<0.05), and if it contains the same letters, itmeans there is no significant difference (p-value>0.05).

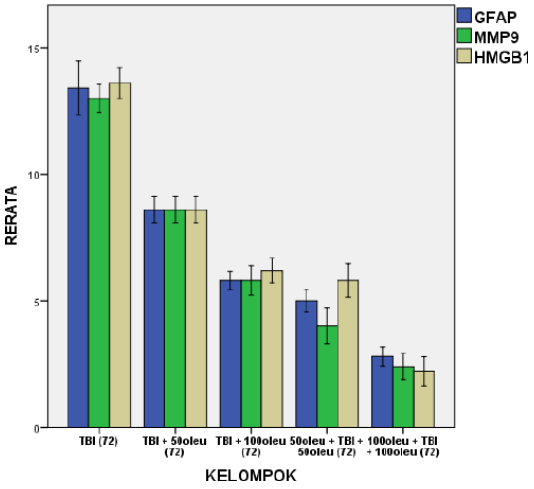
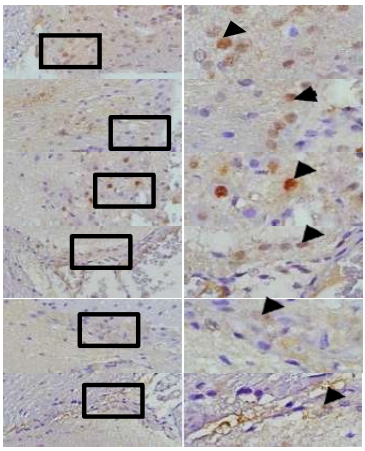


Figure 7. Mean histogram and analysis results of GFAP, MMP9, and HMGB1 expression in the brain tissue of TBI-induced rats treated with OLE at 72 hours post-treatment.

4. Administration of Oleuropein and Duration of Administration Reduces the Incidence of Neuronal Cell Apoptosis in Brain Tissue with TBI Treatment.

OLE's role in reducing apoptosis was investigated in neurons using immunohistochemical techniques to observe AIF and a TUNEL assay for apoptosis. The results showed a significant decrease in the number of apoptotic neurons in the TBI group with OLE treatment compared to the TBI treatment group ($p < 0.05$).



400X **1000X**

Figure 8. represents the results of TUNEL staining on mouse brain tissue samples with TBI and OLE treatment, stained with immunoperoxidase technique using a TUNEL assay kit photomicrographs at 400x and 1000x. Neurons undergoing apoptosis are marked with a brown colourin the nucleus of the cell (arrow). The TBI group represents mice with TBI treatment; B, C, D, E, and F represent the OLE treatment groups with various doses.

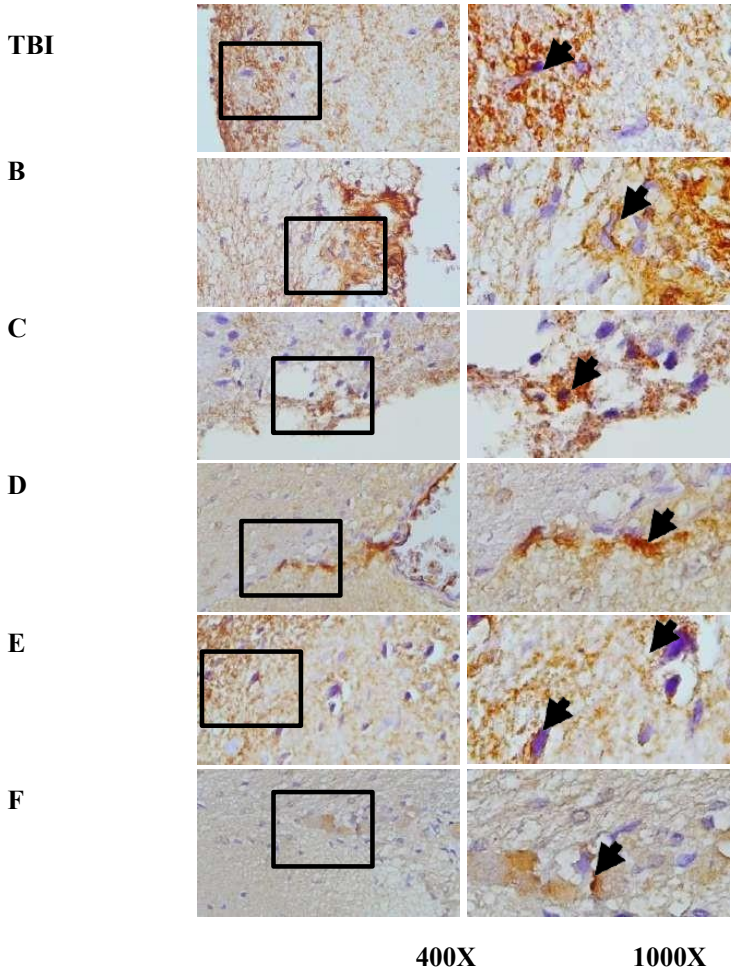


Figure 9. represents the immunohistochemical staining results of brain tissue samples from rats with TBI treatment and OLE treatment, using anti-AIF antibodies and the immunoperoxidase technique, photographed at 400x and 1000x magnification. Neurons expressing AIF are marked with brown colour in the cytoplasm (arrow). TBI group with TBI treatment; B, C, D, E, and F groupswith OLE treatment at various doses.

Table 3. The results of the comparative test of the control group were negative.

Observational group	Mean ± standard deviation	
	apoptosis	aif
B (24)	10,20±1,788	9,80±1,304
C (24)	9,60±1,816	5,20±1,304
D (24)	8,20±1,643	6,60±1,817
E (24)	5,00±1,224	6,40±1,949
F (24)	4,20+0,836	4,40+1,517
p-value	0,000	0,000

Note: The results of the ANOVA test are shown in the column of mean±SD. If it contains different letters, it means there is a significant difference (p-value<0.05), and if it contains the same letters,it means there is no significant difference (p-value>0.05).

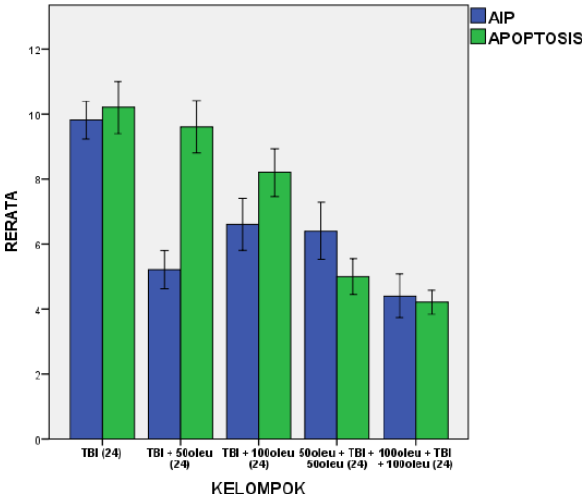


Figure 10. Mean histogram and analysis results of AIF expression and apoptosis incidence in the mouse brain tissue with TBI model and OLE administration at 24 hours post-treatment.

Table 4. The results of the comparative test of the control group were negative

Observational group	Mean ± standard deviation	
	apoptosis	aif
B (72)	13,40±1,140	13,00±1,870
C (72)	11,60±1,140	8,80±1,870
D (72)	6,60±1,816	6,40±1,870
E (72)	3,20±0,837	5,00±1,870
F (72)	2,20+0,836	2,20+1,870
p-value	0,000	0,000

Note: The ANOVA test results are shown in the column of mean ± S.D.; if different letters are shown, it means there is a significant difference (p-value <0.05), and if the same letters are shown,it means there is no significant difference (p-value> 0.05).

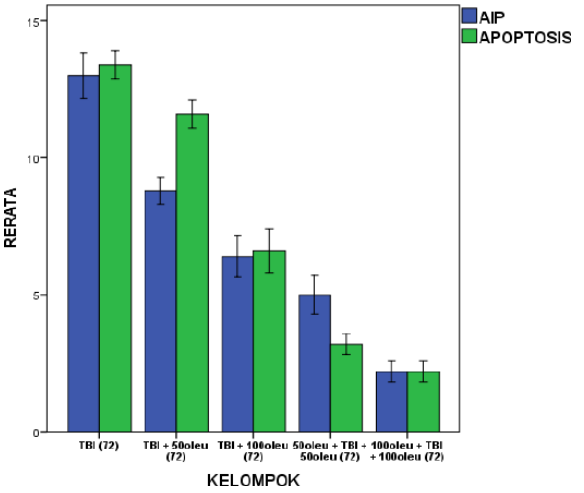


Figure 11. Mean histogram and analysis results of AIF expression and apoptosis incidence in mouse brain tissue with TBI model treated with OLE at 72 hours post-treatment.

5. The Role of HMGB1 as A Marker of Acute TBI Occurrence

HMGB1 is a pro-inflammatory cytokine involved in TBI and EBI after SAH. HMGB1 levelssignificantly increased in the TBI rat group within the first 24 hours and 72 hours after induction.In a clinical study, increased HMGB1 levels in ventricular CSF correlate with worse outcomes after TBI in children. Peak HMGB1 levels have an inverse and independent correlation with GOSat six months after TBI [16].

HMGB1 is not detected in the CSF of normal pressure hydrocephalus (NPH) patients. Still, its expression is high in the CSF of TBI patients, supporting its potential as a biomarker in TBI cases.Additionally, plasma concentration of HMGB1 is higher in TBI patients than in healthy controls upon admission, and its level upon admission is associated with a poor prognosis [16].

It can be concluded that HMGB1 can be used as a biomarker for TBI occurrence. In addition,we provide evidence of the potential biomarker of HMGB1 and the significance of its nucleocytoplasmic translocation during TBI.

Discussion

Induction of Apoptosis through AIF Pathway on HMGB1 Levels in Traumatic Brain InjuryRat Model.

Apoptosis is programmed cell death mediated by mitochondria, resulting in internucleosomal DNA fragmentation, which can be detected in situ using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL). Neuronal apoptosis death is a mechanism for eliminating unnecessary neurons with minimal immune system activation. Thus, neuronal apoptosis following traumatic injury can represent a physiological and protective response to damage [17], [18]. However, transgenic mice overexpressing anti-apoptotic proteins show a significant decrease in cortical and hippocampal damage following TBI. Therefore, excessive apoptosis-related pathway activation can be hazardous, particularly under pathological conditions. Accumulated pre-clinical studies revealed neuronal cell death in the pericontusional and hippocampus [19], [20].

Using a moderate lateral fluid percussion brain injury model, apoptotic cells in the injured cortex were recorded as early as 24 hours, while in the hippocampus and thalamus, apoptotic response was delayed, peaking at 48 hours and two weeks after injury, respectively. Mitochondria trigger various apoptotic signalling pathways through interaction among the bcl-2 family of proteins, such as cytochrome c, second mitochondria-derived activator of caspases (Smac), apoptosis-inducing factor (AIF), endonuclease G (Endo G), and mitochondrial-derived activator of caspases (Smac), to release pro-apoptotic proteins from the mitochondrial intermembrane space,resulting in apoptosis. This pathway is called the intrinsic pathway [21].

One of these is the downstream interaction protein of the Bcl-2 family mechanism in the intermembrane space, including cytochrome c, a second mitochondrial-derived activator of caspases(Smac), endonuclease G (Endo G), and AIF, released and interact with each other after TBI. Theirinteraction then results in the release of pro-apoptotic proteins. Cytochrome c interacts with procaspase-9 and apoptosis protease-activating factor-1 (apaf-1) and forms the apoptosome, activating procaspase-9. Caspase-9 activates procaspase-3, then caspase-3 degrades the activated caspase DNase inhibitor, causing DNA fragmentation and apoptosis. Caspase-3 can also activate other enzymes after TBI that repair damaged DNA, such as poly(ADP-ribose) polymerase (PARP) [22], [23]. Although involved in necrosis and apoptosis, PARP 89- and 21- kDa fragments are cleaved by caspases and associated with apoptosis after cerebral ischemia. Smac is also involved in caspase activation. Following TBI, Smac released from mitochondria binds and neutralizes the effects of X-linked inhibitors of apoptosis protein, resulting in further apoptosis.

Other studies have shown the importance of the caspase-independent pathway. Following TBI,apoptosis-inducing factor (AIF), a flavoprotein with NADH oxidase in the intermembrane space of mitochondria, is released into the cytosol through membrane permeabilization. AIF then translocates to the nucleus and induces apoptosis. This apoptotic pathway also does not depend oncytochrome c, Apaf-1, and caspases. PARP-1, cyclophilin A, and HSP-70 also regulate AIF release from mitochondria and translocation to the nucleus. Inhibiting PARP-1 has a neuroprotective effect after TBI [24], [25].

Cyclophilins are a family of peptidylprolyl cis-trans isomerases. Cyclophilin A participates inthe nuclear translocation of AIF from the cytosol to the nucleus and facilitates the chromatinolyticeffects of AIF. Heat shock proteins from the HSP70 family have a neuroprotective effect through their chaperone function. HSP70 binding to Apaf-1 and AIF counteracts their pro-apoptotic effects by inhibiting apoptosome formation and nuclear translocation of AIF. Overexpressionof HSP70 weakens ischemic brain injury by sequestering AIF and inhibiting caspase-dependent pathways. Unlike caspase-dependent cell death, AIF-mediated apoptosis can occur under disrupted bioenergetic status; this can easily be seen in nuclear lesions after cerebral ischemia [24], [26].

In fact, under conditions of energy deficiency, mitochondria are more likely to release AIF, resulting in caspase-independent apoptosis. Therefore, AIF-induced apoptosis may be more common in severe TBI, where bioenergetic disturbance is more likely to occur. Conversely, caspase-mediated apoptosis is more likely to occur in mildTBI, where mitochondrial energy utilization is less of an issue. Endo G is also known to translocate to the nucleus, causing DNA fragmentation ina focal cerebral ischemia animal model, which may also apply to TBI injury [27], [28].

This study found that treatment of traumatic brain injury (TBI) in rats increases the number of apoptotic neurons through the AIF pathway, which is linked to increased HMGB1. The response to TBI changes the proteomic transcription profile of microglia, which directly causes changes in morphology and cytokine and chemokine secretion associated with neuron cellviability. This study examined the expression of HMGB1 in microglia (GFAP) in the brain tissueof rats induced with TBI. HMGB1 expression was observed in microglial cells using specific antibodies labelled with brown colour on the cytoplasm of cells [29], [30]. The study also observed the effect on neuron cell apoptosis through AIF expression and DNA fragmentation (point of cell apoptosis). AIF expression was observed using specific antibodies in immunohistochemistry, andapoptosis was examined using the TUNEL assay to observe DNA fragmentation. AIF expressionwas observed with a brown colour in the cytoplasm of neurons undergoing apoptosis, and apoptosis was observed with a brown colour in the nucleus of cells [31].

Our research showed that HMGB1 expression significantly increased in the TBI groupcompared to the normal group ($p < 0.05$). Observation of neuron cells showed a significant increasein apoptosis incidence (in AIF expression and DNA fragmentation) in the TBI group compared tothe normal group ($p < 0.05$).

HMGB1 was shown to promote nerve inflammation response after TBI, which leads to secondary damage, as demonstrated by its upregulated expression and release after experimental TBI induction. Similarly, HMGB1-mediated nerve inflammation response contributes to early brain injury (EBI) after SAH. The active translocation of HMGB1 from the nucleus to the extracellular space after TBI and SAH was shown to activate the neuroinflammatory cascade andbreak down the BBB. Interestingly, HMGB1-targeted therapy, such as anti-HMGB1 monoclonal

antibodies (mAbs) and pharmacological inhibitor Glycyrrhizin, were effective experimentally in inhibiting post-TBI nerve inflammation response and minimizing EBI after SAH. This suggests that HMGB1 may be a promising extracellular target for these conditions and has the potential tobecome a new therapy [32], [33].

This study found a significant improvement in the group of rats with TBI in the first 24 hourscompared to the control group during the same observation period and at 72 hours after TBIinduction. On the other hand, when comparing the TBI group at 24 hours with 72 hours, HMGB1 expression in the first 24 hours was significantly higher compared to the control group and no longer significantly different from the 72-hour TBI group. In this study, AIF continued to increase at 24 and 72 hours of TBI treatment. In a previous study, HMGB1 levels were similar to the GCS score, suggesting the potential for a biomarker that appears in TBI cases. This clinical study showed that increased HMGB1 levels in ventricular CSF correlate with worse outcomes after TBI in children. In addition, peak levels of HMGB1 have an inverse and independent correlation with the Glasgow Outcome Scale (GOS) score six months after TBI [16].

The fact is that HMGB1 is not detected in the CSF of patients with normal pressurehydrocephalus (NPH). High expression of HMGB1 is shown in the CSF of TBI patients, indicatingthat the release of HMGB1 may be driven by the traumatic process, supporting the potential of HMGB1 as a biomarker in CSF for TBI cases. Upon admission, this study also obtained a higher detected plasma concentration of HMGB1 in TBI patients than in healthy controls. The HMGB1 level upon admission was higher in those who did not survive or had a poor prognosis compared to those who did [16].

HMGB1 can be used as a biomarker for TBI occurrence. In some animal models, HMGB1 has been significantly associated with the adverse effects of traumatic and non-traumatic brain injury. It has been shown to increase chemotaxis and leukocyte activation ex vivo. Furthermore, it triggers microglial activation, increases neuroinflammation, and subsequently worsens neurocognitive impairments, at least in part through TLR-dependent mechanisms in some non-traumatic injury models. TBI-induced microglial activation and increased expression of inflammatory mediators (HMGB1, TNF-α, IL-1β, IL-6) in the brain have been associated with cerebral oedema and neurological deficits [32], [34]. HMGB1 release, mediated by NR2B from neurons undergoing necrosis, triggers post-TBI brain oedema and correlates with increased Intracranial Pressure (ICP) observed in human patients. The damaging effects of HMGB1 are mediated, at least in part, by TLR4 stimulation in microglia and gradually increasing expression of aquaporin-4 (AQP4). HMGB1 protein has been extensively studied in pre-clinical TBI models, where it can interact with TLRs and activate mitogen-activated protein kinase (MAPK) and NF-κB pathways,resulting in excessive release of several pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 [35], [36].

In particular, these factors can influence the local brain environment after CNS injury and worsen brain damage after TBI, indicating that modulation and targeting of the inflammatory response after TBI could represent promising therapeutic strategies to minimize secondary damage. In a mouse TBI model, HMGB1 expression decreased significantly below basal levels 6 hours post-TBI, gradually reaching basal levels two days after trauma [37], [38]. Similarly, RAGE expression increased at 6 hours and peaked at 24 hours post-insult, gradually declining and maintaining higher expression than the sham group at day 6. The observed HMGB1 and RAGE expressions after TBI suggest the contribution of HMGB1 in the secondary post-TBI inflammatoryprocesses [39], [40].

Although HMGB1 is known to interact with two primary receptors, RAGE and TLR4, RAGE is the primary mediator of HMGB1 effects in TBI cases. Interestingly, age-dependent HMGB1 release was demonstrated in an experimental model of controlled cortical impact (CCI)-induced pediatric (3 weeks) and adult (8-10 weeks) TBI. Specifically, after TBI, extracellular HMGB1 was increased in the lesional and perilesional neocortex of both young and adult mice, compared to sham controls [41], [43]. However, statistically significant increases in HMGB1were only observed in the perilesional neocortex of adult mice, indicating that TBI-induced nerve damage may be accompanied by HMGB1 release into the extracellular environment. Regarding circulating HMGB1 levels, fluctuations in serum HMGB1 concentration were observed in pediatric TBI, while it was relatively stable in adult TBI. These findings suggest that developing pediatric brains may represent better candidates for therapeutic targeting/inhibition of HMGB1 toprevent the harmful effects of TBI-induced nerve inflammation compared to adult brains [42], [43].

The Effect of Oleuropein Administration and Administration Duration on HMGB1 Expression of Microglia Cells in the Brain Tissue of Traumatic Brain Injury Rat Model.

This study observed the role of OLE on HMGB1 expression in rat brain tissue following TBIinduction and its association with apoptosis of neighbouring neuron cells. As mentioned earlier, thesame method of immunohistochemistry was used. The study results showed that HMGB1 expression also significantly decreased in the TBI and OLE treatment group compared to theTBI treatment group ($p < 0.05$).

This study found increased HMGB1 expression in the brain tissue of a traumatic brain injury (TBI) mouse model. This can be explained by the fact that extracellular HMGB1 induces complex signalling cascades through binding to its receptors, including RAGE, TLR2, andTLR4. HMGB1 can trigger inflammation, heart regeneration, and neurite development. Many of these cascades are mediated by well-conserved pathways such as the MAP kinase, NF-κB, and A.P.- 1 transcriptional response. HMGB1 release occurs as early as 30 minutes after ischemia [43].

Previous studies have shown that HMGB1 upregulates MMP-9 in cultured neurons, astrocytes, and mouse brains. This phenomenon was mainly mediated through the TLR4 receptor, which was highly expressed in neuronal and astrocyte cultures and mouse brains. Upregulation of MMP-9 via HMGB1 expression was attenuated in TLR4 missense mutant neuronal cells compared to wild-type control cells after HMGB1 therapy. Finally, MMP-9expression in the cerebral cortex of TLR4 mutant mice was lower than in wild-type controls after focal cerebral ischemia. Our findings suggest that after stroke onset, HMGB1 released from rapidly dying cells may bind to constitutively expressed TLR4 receptors in the adjacent brain, thereby upregulating MMP-9 and expanding neurovascular damage and ischemic brain injury. To date, three putative HMGB1 receptors have been reported - RAGE, TLR2 and TLR4. Baseline RAGE expression is low in the brain.

Regarding the long-term effects, the impact of Oleuropein can be explained by HMGB1's participation in many overlapping pathways, making it challenging to dissect the exact signalling pathway. HMGB1 can also induce many other cytokines, such as TNFα and IL-1β. Our study used TNFα knockout mice to control for one alternative pathway. Stereotactic injection of recombinant HMGB1 strongly increased MMP-9 expression in TNFα knockout mice, indicating that, at least in the mouse model, induction of MMP-9 by HMGB1 can be TNFα-independent [44], [45]. However, the interaction between HMGB1, MMP-9, and other cytokines should be carefully elucidated in future studies. Along with the downregulation of MMP-9, a significant reduction ininfarct volume was observed in TLR4 mutant mice after focal cerebral ischemia.

The Effect of Oleuropein Administration and Administration Duration on Neuronal Cell Apoptosis in Brain Tissue with Traumatic Brain Injury Treatment

Oleuropein, generally known as one of the most prominent phenolic compounds in olive plants, has been proven to have several pharmacological functions consisting of anti-cancer,antioxidant, anti-inflammatory, and antiviral properties. Previous studies have shown that Oleuropein induces apoptosis activity and suppresses tumour growth in different cancer cells, including hepatocellular carcinoma and promyelocytic leukaemia.[11,46] In this study, apoptosis was observed on neuronal cells to investigate the role of OLE in apoptosis occurrence. The technique used was the same as in the previous experiment, using immunohistochemistry to observe AIF and tunnel assay for apoptosis. The results showed a decrease in AIF expression in the TBI group with OLE treatment compared to the TBI treatmentgroup ($p < 0.05$). The tunnel assay data calculation results showed values ared with the AIF expression. OLE administration in the TBI model rats significantly reduced ($p <0.05$) the number of neuronal cells undergoing apoptosis compared to rats that only received TBItreatment. Another study also stated that OLE improved neurological and cognitive outcomes, relieved brain oedema, increased neurotrophic factors, and reduced neuronal cell apoptosis by regulating the Akt/GSK-3b signalling pathway in a classic experiment of a rat modelof IRI. OLE exhibited an anti-apoptotic response through anti-inflammatory actions and suppression of lipid peroxidation and neutrophil infiltration in a rat model of spinal cord trauma.In addition, oral administration of OLE improved cerebral injury in a rat model of IRI by inhibiting Bax expression related to apoptosis, thus suppressing Bcl-2 activation.[47]

Protein X promotes apoptosis in glioma cells by increasing caspase-3 and nine expression. Baxand Bcl2 regulate the mitochondrial apoptosis pathway, with Bax being pro-apoptotic and Bcl2 being anti-apoptotic. Ole increases the Bax/Bcl2 ratio, supporting apoptosis in various cancer cell lines, including MIA PaCa-2 pancreatic cancer cells. Ole affects the pro-apoptotic protein P53 andthe Bax/bcl2 ratio, causing apoptosis in breast and colon tumours but not in glioma cells. Ole also induces apoptosis in MIA PaCa-2 pancreatic cancer cells through c-Jun and c-Fos dimerization.[47,48]

Ole (200 μM) promotes apoptosis in vitro in various cancer cells through different mechanisms, such as dimerizing c-Jun and c-Fos into AP1 in MIA PaCa-2 pancreatic cancer cells, increasing the pro-apoptotic potential in TCAM-2 and SEM-1 cells with BAX overexpression, and activating the p38/ATF-2 pathway in NSCLC H1299 lung cancer cells. Ole also decreased the expression of HIF-1α protein in HT-29 human colon adenocarcinoma cells. However, Ole atdoses of 200 or 400 did not affect the p38, ERK, or JNK pathways in glioma cancer cells.[46]

Ethical Approval

This study has been approved by the Universitas Sumatera Utara research ethics committee with Number 1174/KEP/USU/2021

Source of Funding

None

References

1. Bianchi, M.E. and Manfredi, A.A., 2007. High-mobility group box 1 (HMGB1) protein at the

crossroads between innate and adaptive immunity. *Immunological Reviews*, *220*(1), pp.35-46.

BJA: British Journal of Anaesthesia, *99*(1), pp.4-9.

2. Harris, H.E., Andersson, U. and Pisetsky, D.S., 2012. HMGB1: a multifunctional alarmin driving autoimmune and inflammatory disease. *Nature Reviews Rheumatology*, *8*(4), p.195.
3. Okuma, Y., Liu, K., Wake, H., Liu, R., Nishimura, Y., Hui, Z., Teshigawara, K., Haruma, J.,Yamamoto, Y., Yamamoto, H. and Date, I., 2014. Glycyrrhizin inhibits traumatic brain injuryby reducing HMGB1–RAGE interaction. *Neuropharmacology*, *85*, pp.18-26.
4. Webster, K.M., Shultz, S.R., Ozturk, E., Dill, L.K., Sun, M., Casillas-Espinosa, P., Jones, N.C.,Crack, P.J., O'Brien, T.J. and Semple, B.D., (2019). Targeting high-mobility group box protein 1 (HMGB1) in pediatric traumatic brain injury: Chronic neuroinflammatory, behavioural, and epileptogenic consequences. *Experimental neurology*, *320*, p.112979.
5. Balosso, S., Liu, J., Bianchi, M.E. and Vezzani, A., 2014. Disulfide-containing high mobility group box-1 promotes N-methyl-D-aspartate receptor function and excitotoxicity by activating Toll-like receptor 4-dependent signalling in hippocampal neurons. *Antioxidants & redox signaling*, *21*(12), pp.1726- 1740.
6. Meshkini, M., Meshkini, A. & Sadeghi-Bazargani, H. (2019). Use of Neuroprotective Agentsfor Traumatic Brain Injury. In *Traumatic Brain Injury*. IntechOpen.
7. Hashmi, M.A., Khan, A., Hanif, M., Farooq, U. & Perveen, S. (2015). Traditional uses, phytochemistry, and pharmacology of Olea europaea (olive). *Evidence-Based Complementaryand Alternative Medicine*, *2015*.
8. Khalatbary, A.R. and Ahmadvand, H., (2012). Neuroprotective effect of Oleuropein following spinal cord injury in rats. *Neurological research*, *34*(1), 44–51.
9. Kim, M.H., Min, J.S., Lee, J.Y., Chae, U., Yang, E.J., Song, K.S., Lee, H.S., Lee,H.J., Lee, S.R.and Lee, D.S., 2018. Oleuropein isolated from Fraxinus rhynchophylla inhibits glutamate-induced neuronal cell death by attenuating mitochondrial dysfunction. *Nutritional neuroscience*,*21*(7), pp.520-528.
10. Omar, S.H., (2010). Oleuropein in olive and its pharmacological effects. *Scientia Pharmaceutica*, *78*(2), 133–154.
11. Zhang, W., Liu, X. & Li, Q. (2018a). 'Protective effects of oleuropein against cerebral ischemia/reperfusion by inhibiting neuronal apoptosis', *Medical Science Monitor*, 24, pp. 6587–6598. Available at: https://doi.org/10.12659/MSM.912336.
12. Notoatmodjo, S, 2003. Metodologi Penelitian Kesehatan, Binarupa Aksara.
13. Lopez-Rodriguez, A.B., Acaz-Fonseca, E., Viveros, M.P. and Garcia-Segura, L.M., 2015. Changes in cannabinoid receptors, aquaporin 4 and vimentin expression after traumatic brain injury in adolescent male mice. Association with oedema and neurological deficit. *PloS one*, *10*(6).
14. Chenna, A., Petropoulos, C. & Winslow, J. (2019). Highly Sensitive Single Molecular ArrayImmunoassay Measurement of t-Tau, NF-L, GFAP, and UCH-L1 Biomarkers in Acute Concussion/Mild Traumatic Brain Injury (mTBI) Patient Serum and Saliva Samples (P2. 9–047)
15. Ba, X.H., Cai, L.P. and Han, W., (2014). Effect of cilostazol pretreatment on the PARP/APOPTOSIS INDUCING FACTOR-mediated apoptotic pathway in rat cerebral ischemia-reperfusion models. *Experimental and therapeutic medicine*, *7*(5), 1209–1214.
16. Au, A.K., Aneja, R.K., Bell, M.J., Bayir, H., Feldman, K., Adelson, P.D., Fink, E.L., Kochanek, P.M. and Clark, R.S., 2012. Cerebrospinal fluid levels of high-mobility group box1 and cytochrome C predict outcomes after pediatric traumatic brain injury. *Journal of neurotrauma*, *29*(11), pp.2013- 2021.
17. Ladak, A.A., Enam, S.A. and Ibrahim, M.T., 2019. A review of the molecular mechanisms ofTraumatic Brain Injury. *World neurosurgery*, *131*, pp.126- 132.
18. Yang, X.J., Ling, S., Zhou, M.L., Deng, H.J., Qi, M., Liu, X.L., Zhen, C., Chen, Y.X., Fan, X.R., Wu, Z.Y., Ma, F.C., Rong, J., Di, G.F. and Jiang, X.C. (2022). 'Inhibition of TRPA1 Attenuates Oxidative Stress-induced Damage After Traumatic Brain Injury via the ERK/AKT Signaling Pathway', *Neuroscience*, 494, pp. 51–68. Available at: https://doi.org/10.1016/j.neuroscience.2022.02.003
19. Stringer, M., Stefovska, V., Zentner, I., Hansen, B., Stepulak, A., Knaute, C., Marzahn, J. andIkonomidou, C. (2007). 'The role of matrix metalloproteinases in infant traumatic brain injury', *Neurobiology of Disease*, 25(3), pp. 526–535. Available at: https://doi.org/10.1016/j.nbd.2006.10.019.

20. Cruz-Haces, M., Tang, J., Acosta, G., Fernandez, J. and Shi, R. (2017). 'Pathological correlations between traumatic brain injury and chronic neurodegenerative diseases', *Translational Neurodegeneration*. BioMed Central Ltd. Available at: https://doi.org/10.1186/s40035-017-0088-2.

21. Xu, X.J., Yang, M.S., Zhang, B., Niu, F., Dong, J.Q. & Liu, B.Y. (2021). 'Glucose metabolism: A link between traumatic brain injury and Alzheimer's disease', *Chinese Journal of Traumatology - English Edition*, 24(1), pp. 5–10. Available at: https://doi.org/10.1016/j.cjtee.2020.10.001.

22. Simon, D.W., McGeachy, M.J., Baylr, H., Clark, R.S.B., Loane, D.J. & Kochanek, P.M. (2017). 'The far-reaching scope of neuroinflammation after traumatic brain injury', *Nature Reviews Neurology*. Nature Publishing Group, pp. 171–191. Available at: https://doi.org/10.1038/nrneurol.2017.13.

23. Ostrowski, R.P., Pucko, E. and Matyja, E. (2020). 'The effectiveness of hyperbaric oxygen modalities against vascular component of traumatic brain injury', *Brain Hemorrhages*. KeAi Communications Co., pp. 118–123. Available at: https://doi.org/10.1016/j.hest.2020.04.002.

24. Sowers, J.L., Sowers, M.L., Shavkunov, A.S., Hawkins, B.E., Wu, P., DeWitt, D.S., Prough,

    D.S. & Zhang, K. (2021). 'Traumatic brain injury induces region-specific glutamate metabolism changes as measured by multiple mass spectrometry methods', *iScience*, 24(10).Available at: https://doi.org/10.1016/j.isci.2021.103108.

25. Vaghebin, R., Khalili, M., Amiresmaili, S., Roghani, M., Esmaeili Saber, S.S. & Namdar, H.(2022). 'Saphenous vein phlebotomy alleviates neuroinflammatory response and oxidative stress following traumatic brain injury', *Interdisciplinary Neurosurgery: Advanced Techniques and Case Management*, 30. Available at:https://doi.org/10.1016/j.inat.2022.101626.

26. Nakashima, M., Gotoh, M., Hashimoto, K., Endo, M., Murakami-Murofushi, K., Ikeshima-Kataoka, H. & Miyamoto, Y. (2021). 'The neuroprotective function of 2-carbs-cyclic phosphatidic acid: Implications for tenascin-C via astrocytes in traumatic brain injury',*Journal of Neuroimmunology*, 361. Available at: https://doi.org/10.1016/j.jneuroim.2021.577749.

27. Borumand, M.R., Babaloii, F., Mirmotahari, S.A., Maghsoudi, A.S., Torabi, R., Mojtahedzadeh, M., Norouzi, P., Rad-Malekshahi, M., Javar, H.A. & Hassani, S. (2022). 'Recent trends and innovations in biosensors development for biomarkers towards monitoring traumatic brain injury', *Biosensors and Bioelectronics: X*, 12. Available at: https://doi.org/10.1016/j.biosx.2022.100247.

28. Sezer, C., Zırh, S., Gokten, M., Sezer, A., Acıkalın, R., Bilgin, E. and Zırh, E.B. (2022). 'Neuroprotective Effects of Milrinone on Acute Traumatic Brain Injury', *World Neurosurgery*[Preprint]. Available at: https://doi.org/10.1016/j.wneu.2022.11.072.

29. Rosyidi, R.M., Priyanto, B., Islam, A.A., Hatta, M., Bukhari, A., Prihastomo, K.T., Nasution,R.A., Rozikin and Prihatina, L.M. (2020). 'Role of MLC901 in increasing neurogenesis in ratswith traumatic brain injury', *Annals of Medicine and Surgery*, 60, pp. 36–40. Available at: https://doi.org/10.1016/j.amsu.2020.10.013.

30. Dai, Y., Jin, W., Cheng, L., Yu, C., Chen, C. & Ni, H. (2018). 'Nur77 is a promoting factor in traumatic brain injury-induced nerve cell apoptosis', *Biomedicine and Pharmacotherapy*, 108, pp. 774–782. Available at: https://doi.org/10.1016/j.biopha.2018.09.091.

31. Xu, X.J., Yang, M.S., Zhang, B., Niu, F., Dong, J.Q. & Liu, B.Y. (2021). 'Glucose metabolism: A link between traumatic brain injury and Alzheimer's disease', *Chinese Journal of Traumatology - English Edition*, 24(1), pp. 5–10. Available at: https://doi.org/10.1016/j.cjtee.2020.10.001.

32. Akamatsu, Y. and Hanafy, K.A. (2020b). 'Cell Death and Recovery in Traumatic Brain Injury,

    Neurotherapeutics. Springer, pp. 446–456. Available at: https://doi.org/10.1007/s13311-020-00840-7.

33. Padmakumar, S., Kulkarni, P., Ferris, C.F., Bleier, B.S. and Amiji, M.M. (2022). 'Traumatic brain injury and the development of parkinsonism: Understanding pathophysiology, animal models, and therapeutic targets', *Biomedicine and Pharmacotherapy*. Elsevier Masson s.r.l. Available at: https://doi.org/10.1016/j.biopha.2022.112812.

34. Ng, S.Y. & Lee, A.Y.W. (2019a). 'Traumatic Brain Injuries: Pathophysiology and PotentialTherapeutic Targets', *Frontiers in Cellular Neuroscience*. Frontiers Media S.A. Available at:https://doi.org/10.3389/fncel.2019.00528.

35. Slemmer, J.E., Zhu, C., Landshamer, S., Trabold, R., Graham, J., Ardeshiri, A., Wagner, E., Sweeney, M.I., Blomgren, K., Culmsee, C., Weber, J.T. & Plesnila, N. (2008). 'Causal roleof apoptosis-inducing factor for neuronal cell death following traumatic brain injury', *American Journal of Pathology*, 173(6), pp. 1795–1805. Available at: https://doi.org/10.2353/ajpath.2008.080168.

36. Yu, C., Boutté, A., Yu, X., Dutta, B., Feala, J.D., Schmid, K., Dave, J., Tawa, G.J., Wallqvist,

A. and Reifman, J. (2015). 'A systems biology strategy to identify molecular mechanisms of action and protein indicators of traumatic brain injury', *Journal of Neuroscience Research*, 93(2), pp. 199–214. Available at: https://doi.org/10.1002/jnr.23503.

37. Saikumar, J., Byrns, C.N., Hemphill, M., Meaney, D.F. & Bonini, N.M. (2003). 'Dynamic neural and glial responses of a head-specific model for traumatic brain injury in Drosophila', *PNAS*, 117(29), pp. 17269–17277. Available at: https://doi.org/10.21203/rs.3.pex-949/v1.

38. Mendes Arent, A., Souza, L.F. de, Walz, R. and Dafre, A.L. (2014). 'Perspectives on molecularbiomarkers of oxidative stress and antioxidant strategies in traumatic brain injury', *BioMed Research International*. Hindawi Publishing Corporation. Available at: https://doi.org/10.1155/2014/723060.

39. Ng, S.Y. & Lee, A.Y.W. (2019b). 'Traumatic Brain Injuries: Pathophysiology and PotentialTherapeutic Targets', *Frontiers in Cellular Neuroscience*. Frontiers Media S.A. Available at:https://doi.org/10.3389/fncel.2019.00528.

40. Ray, S.K. and Banik, N.L. (2002). 'Molecular mechanisms in the pathogenesis of traumatic brain injury Studies on Ge/Si Nanostructures for Electronic and Optoelectronic Devices Viewproject Luminescent silicon nanocrystals View project', *Article in Histology and Histopathology* [Preprint]. Available at: https://doi.org/10.14670/HH-17.1137.

41. Graham, N.S.N. & Sharp, D.J. (2019). 'Understanding neurodegeneration after traumatic brain injury: From mechanisms to clinical trials in dementia', *Journal of Neurology, Neurosurgery and Psychiatry*. BMJ Publishing Group, pp. 1221–1233. Available at: https://doi.org/10.1136/jnnp-2017-317557.

42. Lööv, C. (2014). *Cellular and Molecular Responses to Traumatic Brain Injury*. Acta Universitatis Upsaliensis.

43. Van den Bedem, H. and Kuhl, E. (2017). 'Molecular mechanisms of chronic traumatic encephalopathy', *Current Opinion in Biomedical Engineering*. Elsevier B.V., pp. 23–30. Available at: https://doi.org/10.1016/j.cobme.2017.02.003.

44. Tajik, M., Danielli, E., Sharma, B. & Noseworthy, M.D. (2022). 'A review of molecular andgenetic factors for determining mild traumatic brain injury severity and recovery', *Brain Disorders*, p. 100058. Available at: https://doi.org/10.1016/j.dscb.2022.100058.

45. Nath, J., Roy, R., Sathyamoorthy, Y.K., Paul, S., Goswami, S., Chakravarty, H., Paul, R. &Borah, A. (2022). 'Resveratrol as a therapeutic choice for traumatic brain injury: An insight into its molecular mechanism of action', *Brain Disorders*, 6, p. 100038. Available at: https://doi.org/10.1016/j.dscb.2022.100038.

46. Rishmawi, S., Haddad, F., Dokmak, G. & Karaman, R. (2022). 'A Comprehensive Review on the Anti-Cancer Effects of Oleuropein', *Life*. MDPI. Available at: https://doi.org/10.3390/life12081140.

47. Zhang, W., Liu, X. & Li, Q. (2018b). 'Protective effects of oleuropein against cerebral ischemia/reperfusion by inhibiting neuronal apoptosis', *Medical Science Monitor*, 24, pp. 6587–6598. Available at: https://doi.org/10.12659/MSM.912336.

48. Bossio, S., Perri, A., Malivindi, R., Giordano, F., Rago, V., Mirabelli, M., Salatino, A., Brunetti, A., Greco, E.A. and Aversa, A. (2022). 'Oleuropein Counteracts Both the Proliferation and Migration of Intra-and Extragonadal Seminoma Cells', *Nutrients*, 14(11). Available at: https://doi.org/10.3390/nu14112323.