AMERICAN UNIVERSITY OF BEIRUT

NEURODEGENERATION AND BEHAVIORAL CHANGES FOLLOWING TRAUMATIC BRAIN INJURY

by
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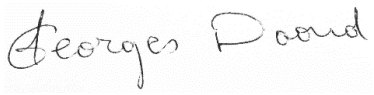

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ABSTRACT
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Traumatic Brain Injury (TBI) is one of the leading causes of disability and mortality in patients. Recent studies have shown a significant association between TBI and various neurological, cognitive, emotional, sensory, and motor deficits, which render patients at a higher risk for developing neurodegenerative diseases. In this study, we aimed to characterize the cellular and behavioral changes associated with closed head injury in rodents. Male Sprague-Dawley rats were subjected to a head injury with the skull intact, using a modified version of the weight drop model. All animals were tested for sensorimotor and cognitive behavioral changes prior to and weekly for one month after injury. At the end of each experiment, brains were collected to examine the extent of injury and the presence of cellular damage in the somatosensory cortex and hippocampus using pathogreen staining and LDH assay. Our results have shown that rats with TBI injury displayed significant changes in sensory, motor and cognitive behaviors when compared to their controls. In addition, histological analysis has confirmed that neural degeneration occurred not only in the somatosensory cortex but had spread to the hippocampus as well. These findings suggest that closed head injury is detrimental to the integrity of neural tissue not only at the site of injury but also in deep brain structures.

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ABBREVIATIONS

TBI	=	Traumatic Brain Injury
IACUC	=	Institutional Animal Care and Use Committees
ACF	=	Animal Care Facility
AUB	=	American University of Beirut

CHAPTER I

INTRODUCTION

A. Traumatic Brain Injury

1. Definition and Statistics

Traumatic brain injury (TBI) is a global burden with serious ramifications that impact life expectancy and morbidity rates. Some argue that it is in fact a chronic process due to its persistent effects (1). A longitudinal study revealed that 8 years following a TBI, 19.8% of the patients remained very impaired while 46.5% stayed slightly impaired (2). The term TBI replaced its more general antecedent "head injury" in order to highlight the damage sustained by the brain, yet there is no agreement on a single and clear definition of TBI (3). According to the Centers for Disease Control and Prevention (CDC), TBI is defined as an invasive or noninvasive hit to the head that can alter normal brain function depending on the severity of the damage (4). The National Institute of Neurological Disorders and Stroke states that TBI is an acquired brain trauma that can damage the brain and requires immediate hospitalization (5). Its symptoms vary according to the extent of the damage and range from headache, memory problems, behavioral changes, sleep pattern changes, and blurry vision to motor impairment, speech problems, and even seizures (5). More importantly, TBI is a serious injury that has led to 61000 TBI-connected deaths in 2019 as reported by the CDC (6). In the United States, 52000 people die each year out of 17 million TBI cases (7). Thus, there is substantial evidence that showcases the danger of TBIs. There are different types of TBIs. 70% to 90% of TBIs are mild (mTBI) while others are

classified as moderate or severe (5, 8). A mTBI or concussion causes loss of consciousness that lasts for less than 30 minutes, altered brain function for less than 24 hours, and a score of 13-15 on the Glasgow Coma Scale (9). On the other hand, moderate or severe TBIs result in consciousness loss for more than 30 minutes with complications like coma or amnesia that can last more than 24 hours and possibly a lifetime (9). Also, such patients would score a 3 on the Glasgow Coma Scale and have focal neurologic signs (9). The causes of TBI are numerous but most commonly include motorcycle or car accidents in addition to falls (8, 10). Most adults aged between 20 and 24 years sustain traffic related TBIs (7). Recently, falls have been become the main cause of TBIs (3). For instance, in the United States, the number of deaths from fall-induced TBIs has increased from 2008 to 2017 by 17% to reach a staggering 17500 deaths (11). Also, males are twice as likely to suffer a TBI as women (8, 10). TBIs most likely occur in teenagers and young adults; however, the age group has been increasing (3, 8). This highlights the cases of TBIs sustained by the elderly that are often overlooked due to misdiagnosis with age-related conditions like dementia (12). In fact, patients aged 75 years old and above exhibit the greatest rate of death and hospitalization following a TBI (7). Hence, TBIs impose a serious health burden and require careful assessment.

2. Categories of TBI

a. Penetrating head injury

Penetrating head injuries (PHIs) have the worst prognosis among other categories of TBIs as 66-90% of patients with PHI die before arriving at a hospital and only 51% survive following hospitalization (13). A PHI occurs whenever the brain is

injured by any mechanism except a blunt one (14). An injury caused by an object traveling at a high speed has a worse prognosis than that caused by slow one (14). The kinetic energy formula ($E=0.5\ mv^2$) highlights the contribution of velocity to the energy transferred to the brain (14). Also, the closer the object to the head, the worse the injury due to the lesser amount of air resistance experienced by the traveling object (14). Most PHIs are caused by a gunshot, which makes suicide using a gun fired at a close range a major cause of PHI (14). Also, the survival rate following suicide by a gunshot is only 16% (15). Other sources of PHI include slower objects like an electric drill, knife, and a nail gun (16-18). Additionally, the path followed by the object across the brain is a vital contributor to the severity of damage (14). More specifically, an injury can be penetrating if the object is stuck in the intracranial cavity, tangential if bone pieces pierce the brain tissue as the object brushes against the skull, and piercing when the object exits the skull from the opposite side (14).

b. Closed head injury

A closed head injury (CHI) is more common than PHI and is the result of a hit to the head that affects the brain without injuring the skull nor the dura mater (19). CHIs are caused by warfare explosives, falls, and car or motorcycle accidents (20). Explosives and war weapons lead to a blast-induced head injury that is unique in the damage it inflicts on the brain (20). Therefore, some argue that blast or explosive head injury serves as a separate category of TBI (21). On the other hand, 35% of CHIs are caused by falling (19). Sports such as Rugby also constitute a major cause of CHIs that range from mild abrasions to fatal cases of intracranial hemorrhages (22). As a result, carefully assessing the severity of the CHI becomes imperative to the patient's survival (22). CHI diagnosis is usually carried out by a non-contrast computed tomography (CT)

scan of the head (23). However, there is a shift towards more sensitive and advanced neuroimaging techniques such as perfusion imaging (23). With respect to prognosis, patients with CHI exhibit an impaired quality of life that is reflected in their socialization and activities during free time (24). Also, CHIs led to an increased tendency to adapt a depressive coping mechanism in patients (24). Therefore, severe or mild cases of CHIs require careful examination and monitoring with time.

c. Explosive injury

Explosive or blast head injuries (EHI) are common during warfare, civil unrest, or terrorist attacks. The most common source of such injuries is explosive devices, which were responsible for 52% of TBI injuries in soldiers involved in Operation Iraqi Freedom (25). Moreover, explosive devices impacted 62% of soldiers wounded by action and 53% of those killed while gunshots and mortar blasts accounted for 7-9% of the TBIs sustained (25). Blast injuries are divided into 4 subgroups that include primary, secondary, tertiary, and quaternary blast injuries (26). The main mechanism through which EHIs generally occur is the shock pressure sustained by the brain due an explosive (21). In primary blast injuries, a blast shock wave directly affects the body while in secondary blast injuries, explosive fragments cause milder trauma (26). Moreover, tertiary injuries involve a hurling force exerted by the blast pressure while quaternary injuries constitute any indirect injury sustained such as burns (26).

3. Pathophysiology of TBI

Following head injury, primary or secondary neural injuries can impact normal brain function. A primary neural injury occurs directly after an external force is applied to the brain and results in both focal and diffuse injuries (27). On the other hand, a

secondary neural injury is a progression of the damage caused by the primary injury that holds delayed clinical manifestations ranging from short to long term side effects (28). Secondary neural injures are sensitive to therapeutic interventions unlike primary injuries that can only be managed through preventive methods (28). Numerous mechanisms have been postulated to explain the morphological and behavioral changes induced by TBIs. These include neuroinflammation in the brain, oxidative stress, axonal degeneration, and apoptosis (27).

a. Neuroinflammation:

Neuroinflammation is a main contributor to the progression of secondary neural injuries (29). In fact, it has been termed "sterile inflammation" to account for the inflammatory changes that occur in the brain due to an accumulation of dead cells and molecular aggregates (30). At the site of injury, dead cells create cellular debris that can activate microglia and astrocytes, which in turn trigger an inflammatory cascade (28). In addition to glial cells, immune cells are also activated following brain injury (28). Peripheral immune cells infiltrate the brain when the integrity of the blood-brain-barrier (BBB) is compromised (31). Studies have shown that TBIs cause BBB dysfunction which allows any peripheral immune cells and molecules carried by the blood to access the brain parenchyma (31). Microglia constitute the first line of defense against foreign molecules in the brain (32). As the immune cells of the nervous system, microglia have a phagocytic function (32). Once activated, they transform from a ramified shape into an amoeboid one and upregulate the expression of ionized calcium-binding adaptor protein-1 (Iba-1), which acts as a marker (33). Another marker of microglia activation is CD68 (34). Both Iba-1 and CD68 were shown to increase following TBI (35). Microglia produce anti-inflammatory cytokines to protect the brain after injury;

however, they also release excessive pro-inflammatory cytokines which further worsen brain injury and activate other neuronal cells triggering an inflammatory cycle (36). Astrocytes are vital for maintaining homeostasis and the structure of the BBB (31, 33). In the BBB, they attach their end feet to the resident endothelial cells which contributes to the barrier function of the BBB (37). Following injury, cell debris or inflammatory cytokines activate astrocytes to release adenosine triphosphate (ATP) needed to trigger multiple other signaling pathways (38). The increase in the reactivity of astrocytes leads to astrogliosis, a major marker of central nervous system (CNS) damage (38). Astrogliosis entails many morphological and functional changes such as astrocyte proliferation and hypertrophy along with an increase in the expression of intermediate filaments such as glial fibrillary acidic protein (GFAP) and vimentin (39). Peripheral immune cells infiltrate the BBB when it is disrupted by TBI (40). BBB disruption is caused by the swelling of astrocyte end feet, thickening of the basal lamina, pericyte degeneration, reduction of tight junctions, and other factors that compromise its microvascular structure (41, 42). Chemokines secreted by activated microglia and astrocytes upon injury also attract immune cells to the injury site across the compromised BBB (43). BBB dysfunction, following TBI, has been recently suggested to be long lasting as it was evident up to 10 months post injury in TBI rats (44). It was also accompanied with an increase in neuroinflammation through the activation of monocytes, astrocytes, and microglia (44).

b. <u>ROS and free radical activation:</u>

Reactive oxygen species (ROS) are free radicals capable of stealing electrons from lipids found in cell membranes through lipid peroxidation (45). Normally, the body's antioxidant system, which includes various enzymes like glutathione, catalase,

and superoxide dismutase, controls the amount of ROS in the body (46). However, injuries or pathogens create an imbalance in the oxidant and anti-oxidant systems leading to oxidative stress (46). After TBI, an increase in the concentration of ROS induces oxidative stress that has detrimental effects on the brain (47). Moreover, the damage caused by ROS correlates with the severity of the brain injury (48). TBI induces the production of nitric oxide synthase which promotes the formation of nitric oxide (NO•) (49). Additionally, TBI increases excitotoxicity through glutamate release, mitochondrial dysfunction in neutrophils along with cyclooxygenase release, which all encourage the production of superoxide (O2•-) (49). NO• and O2•- react together to form peroxynitrite (NOO-) that causes the production of multiple free radicals ultimately leading to lipid peroxidation, DNA damage, and protein oxidation (49). A study that measured oxidative stress markers in TBI mice showed an increase in levels of thiobarbituric acid reactive species (TBARS) in the cortex 24 hours following TBI (50). Additionally, 14 days after injury, an elevation in the activity of glutathione peroxidase in the hippocampus was observed (50). However, there no correlation between the severity of TBI and the oxidative stress markers (50).

c. Axonal degeneration:

Axonal degeneration is a common indicator of damage in the CNS and peripheral nervous system (51). A common type is Wallerian degeneration that arises from damage or trauma to part of the axon distal to the cell body leading to its disintegration (51). Injuries exert a mechanical force on axons leading to their breakage through shearing and stretching (52). More specifically, the microtubules found in axon projections of white matter break upon TBI (52). Since microtubules are vital for transporting proteins retrogradely and anterogradely, the proteins cluster and form

swellings after microtubules break (52). Tau proteins are responsible for stabilizing microtubules as they stretch (53). However, it is suggested that when a large mechanical force is exerted on the axon, the tau proteins fail to hold on to the sliding microtubules leading to their breaking (53). Another factor which could be involved in axonal degeneration is the role of sodium and calcium channels (54). Stretch injuries affect these channels which in turn changes the ionic concentration gradients resulting in high intracellular sodium and calcium levels (54). Consequently, the propagation of action potentials is affected and calcium-dependent proteases are activated (54).

d. Apoptosis

Apoptosis is programmed cell death (55). It is a process triggered by extracellular or intracellular factors leading to the activation of caspase dependent pathways (55). Caspases are regulated by the anti-cell death (Bcl2) and inhibitor of apoptosis (IAP) families (55). Following TBI, apoptosis occurs in neuronal or glial cells (56). A single mTBI can lead to a decrease in Bcl2 levels only 2 hours after injury (57). Preclinical studies have shown that TBI causes an increase in activated caspase-3 and caspase-3 cleaved tau in the corpus callosum (58). Notably, this upregulation was accompanied by an increase in neuroinflammatory markers such as GFAP and CD68 (58). Thus, the mechanism through which TBIs exert CNS damage is multifactorial and does not rely on a single pathway. In addition, it was demonstrated that axonal degeneration precedes cell death in the cortex and thalamus of infant mTBI mice (59). Free radical production post TBI triggers DNA damage that in turn increases p53 levels leading to apoptosis (56). Therefore, apoptosis is carried out along with other mechanisms to induce the primary and secondary injuries.

4. Symptoms of TBI:

a. Mild symptoms

Mild symptoms of TBI can be divided into physical, cognitive and affective symptoms in addition to sleep problems (60). Physical or somatic symptoms include light or noise irritation, dizziness, difficulty maintaining balance, fatigue, headache, nausea, vomiting, and vision impairment (60). Dizziness is a serious symptom that persists following TBI and requires rehabilitation and vestibular examination (61). It has been shown that dizziness is prevalent among veterans exposed to blasts and examined after 72 hours while vertigo is more common in veterans who were examined more than 4 to 30 days following blast exposure (62). Furthermore, different types of dizziness can be identified in patients with TBI such as posttraumatic vestibular migraines, posttraumatic positional vertigo, and posttraumatic spatial disorientation (61). Fatigue is another important symptom that is associated with acute injury and cognitive or sleep problems (63). Indeed, 68.5% of patients with TBI experience fatigue that is more significant as a mental fatigue rather than a physical one (63). On the other hand, a study on 5416 patients with TBI found that 7% of adults and 12% of children exhibited vomiting following TBI (64). Also, vomiting post TBI was associated with an increased risk for having a skull fracture (64). Cognitive symptoms such as concentration difficulty, inability to focus, and short or long term memory problems can arise after TBI (60). TBI has been shown to decrease connectivity in multiple brain areas vital for working memory like the parietal lobe, frontal lobe, occipital lobe, and cerebellum (65). The resultant reduction in working memory was reversed by the administration of the neurocognitive enhancer, methylphenidate (65). Other types of memory affected include short term memory which results in patients forgetting the

location for their belongings, conversation details and losing track of time (66). Prospective memory, which is remembering to remember, can also in impaired in addition to the memory of the injury in what is termed posttraumatic amnesia (66). Another main group of TBI symptoms is affective or emotional symptoms that are comprised of anxiety, depression, and irritability (60). Depression is highly prevalent among TBI patients especially those with worse post-concussion symptoms (67). It is also persistent as a study found that 3 months after TBI, 56.3% of TBI patients exhibited depression (67). This highlights the importance of follow up even after mTBI also due to the fact that after TBI minor depression can develop into major depression (68). Sleep troubles are also common among patients with TBI (60). After acute TBI, it is estimated that 30% of patients develop insomnia while 12 % experience disturbed sleep quality (69). Such sleep disturbances increase a patient's susceptibility to develop neurocognitive disorders in addition to delaying recovery after TBI (70). Even so, sleep disorders were found to be greater in TBI patients aged 65 years old and above compared to non TBI elders (71). Also, insomnia and obstructive sleep apnea were the most prevalent disorders diagnosed (71).

b. Moderate to severe symptoms

The moderate or severe symptoms of TBI involve thinking problems, trouble retaining information, and difficulty learning new skills (72). Also, patients may experience weakness in the extremities, sensory impairment in addition to troubles in motor skills and coordination (72). Similar to the mild symptoms, depression, anxiety, and nervousness can arise along with feelings of increased aggressiveness and anger (72). In fact, behavioral changes can occur with reported personality changes and impaired decision-making skills (72).

c. Motor problems:

Motor deficits constitute a major symptom following TBI. Children with TBI have a significantly worse motor performance than normal children especially in tasks that require gross motor skills (73). More specifically, motor tasks that require speed were the most challenging to perform by children with TBI that demonstrated persistent motor impairment (73). In addition to speed, factors such as balance and gait were associated with motor deficits that persisted 12 months after TBI (74). Moreover, a multicenter study on veterans with TBIs showed that even 2 years following motor rehabilitation, more than one third of patients continued to display motor deficits (75). Tandem gait was the most prevalent deficit, which emphasizes the importance of posture rehabilitation (75). Diffuser tensor imaging (DTI) of the corticospinal tract is used to identify the mechanism of motor impairment following TBI (76). In fact, DTI has revealed that 58.5% of patients with TBI have diffuse axonal injury with a mean of 3.6 lesions (76). Additionally, 61% of these lesions are in the pons, 50% in the cerebral peduncles, 40% in the medulla, 17% in the posterior limb of the internal capsule, and 13% in the corona radiata (76). Developing DTI to analyze other motor tracts such as the reticulospinal tract or the vestibulospinal tract might also be helpful to identify the cause of motor weakness in patients with TBI (76).

d. Cognitive symptoms:

TBIs can result in neurocognitive impairments that affect working memory, attention, and concentration with an estimated recovery of one-year post TBI (77). Cognition is an important factor that determines how soon patients with TBI can return to the workplace (78). Given that 58% patients with moderate or severe TBI are employed 10 years after their injury supports the notion of recovery from the possible

cognitive problems ensued (79). However, the neurocognitive deficits displayed post TBI are vital to address and understand especially in elders. Patients with moderate or severe TBI suffer from attention and learning deficits along with possible dysfunction in the cholinergic system similar to the profile of mild Alzheimer's disease (80). In men aged between 19 and 78 years old, TBI is associated with a high risk of dementia (81). One study determined that age impacts the severity of dementia following TBI (82). Its results showed that dementia was a risk factor following moderate or severe TBI in patients older than 55 years old while in patients older than 65 years old, mTBI was sufficient to increase dementia risk (82).

e. Chronic pain:

Chronic pain is an important symptom afflicting many patients who suffer from TBI (83). It has been shown that chronic pain increases with the severity of TBI in veterans (84). This pain is not associated with depression or posttraumatic stress disorder (PTSD) but is highest in veterans who present the three factors consisting of TBI, depression, and PTSD (84). In support of this result is a systematic review that also demonstrated the prevalence of chronic pain after TBI as a risk factor independent of depression or PTSD (85). Moreover, post traumatic headache is a one of the most common adverse effects of TBI and that is more prevalent following milder types of TBI (86, 87). For example, 80% of patients with mTBI develop post traumatic headache while only 27% complain of headache following moderate or severe TBI (87). It is also long lasting as 58% of patient with TBI still complained of headache 1 year after injury (88). Interestingly, 6 months following TBI, patients displayed the greatest prevalence of TBI with a value of 69% while 62% reported headaches after just 3 months (88).

Thus, although the headache is persistent, signs of recovery are present 1 year after injury.

5. Animal models of TBI

Choosing the right animal model is vital to ensure the translational aspect of preclinical research. Animal models of TBI help scientists understand the pathophysiology of TBI and evaluate different treatments. These models involve the use of large animals such as pigs, monkeys, and sheep in addition to smaller animals like Drosophila and zebrafish (89). However, rodents are the most commonly used animal models of TBI due to their ease of use and shared similarities with humans (89). Different types of TBI models can be classified according to the type of injury and clinical relevance (90).

a. Open head model:

In open head injury models, a force is applied directly to the brain meninges exposed after performing craniotomy (91). Such models are classified into controlled cortical impact (CCI) and fluid percussion (FP) models (91). In CCI models, a piston is applied directly to the dura mater to induce a focal injury with subsequent cell death (92). The advantage of using this model is the ability to control the severity of the TBI by controlling the size of the piston, its velocity, and depth of injury (90, 91). However, since CCI induces widespread destruction in the cortex, thalamus, and hippocampus, it is mainly used to induce severe TBI (91, 93). Cognitive deficits assessed by the Morris water maze test are evident in mice subjected to CCI and are dependent on the severity of injury (94). However, emotional deficits following CCI are not associated with the severity of injury (94). Thus, CCI is good model to study cognitive impairment

following severe TBI. The second type of closed head injury model is the FP model in which a pressure pulse is applied to the exposed dura mater (92). To create this pressure pulse, a pendulum released from a predetermined height strikes a saline filled cylinder that injects the fluid into the intracranial cavity of the rodent (92). Similar to the CCI, the FP model allows controlling of the severity of the TBI by adjusting the velocity and height from which the pendulum is released (90). A high pressure threshold results in focal and diffuse injury while a low threshold pressure leads to a diffuse injury (95). However, FO model has a high mortality rate in animal since it causes apnea (90).

b. Closed head model:

In closed head injuries (CHI) models, a force is applied directly or indirectly to the skull to induce TBI (91). Direct injury to the brain is carried out by dropping a specific weight on the intact skull while an indirect injury occurs via a blast (91). A blast CHI model is usually diffuse and representative of battlefield injuries (90). Thus, its key advantage lies in being an accurate model of blast injuries sustained by soldiers (90). This model uses compressed gas to generate overpressure from a shock tube in such a way that only the rodent's head is exposed (96). After the blast, rodents usually exhibit instant apnea that lasts for seconds in addition to rapid heartbeat (97). Motor performance and cognitive ability are also impaired as assessed by beam walking and Morris water maze tests (97).

c. Weight drop model:

The weight drop model is widely used in research to investigate the effects and mechanisms of TBI (91). There are different weight drop models which vary in the type of injury created (90). For example, Marmarou's method uses a disk above the skull to create a diffuse injury whereas Shohami's method creates a focal injury by dropping the

weigh on one side of the skull (90). One of the advantages of the weight drop model is the ability to control the severity of the injury by adjusting the height from which the weight is dropped (91). Moreover, it is an easy and inexpensive way to emulate TBI in humans that could occur as a result of falling or car accidents (90). However, it is not an accurate method to create an injury in a specific brain area (90). The rodent's head may also suffer a second injury upon rebound and there is a high fatality risk due to resultant apnea (90). A more important limitation lies in the reproducibility of the behavioral results between different labs given the numerous parameters involved from the impactor to the rodent's head response (91).

6. Aim of the study

The aim of the study is to assess the long term effects of closed head injury, using the weight drop model, on the development of sensory hypersensitivity, motor coordination and cognitive functions in rodents.

CHAPTER II

METHODS AND MATERIAL

A. Material

1. Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut, and the ethical guidelines for experimental pain on conscious animals were followed. Male Sprague-Dawley rats weighing between 200 and 280 g were ordered from the Animal Care Facility at AUB, and were group-housed in a temperature-controlled room at 25 °C with a 12-12-hour light-dark cycle with free access to food and water, ad libitum. Rats were randomly divided into two groups: sham (n=4) and TBI (n=4). Animals were habituated to the experimental rooms and to the experimenter's presence prior to any manipulation or testing.

2. TBI (weight drop model):

A bullet shape lead weight (100 grams) that can fall freely via gravity was used in this study. A 30 cm tube was affixed to the stereotaxic frame to guide the trajectory of the fall. The impact generated by the drop was aimed at the right parietal bone and was calibrated to hit an area with the following coordinates (3mm posterior to bregma and 3.5 mm lateral to midline).

B. Methods

1. Surgery:

Rats were anesthetized with an intraperitoneal injection of a mixture of Ketamine/Xylazine (50mg/kg-15mg/kg). Each rat was then placed on a stereotaxic frame with its head fixed between a nose clamp and its ears secure by blunt bars to ensure more stability. The head was shaved and disinfected with an iodine solution, and a midline longitudinal skin incision was made to expose the Bregma and Lambda fissures of the skull.

The two fissures were used to find the coordinates of the targeted brain area. The weight was then dropped to hit the somatosensory cortex and cause traumatic brain injury. After careful monitoring of the animal's vital signs, the skin overlying the impacted site was sutured and the animal was returned to its home cage.

For the sham group, the animals were subjected to the same surgical procedure without the weight drop.

Figure 1: TBI weight drop model

2. Baseline and behavioral tests:

a. Heat Hyperalgesia:

This test was performed to assess the animal's sensitivity to noxious thermal stimulation. A radiant heat source was applied to the plantar surface of the rat's hind paw, and the time it took the animal to withdraw its paw was recorded. For accommodation, rats were placed over an elevated 3-mm thick glass plate in separate plexiglass cubicles 20 minutes prior to testing. The intensity of the infrared heat source (Ugo Basile) was adjusted so it evokes a withdrawal response after approximately 10–15 s in naïve animals.

The radiant heat was applied 5 times separated by 5 min time interval to avoid conditioning limb withdrawal. Baseline values were obtained prior to TBI. After surgery, testing was performed weekly for one month.

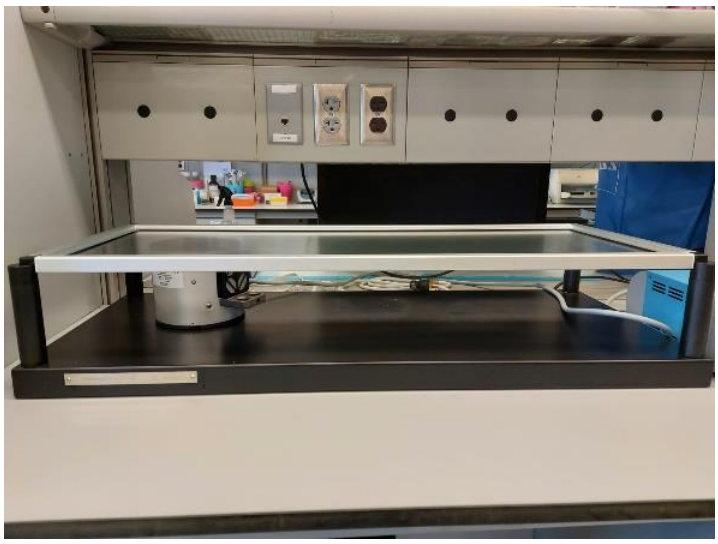

Figure 2: Heat hyperalgesia

b. Mechanical allodynia

Non-noxious stimulus was applied to the plantar surface of the animal's hind paw using a von Frey filament with a bending force of 2g. For accommodation, rats were placed over an elevated metal wire mesh floor for 20 minutes prior to testing.

The tip of the filament was used to poke the medial plantar surface of the hind paw from below the mesh grid, and was pressed until it bent for 2 seconds and a withdrawal was observed. Testing was done 5 times with a 5-min resting period between trials. Baseline values were obtained prior to TBI. After surgery, testing was repeated once every week for one month. Measurements were averaged for each animal and the responses of both the left and the right paws of all rat groups were recorded.

c. Mechanical hyperalgesia

Von Frey filament with a bending force of 15 g (0.15N) was applied to the plantar surface of the hind paw. The tip of the filament was used to poke the medial plantar surface of the hind paw from below the mesh grid 5 successive times until the animal elicits a withdrawal response. Five trials were conducted separated by 5-min resting period. Baseline values were obtained prior to TBI. After surgery, testing was repeated once every week for one month. Measurements were averaged for each animal and the responses of both the left and the right paws of all rat groups were recorded.

Figure 3: Mechanical allodynia and hyperalgesia

d. Rotarod

RotaRod (Ugo Basile) consists of a rotating rod that provide grip to the rats and rotates at a constant or increasing speed from 10 to 40 rpm. Under the rod, fall sensors are present and the height to fall is 30 cm. Rats were placed simultaneously on the cylinder but separated from each other by plastic spacer disks. Rats had to try to maintain their balance and avoid falling onto the platform.

The latency to fall off the rotating rod was recorded. Rats were trained to walk on the rotarod few days prior to testing. Baseline values were obtained before TBI. After surgery, testing was repeated once every week for one month. Three trials were performed; each separated by at least a 10 min resting period.

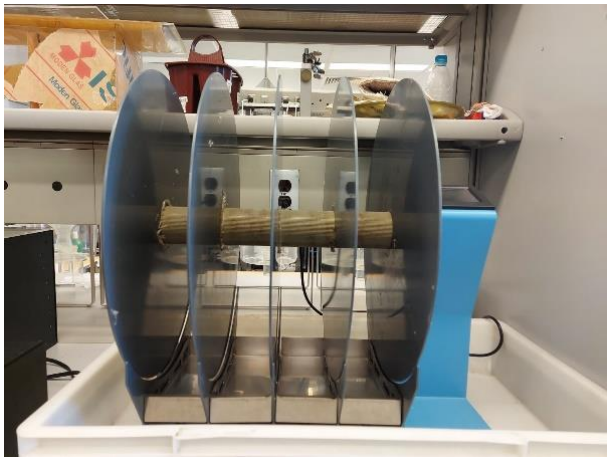

Figure 4: RotaRod

e. T-maze

All rats were left to accommodate in the experimental room for 30 min prior to testing. Spontaneous alternation T-maze test was conducted to assess the rats' cognitive functions.

The maze was set so that the central partition is in place and goal arms doors are open. The animal was placed in the start area and was allowed to choose a goal arm. After

entering to the chosen arm, the rat was confined by quietly sliding the door down. After 30 s, the door was open and the rat was removed and placed in the start area facing away from the goal arms and allowed to choose between the two open goal arms again. Test was repeated 3 times with gaps between exposures of at least 10 min; if an animal failed to run within 90 s, it was removed and tested again later.

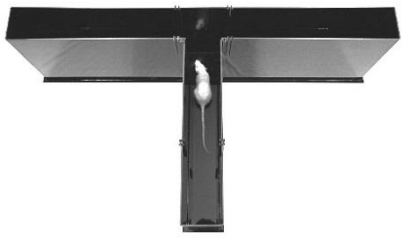

Figure 5: T-maze

f. Ladder climbing test:

The ladder climbing test was performed to assess the locomotor activity of rats. Animals were trained to ascend and descend a staircase runway few days before testing. The assessment was done by observing to animal's attempt to climb up and down the ladder. The performance of rats was noted and given a score according to the following table:

Table 1: Ladder scoring technique

0 points	No attempt to climb up the platform
2 points	Mild attempt to climb up the platform

4 points	Weak attempt to climb up the platform with slips and significant change in time
6 points	Good attempt to climb up the platform with slips and significant change in time
8 points	Good attempt to climb up the platform without any slips and with significant change in time
10 points	Good attempt to climb up the platform with the performance near to normal animals

Figure 6: Ladder Climbing Test

3. Animal perfusion

Perfusion was conducted one-month post-surgery. Rats were deeply anesthetized with Ketamine (0.8 mL) and Xylazine (0.2 mL) mixture and then perfused with 0.9% saline solution followed by 40% formalin. Brains were then removed and tissues were fixed overnight in PFA and then transferred to 30% sucrose at 4 °C or until processing time.

4. Cutting and tissue processing

Fixed brains were sectioned coronally using a freezing microtome from the rostral to the caudal extent of the hippocampus. Sections were cut at 7 μm and put directly on Superfrost Plus slides for PathoGreen Histofuorescent staining.

5. Pathogreen staining

Brain sections were left at room temperature to dry. Sections were first fixed in basic alcohol for 5 minutes and then incubated in 70% ethanol for 2 minutes followed by dH2 O for 2 minutes. Slides were then incubated in 0.06% potassium permanganate in dH2 O for 10 minutes then rinsed twice with dH2 O and incubated in dH2 O for 2 minutes.

1X PathoGreen™ staining solution was prepared by diluting 1000X PathoGreen™ stock solution 1:1000 in 0.1% acetic acid in dH2 O and slides were incubated in 1X PathoGreen™ staining solution for 10 minutes then rinsed 3 X for 1 minute in dH2 O.

Slides were air dried on a slide warmer at 50-60 ºC for at least 5 minutes then incubated in xylene for 1-5 minutes and finally coverslips were put on slides with DPX mounting medium.

6. LDH

a. Sample Preparation:

Brain tissue was rinsed in phosphate buffered saline (pH 7.4) to remove blood. 0.01 grams of the hippocampus was removed and homogenized in 5 mL buffer containing 100 mM potassium phosphate (pH 7.0) and 2 mM EDTA, per gram tissue.

Mixture was centrifuged at 10,000 x g for 15 min at 4°C to remove supernatant for assay.

b. Reagent Preparation:

Working Reagent (WR) was prepared for all sample wells by mixing, for each well: 14 µL MTT Solution, 8 µL NAD Solution, 1 µL Diaphorase and 175 µL Substrate Buffer.

c. Assay Procedure:

In the 96-well plate, wells were divided into: H2O well (200 µL), Calibrator solution (200 µL), control (10 µL) and TBI (10 µL) wells.

190 µL of WR was then added to each sample well and reading started immediately at time=0 (ODS0), and again after 25 min (ODS25) on a plate reader.

LDH activity was then calculated as follows:

$$\text{LDH Activity} = \frac{OD_{S25} - OD_{S0}}{\varepsilon_{mtt} \cdot l} \times \frac{\text{Reaction Vol (µL)}}{\text{Time} \cdot \text{Sample Vol (µL)}} \times n$$

$$= 43.68 \times \frac{OD_{S25} - OD_{S0}}{OD_{CAL} - OD_{H20}} \times n \quad \text{(IU/L)}$$

7. Statistical analysis:

Statistical analysis was done using the GraphPad Prism version 9.1.2 software. Data were presented as means ± standard errors of the means (SEM). Unpaired T-tests wwere performed in order to compare groups at each time point. Moreover, paired T-tests were done to compare each time point within group to its baseline value, while

repeated measures analysis of variance (ANOVA) was used to determine the change within the same group over time. The level of significance was set at $p < 0.05$.

CHAPTER III

RESULTS

A. Effect of TBI on heat hyperalgesia:

The effect of TBI on the development of heat hyperalgesia was assessed by measuring the paw withdrawal latency to radiant heat. Our results showed that TBI rats developed an increased sensitivity to the painful stimulus as they took significantly less time to withdraw their paw when compared to the sham group. TBI rats showed a statistically significant decrease in paw withdrawal latency at weeks 1, 2, 3 ($p<0.0001$) and 4 ($p<0.0002$) post-surgery compared to sham rats. Moreover, TBI rats showed a statistically significant change from baseline within the same group at weeks 1 and 2 ($p<0.05$). More importantly, the left hind paw showed a more pronounced effect compared to the right side.

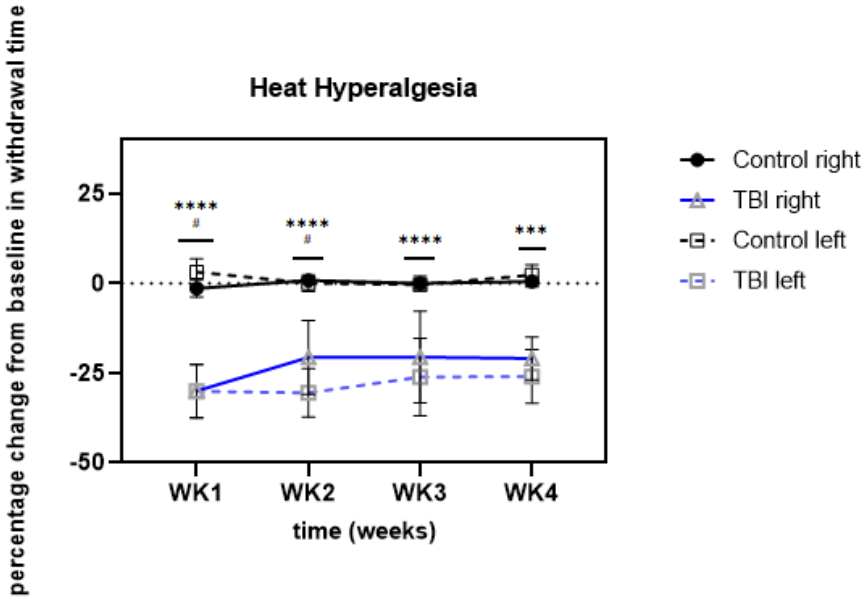

Figure 7: Effect of TBI on paw sensitivity to noxious heat

Data presented in figure 7 were expressed as percentage change from baseline and presented as mean ± SEM. Significant differences between TBI and control rats are indicated by **** p< 0.0001, *** p < 0.0002, ** p < 0.0021, * p< 0.05 and the significant difference from baseline within the same group is indicated by #### p< 0.0001, ### p < 0.0002, ##p < 0.0021, # p< 0.05.

B. Effect of TBI on motor coordination:

The effect of TBI on motor coordination was evaluated by recording the time taken by the rats to fall off the rotating cylinder, and comparing results between TBI and sham rats. As shown in figure 8, TBI rats showed significant decreases in their motor balance compared to control rats at weeks 1, 2, 3 and 4 post-surgery. (p< 0.0002).

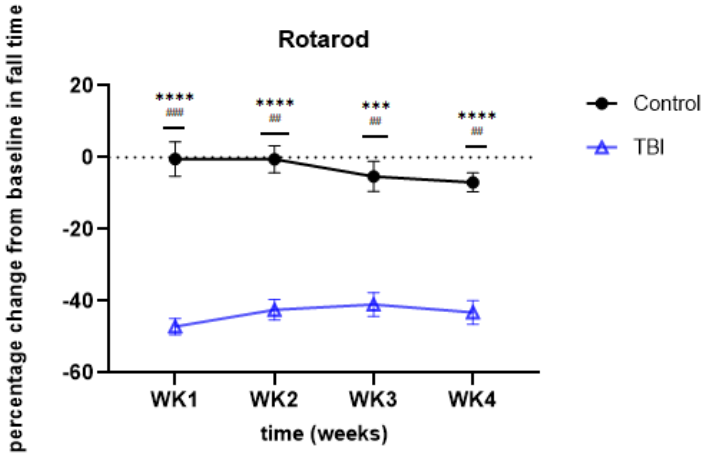

Figure 8: Effect of TBI on motor coordination

Data were expressed as percentage change from baseline and presented as mean ± SEM. Significant differences between TBI and control rats are indicated by **** p< 0.0001, *** p < 0.0002, ** p < 0.0021, * p< 0.05 and the significant difference from

baseline within the same group is indicated by #### p< 0.0001, ### p < 0.0002, ##p < 0.0021, # p< 0.05.

C. Effect TBI on mechanical allodynia and hyperalgesia:

1. Mechanical allodynia:

The development of mechanical allodynia in rats after TBI was assessed using an innocuous stimulus to activate mechanoreceptors i.e. Von Frey filament with a bending force of 2g. No significant change in the frequency of paw withdrawal between control and TBI groups was observed.

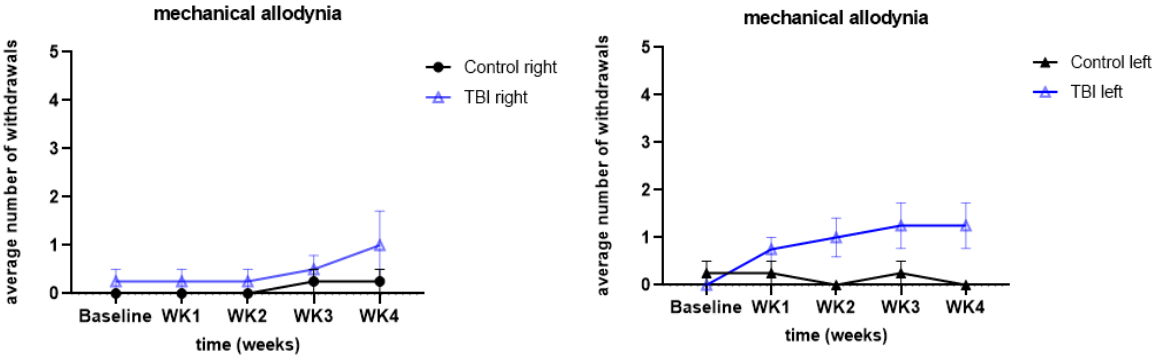

Figure 9 :Effect of TBI on mechanical allodynia

Paw withdrawal frequency was measured in the right (figure 9a) and left (figure 9b) paws of TBI and control rats before surgery (baseline) at weeks 1, 2, 3 and 4 post-surgery. Data were recorded and presented as mean ± SEM. Significant differences between TBI and control rats are indicated by ∗∗∗∗ p< 0.0001, ∗∗∗ p < 0.0002, ∗∗ p < 0.0021, ∗ p< 0.05 and the significant difference from baseline within the same group is indicated by #### p< 0.0001, ### p < 0.0002, ##p < 0.0021, # p< 0.05.

2. Mechanical hyperalgesia:

The effect of TBI on the development of mechanical hyperalgesia was also assessed by measuring paw withdrawal frequency to mechanical stimulation using Von Fry filaments with a bending force of 15g. At week 2 post-surgery, TBI group revealed a significant increase in the number of paw withdrawal compared to the control ($p < 0.5$). Moreover, the left hind paw ($p < 0.0021$) showed a more pronounced effect compared to the right side ($p < 0.05$) and displayed a significant change from baseline within the same group at weeks 2, 3 and 4 ($p<0.05$)

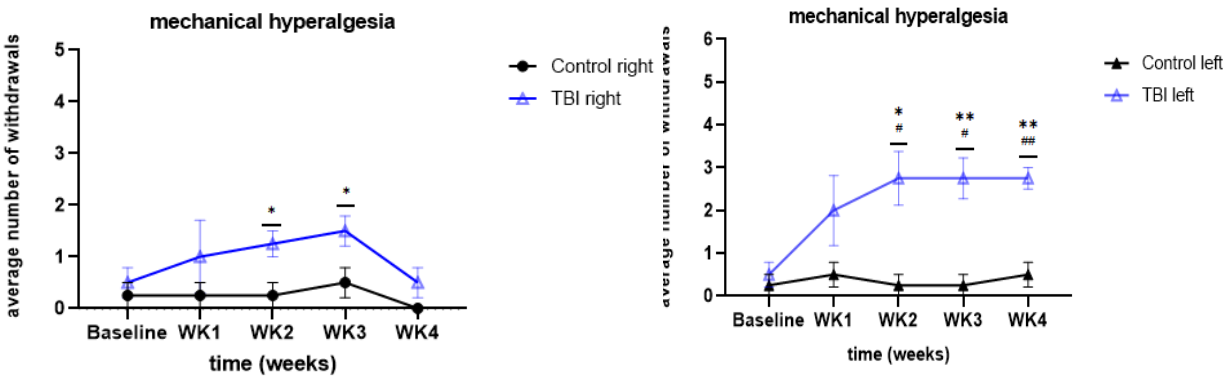

Figure 10: Effect of TBI on mechanical hyperalgesia

Paw withdrawal frequency was measured in the right (figure 10a) and left (figure 10b) paws of TBI and control rats, prior to surgery (baseline) and at weeks 1, 2, 3 and 4 post-surgery. Data were recorded and presented as mean ± SEM. Significant differences between TBI and control rats are indicated by ∗∗∗∗ $p< 0.0001$, ∗∗∗ $p <$

0.0002, ∗∗ p < 0.0021, ∗ p< 0.05 and the significant difference from baseline within the same group is indicated by #### p< 0.0001, ### p < 0.0002, ##p < 0.0021, # p< 0.05.

D. Effect of TBI on T-maze:

The effect of TBI on the cognitive abilities of rats was assessed using the T-maze test by observing and recording the animals' choices and comparing results between TBI and sham rats. As shown in figure 11, TBI rats showed significant decreases in their performance compared to control rats at weeks 2 and 3 post-surgery (p< 0.05).

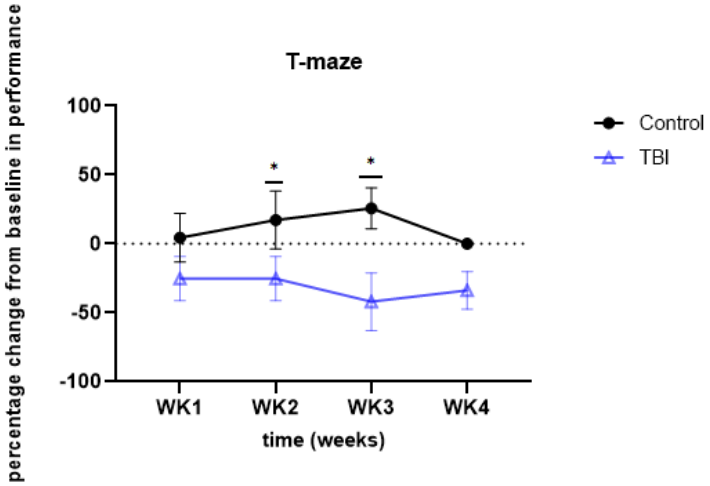

Figure 11 : Effect of TBI on T-maze

Cognitive performance was observed in TBI and sham rats before surgery (baseline) and at weeks 1, 2, 3 and 4 post-surgery. Data were expressed as absolute percentage change from baseline and presented as mean ± SEM. Significant differences between TBI and control rats are indicated by ∗∗∗∗ p< 0.0001, ∗∗∗ p < 0.0002, ∗∗ p <

0.0021, ∗ p< 0.05 and the significant difference from baseline within the same group is indicated by #### p< 0.0001, ### p < 0.0002, ##p < 0.0021, # p< 0.05.

E. Effect of TBI on locomotor activity:

The effect of TBI on the locomotor activity of rats was assessed using the ladder test by observing and recording the animals' performance and comparing results between TBI and sham rats. As shown in figure 12, TBI rats showed statistically significant decreases in their performance compared to control rats at weeks 2 and 4 post-surgery (p< 0.05).

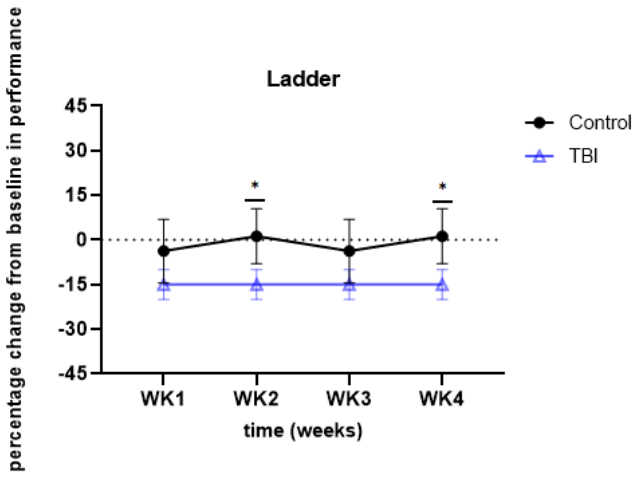

Figure 12 :Effect of TBI on Ladder test

Ladder climbing was evaluated in TBI and sham rats before surgery (baseline) and at weeks 1, 2, 3 and 4 post-surgery. Data were expressed as absolute percentage change from baseline and presented as mean ± SEM. Significant differences between TBI and control rats are indicated by ∗∗∗∗ p< 0.0001, ∗∗∗ p < 0.0002, ∗∗ p < 0.0021, ∗

p< 0.05 and the significant difference from baseline within the same group is indicated by #### p< 0.0001, ### p < 0.0002, ##p < 0.0021, # p< 0.05.

F. Effect of TBI on neural tissue integrity:

LDH assay was used to evaluate the presence of tissue and cell damage. LDH activity was calculated for both control and TBI samples, and as shown in figure 13, TBI rats showed a significant increase in LDH activity compared to control rats. (p< 0.05)

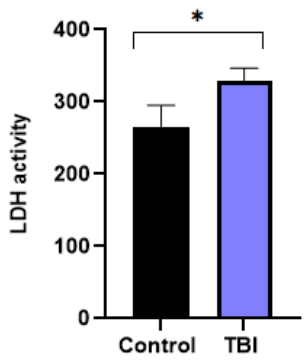

Figure 13 : Effect of TBI on LDH activity

LDH activity was measured and calculated in TBI and sham rats' brain tissue samples (hippocampus). Data were expressed and presented as mean LDH activity ± SEM. Significant differences between TBI and control rats are indicated by ∗∗∗∗ p< 0.0001, ∗∗∗ p < 0.0002, ∗∗ p < 0.0021, ∗ p< 0.05 and the significant difference from baseline within the same group is indicated by #### p< 0.0001, ### p < 0.0002, ##p < 0.0021, # p< 0.05.

G. Pathogreen staining:

Neurodegeneration was assessed by observing pathogreen positive cells. Neurodegeneration at a location close to the injury site, i.e., the sensory cortex and

hippocampus, was significantly noted in the injured group, and it significantly persisted till the 4th week, compared to minimal neurodegeneration in the sham rats.

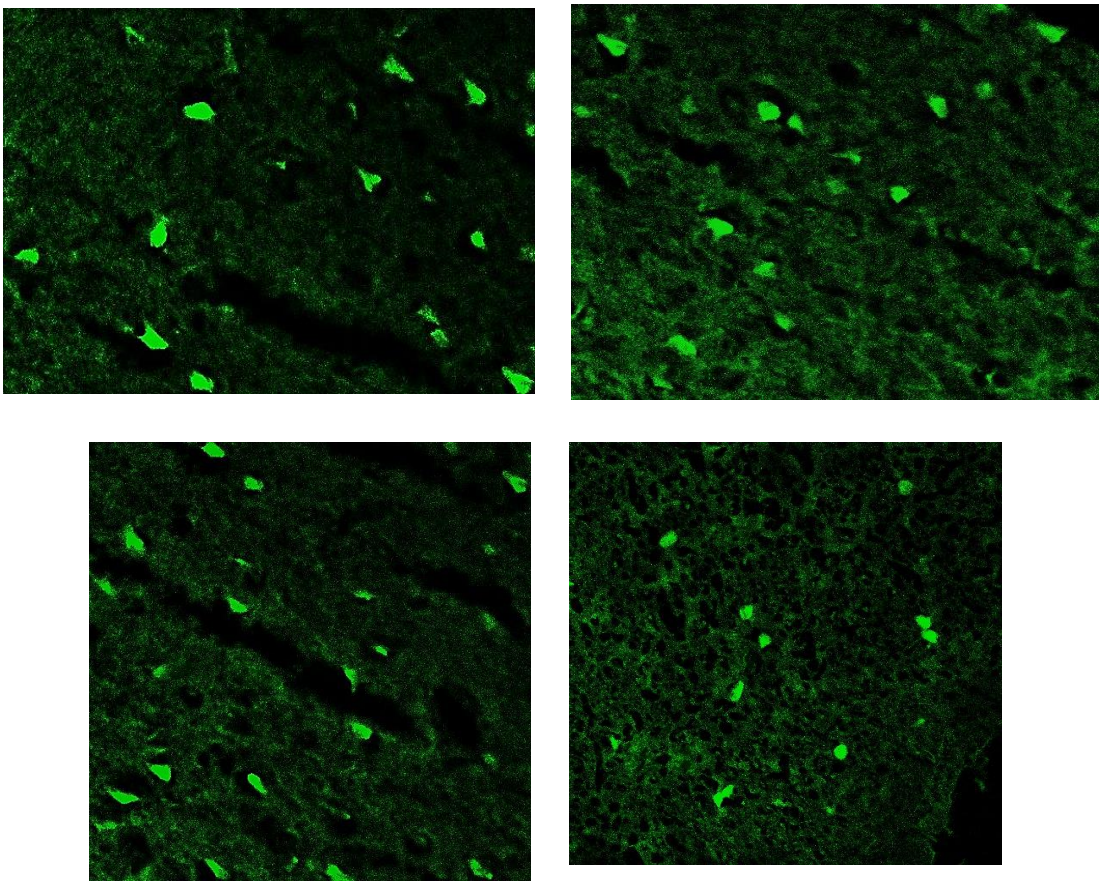

Figure 14 : Cortical Neurodegeneration

Images showing Pathogreen positive cells in the cortex of TBI rats. Apparent neurodegeneration is seen one-month post TBI. Stained images were taken at 40x using a confocal microscope, scale bar 50µm.

CHAPTER IV

DISCUSSION

Traumatic brain injuries have been shown to be associated with developing temporary or permanent neurodegenerative, neurological or neuropsychiatric problems. (98, 99). Moderate or severe TBIs frequently result in permanent neurocognitive and neurobehavioral impairments that include personality changes, problems regulating one's emotions, apathy, disinhibition and loss of awareness. From a neurocognitive perspective, impairments are most notable in attention, concentration, working memory, speed of processing, and memory. (100-105) The neurological and neuropsychiatric complications associated with TBI include: movement disorders (such as tremors and dystonias), balance problems, visual impairments, depression, anxiety disorders, fatigue, sleep disturbance, headaches, motor impairments, personality changes, and decreased motivation. (105-108)

From here, it is essential to explore the neurological, behavioral and molecular outcomes following concussion in animal models in order to understand the pathophysiology and consequences of such injury. In our study, the weight drop model of mild traumatic brain injury was used to test the consequences of such injury at different time points in order to achieve a longitudinal assessment of the effects of TBI over time. This TBI model was compared to sham controls. It is noteworthy to mention that the weight drop model on closed head has been proven to be one of the most reproducible injury models that represents clinical TBI cases. (109)

Our main observations were: (1) Pain-related changes (mechanical nociceptive sensitization) in TBI rats, (2) cognitive performance deficits post-TBI, (3) motor incoordination post-surgery, (4) and neurodegeneration after TBI.

Our findings are in line with other studies that reported pain and motor coordination deficits, 7 to 10 days post-injury, using different animal models of TBI (110). Several studies demonstrated that impaired rotarod performance start to show as early as 24 hours and can persist for a long period after TBI (14-18). Notably, these motor and cognitive deficits are similar to what happens after TBI in human subjects (111, 116).

Further evidence to support our findings comes from clinical studies in which reports of increased sensitivity to painful stimuli, ongoing chronic pain and thermal hypersensitivity was noted in patients for at least 4 weeks after blast injury (117).

With respect to cognitive functions following TBI, our results were in agreement with numerous pre-clinical studies that demonstrated acute cognitive and spatial memory impairments after single TBI maze (113, 116-120). Such impairments can appear 2 to 12 weeks after the last injury (122). In addition, these studies showed that rats displayed mechanical hyperalgesia as well (113, 117, 118, 122-125). In this context, it is noteworthy to mention that the improvement shown in the T-maze performance in our study was in line with improvements mentioned in other studies where TBI animals showed cognitive and spatial learning improvements throughout the testing days (126, 127). Despite the consistency of TBI symptoms, there is some variation in the course of events. This is primarily due to the difference in the extent and severity of injury. Some studies showed no mechanical allodynia and hyperalgesia in

subjects tested 3–4 weeks after blast-injury, where others showed pain sensitivity immediately after (117).

Closed head injury, even under mild conditions, can cause contusion of neural tissue and diffuse axonal injury that can spread to deep brain structures. In the present study, this was demonstrated by the presence of abundant pathogreen positive cells in the cortex and hippocampus of TBI rats. Neurodegeneration was also confirmed by performing LDH assay. Results showed that LDH significantly increased in the hippocampus of TBI rats compared to the sham group. These findings are consistent with those of other studies, which revealed evidence of neurodegeneration after TBI (beyond 7 days) (128-132). This is possibly due to the spillover of excitatory neurotransmitters that occur at the site of injury causing excitoxicity and further neural degeneration. By interrupting the axoplasmic transport, diffuse axonal injury can cause damage to hippocampal neurons and cells located in deep brain structures, as evident in our LDH results. Oxidative stress and inflammatory mediators have shown to play a significant role in the secondary events leading to neuronal damage. Given that Reactive oxygen species (ROS)-mediated axonal degeneration is primarily due to increased extracellular Ca^{2+}, many research studies suggested the involvement of calcium channels in axonal damage by showing that calcium channel blockers can alleviate the secondary damage resulting from the injury. Also, the use of antioxidants had proved effective when given within the neuroprotective time window. All the mechanisms described above may help scientists in finding the best therapeutic approach that can prevent or mitigate the spread of neural damage induced by TBI. However, future research is needed to determine the key molecules that can switch off

hyperexcitability at the site of injury and prevent the damage from spreading and precipitating neurodegenerative diseases.

CHAPTER V

CONCLUSION

In this study, we assessed the effects of closed head injury, using the modified weight drop model, on the sensorimotor and cognitive functions in rats. This was done in one-month time interval for each rat in order to achieve a longitudinal evaluation of the effects of TBI. Our findings provided evidence of increased pain sensitivity on the side contralateral to the injury, with an onset of 1week post TBI and a duration of 28 days. In addition, decreased exploratory behavior and motor coordination followed a similar course. Finally, histological analysis of neural tissue obtained from the somatosensory cortex and hippocampus revealed cellular damage and increased LDH activity. Our findings provide further evidence that a closed head injury, irrespective of its severity, precipitates long-term physical and cognitive changes. In light of current findings, a new, more strategic approach is needed for the treatment of TBI and one that should take into consideration the rapid and long term changes that occur, not only at the site of impact but also in subcortical structures, in which micro damage can go unnoticed.

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