NEUROPHARMACOLOGICAL EFFECTS OF UMBELLIFERONE IN TRAUMATIC BRAIN INJURY MOUSE MODEL

Masters of Science (MS)

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A thesis submitted in partial fulfillment of the requirement for the degree of

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MS Thesis Work

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Dedicated To

My Parents

ACKNOWLEDGEMENTS

All praises for the **Almighty Allah**, the Most Merciful and the Most Gracious, who is the sole provider of all skills and means and without whom we cannot excel in any field of life.

The first people I would extend my deepest thanks to are my parents. It was their immense support, love, trust and faith in me that I have reached these heights. Their support has been the greatest gift I have ever received from anyone. Nothing can be enough to thank them for bestowing me with so much love and trust. I would like to express my deepest gratitude and utmost thanks to my supervisor **Dr. Touqeer Ahmed** for his sheer guidance, constructive criticism and constant support. I have learned a lot under his supervision. With great privilege I extend my heartfelt gratitude to the GEC members, **Dr Saadia Zahid**, **Dr Jawad Hassan** and **Dr Arif-Ullah Khan** for their support. I would like to extend my heartfelt gratitude and thanks to **Dr. Tahseen Alam**, Incharge Animal House, ASAB, for his teaching, guidance and immense support while working with animal model. This project would not have been possible without his help. I would like to thank Maryam Zahoor for her support and her constant drive to push me towards excellence. Special thanks to my lab partners, Momina Shahid and Nafisa Ishaq for their assistance, management and guidance. I would especially like to thank Hina Khan for standing by me when needed the most, for teaching me statistical analysis and helping me with the lab work and write-up whenever needed. Heartfelt thanks to my seniors in the neurobiology

laboratory, Syeda Mahpara Farhat, Habiba Rasheed, Ghazala Iqbal, Sanila Amber, Sumera Khan and Sana Javed, for guiding me devotedly whenever I needed their help and providing such a friendly environment. Special thanks to my senior, Ameema Tariq for being available every time and helping me with the minor of issues. Thanks to Maheen Nawaz and Waqar Iqbal, my juniors, for providing me assistance with my lab work. I would like to thank my friends Ayesha Gauhar, Wardah Akram, Rabail Durrani, Aqsa Ikram, Fatima-tuz-Zahra and Faiza Saleem for always being there for me and helping me with whatever they could offer. In the end, I would like to thank my best friend, my confidante Nosheen Nasir and my little sister, Fatima Hashim, for being my lifelines. Without their undying love, support, care and trust, I would have never reached this stage.

May Allah Almighty bless us all.

Thank you all.

Rabia Hashim

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ABSTRACT

Traumatic Brain Injury (TBI) accounts to be a major concern of mortality worldwide. Patients tend to face various cognitive, emotional and behavioral deficits. These after effects can be long lasting and prove to be devastating for the patient as well his loved ones. Till date, there are very limited therapeutic options available for treatment of traumatic brain injury. Umbelliferone, a coumarin from the Umbelliferae family has been reported to exhibited neuroprotective effects. The neuroprotective effects of Umbelliferone (5mg/kg and 30mg/kg) on an induced mouse model of TBI were evaluated in this study. A mouse model was developed where a weight of 100g was dropped from a height of 4cm three times on an exposed skull. The sensory and motor deficits were evaluated by Neurological severity score (NSS). Behavioral testing analyzed the behaviors. Fear conditioning and fear context assessed the fear learning and memory. Novel object test assesses the recognition memory. Anxiety was analyzed by the light and dark test and elevated plus maze test. The exploratory behaviour was determined by the open field test. Memory and learning were assessed by Morris water maze test. Elevated maze test and light dark test showed mild decrease in anxiety in Umbelliferone treated (5mg/kg and 30mg/kg) groups. Neurological severity scores showed considerable improvement in the Umbelliferone treated groups TBI+Umbelliferone(5mg/kg). Although no significant difference was observed in majority of the other behavioural tests, a trend was witnessed where Umbelliferone treated groups showed improvement. We can conclude that Umbelliferone might have neuroprotective effects on Trauma patients. Further research has to be carried out in order to study the perfect dosage to be administered for promising results.

CHAPTER 1: INTRODUCTION

TBI is defined as injury due to a severe external mechanical force which can be due to a jolt, accident or a simple push. The Glasgow Coma Scale (GCS) assesses the state of consciousness post injury (Udekwu et al., 2004). GCS assesses the weakened conscious levels in response to a stimulus. A GCS score of 14-15, 9-13 and 3-8 classifies TBI to be mild, moderate or severe respectively. Every year approximately 2 million lives are affected by Traumatic brain injury (Heegaard and Biros, 2007). Around 1.5 million people who endure a TBI, do not pay a visit to the emergency department. Annually there are around 50,000 deaths accounted to TBI (Bigler et al., 2009). In the United States alone, estimated 5.3 million individuals endure changing grades of disability post TBI. The incidence rates of males suffering from TBI are higher than females (Dutton and McCunn, 2003). This can be because of the fact that motorcyclists don't wear helmets often, there is no check on the safety requirements of auto passengers and drugs/alcohol intake is frequent. Infants up to 4years, adolescents up to the age of 15 to 19 years and members of the older generation are at high risk to sustain TBI (Dutton and McCunn, 2003).

TBI can occur due to falls, accidents, attacks, severe fights and assaults. In war zone areas, TBI is caused due to blasts. Sports personnel suffer concussions and different forms of TBI as well (Finnie and Blumbergs, 2002). Patients suffering from traumatic brain injury can face different physical, perceptive, communicative, and emotional consequences (Ghajar, 2000). Concussions, which come under the category of mild TBI, can lead to life-long cognitive disabilities. Based on the impact of injury, the level of cognitive dysfunction is assessed. Memory impairment, irritability, dementia, aphasia,

anxiety, depression, aggression are the most observed changes (Greenaway et al., 2006). Patients also tend to start substance abuse and alcohol dependence (Weiner and Lipton, 2009).

There is observed a significant decline in the social circle of TBI patients which leads to loneliness. There is also difficulty in making new friends even while being under the care of their immediate family (Nolan, 2005). A decline in participating in leisure activities has been noted which indicate high levels of anxiety, depression, dejection (Maas et al., 2008). TBI is hence aptly referred to as one of the most damaging injuries.

1.1 Classifications of Traumatic brain injury

The GCS scale assesses the severity by checking three modules – opening of the eye, motor response and verbal responses. The GCS scale is one of the most followed for the management and prognosis of TBI, but it fails to give precise knowledge about the responsible pathophysiologic mechanisms for neurological deficits (Escobedo et al., 2013). Studies are being conducted to pursue enhanced methods to assess the severity of TBI.

Post-traumatic amnesia (PTA) is another vital index used. PTA is the time duration from injury till orientation of the patient, and his ability to create and elicit memories. Moderate TBI has a PTA score of 1-24 hours (Escobedo et al., 2013). Mild TBI includes the following criteria: A PTA score of 24 hours or lesser and losing consciousness within 30 minutes post injury (Maas et al., 2008). Another scale designed to direct the classification of TBI severity is the Mayo Classification System. It includes possible TBI, probable MTBI , and moderate-severe TBI (Malec et al., 2007). The criteria used to

diagnose each include losing consciousness, a fracture in the skull, amnesia and signs of neuroradiological aberrations such as haematomas, hemorrhagic and cerebral contusion (Malec et al., 2007). Paroxysmal sympathetic hyperactivity (PSH) is a syndrome seen frequently in patients with severe TBI. It develops because of dysfunctional autonomic regulation after acquired brain injury and is often also referred to as central dysautonomia, sympathetic storming, paroxysmal autonomic instability with dystonia, and diencephalic seizures. This syndrome includes simultaneous motor and sympathetic activity, which is transient and episodic, in response to obnoxious stimuli. Up to 30% of patients may develop this syndrome after severe TBI (Povlishock, 1993, Greve and Zink, 2009).

A TBI is moderate-severe TBI if the patient dies due to his TBI, for 30 minutes or more he loses consciousness for more than 30 minutes, the PTA score is 24 hours or more, or the GCS score is <13 along with any signs of any neurological injury (Saatman et al., 2008). A possible TBI is identified if the patient faces blurred vision, puzzlement, dizziness, headache, or nausea (Maas et al., 2008). In modern times, clinical methods to assess TBI severity are limited. Marshall and colleagues proposed the need for a new mechanism to assess the level of TBI severity. They proposed focusing on the existence of a mass lesion and by differentiating diffuse injuries by rising or decreasing levels of intracranial pressure (Maas et al., 2008). Though this method is helpful it still lacks the ability to find out the lesions occurring at microscopic level. One of the latest approaches proposed in modern times to classify TBI patients is by prognotic risk. There are available well studied and validated models to assist this approach. It will help in

providing better delivery in health care and help in the clinical trial studies (Saatman et al., 2008).

1.1.1 TBI due to injury mechanism

TBI is further classified into 3 injury mechanisms—blunt, penetrating, or blast (Williamson et al., 1996). Post jolt or hit to the head, the energy transferred to the brain tissue causes the injury. The following formula summarizes this energy transfer. KE = M/2 × V2, where KE indicates kinetic energy; M, mass; and V, velocity (Nolan, 2005). F=ma defines the force involved where F is force; M, mass; and A, acceleration (Nolan, 2005). This transfer of energy creates the pressure and forces that lead to the injury. This injury, also known as primary injury causes various cellular level physiological responses. These responses if not managed lead to secondary injury leading to neuronal cell death and cerebral edema. Secondary injury should be prevented at all costs to control the devastating effects of TBI. To reduce the possibility of progression of secondary injury, hypotension, hypoglycemia, hypoxemia, anemia, and hyperthermia should be managed and controlled (Nolan, 2005).

The most common cause of TBI is Blunt trauma which occurs due to accidents (vehicle crashes and bicycle motor vehicle and bicycle bumps), sport activities, falls and attacks. 50% of the TBIs occur due to the motor vehicles crashes. The mass, acceleration, duration, direction and rate assess the force in a motor vehicle crash. 75% of all deaths occur in motorcycle crashes (Williamson, Scott et al. 1996). This rate can be decreased if the motorcyclists wear a helmet. Penetrating TBI is caused when any razor –sharp or damp object penetrates the skull and scalp, thereby exposing and entering the brain. How

deep and impactful is the injury can be determined by the entry site, force, angle and intensity of entry. Gunshot wounds are an example of penetrating TBI. The size and velocity of the bullet determine the degree of injury (Morales et al., 2005). Blunt and penetrating forces combined result in Blast TBI. Since TBI is more of an issue these days, the nursing care practitioner should be well aware of dealing such cases with utmost care (Morales et al., 2005).

Diffuse injury and focal injury characterize primary injury. Injuries occurring in a confined area are defined as focal brain injuries. Diffuse brain injuries involve a large widespread area (Greve and Zink, 2009). The types of focal brain injuries are subarachnoid, and intracranial hemorrhages (ICHs), epidural hematomas, cortical contusions, and subdural hematomas. Diffuse injury includes diffuse axonal injury and cerebral concussion (Heegaard and Biros, 2007).

1.2. Pathophysiology of TBI

Post injury, closed head injury has the ability to injure the head, scalp, face, neck (stroke soft tissue damage) along with different psychological dysfunctions. As a result of neural injury there are initial complaints of weak concentration levels, absent-mindedness and sleep-wake instabilities (Greve and Zink, 2009). Patients also complain of neck pain which happens because of soft tissue injury of the cervical. Patients also complain of headaches and dizziness, which may be a result of mixture of reasons. Psychological changes in behaviour like anxiety, mood swings, irritatedness, depression occur due to neural injury, pain and or various psychological entities (Alexander, 1995, Dutton and McCunn, 2003, Maas et al., 2008).

The damage caused at the macroscopic level includes white-matter shearing, diffuse swelling, focal contusions and haematomas(Saatman et al., 2008). Stearic conformational changes in proteins, microporation of membranes and leakage of ion channels are the neurotraumatic events that take place at the cellular level. There is also possibility of shearing of blood vessels which lead to minor haemorrhages (Maas et al., 2008). Epidural, subdural hematomas and ICH are the types of haemorrhages that occur due to the focal injuries. Bruises that occur at the cortical tissues are known as Cerebral Contusions (Alexander, 1995). There is observed occurrence of cerebral edema in the brain tissues due to which intracranial pressure (ICP) increases. Elevated intracranial pressure levels result in diminishing levels of blood flow causing ischemia pave the path for secondary injury (Alexander, 1995). Numerous minor lesions in white matter tracts characterize diffuse axonal injury. Patients diagnosed with diffuse axonal injury are in deep coma and exhibit low levels of ICP. Approximately 25-35% of severe TBI patients and 5-10% of moderate TBI patients suffer from traumatic intracranial haematomas (Nolan, 2005). Cerebral concussions can be mild or moderate. In mild cerebral concussions there is neurological dysfunction with the absence of complaints of loss of consciousness or memory. But in moderate concussions there are complaints of loss of memory and consciousness. Most of the energy in still crush injuries and central blows gets absorbed by the skull due to which the brain damage remains external (Hickey, 2013). The pathological mechanism of blast injuries has not yet been understood but they are usually outlined by subarachnoid haemorrhage, acute brain swelling, along with evident angiospasm (Pangilinan).

Secondary mechanisms don't develop overnight. They progress across different time intervals from hours to days. Inflammatory responses, mitochondrial dysfunction, gene activation, calcium- mediated damage, free-radical generation, neurotransmitter release are some of the mechanisms initiated post brain injury (Heegaard and Biros, 2007). Being a prominent provider to brain damage and cell death in TBI, the excitatory neurotransmitter glutamate worsens leakage of ion channels, aggravates swelling of astrocytes and glial cells, causes edema and high levels of ICP (Greve and Zink, 2009, Hawthorne and Piper, 2014). On activation of the NMDA and AMPA receptors, there is normal calcium influx. In TBI, both the receptors observe a high calcium influx and hyper excitability of neurons, high levels of ionic currents, and elevated levels of intracellular calcium. The increased levels of calcium lead to many pathological processes including axonal cessation. Injured axons and damage lead to strong later outcomes of TBI (Greve and Zink, 2009). There is also observed release of caspases and other proapoptic cells with quick gene activation which lead to neuronal apoptosis. Microglia and other neuronal cells release cytokines as a quick inflammatory response within a few hours thereby breaking the blood- brain barrier and initiate apoptosis. Apoptosis and necrosis get triggered also due to the energy failure caused by mitochondrial dysfunction (Crockard et al., 2000). According to Mussack et al., Interleukin(IL-8) and brain injury biochemical marker (S-100B) elevate as a consequence to any acute injury (Mussack et al., 2002). IL-6 is positively related to the clinical outcomes in a way that the higher the peak in cerebrospinal fluid and serum, the better will be the prognosis (Singhal et al., 2002).The inflammatory cascade following acute

injuries can act as a boon or a bane. It can be either a healing mechanism or can be a nuisance by exhibiting an exaggerated inflammatory reaction.

It has been observed that there is considerable improvement 3 months post injury (Clark et al., 2008). But patients who have recovered quickly and are much better still complain of periodic occurrences of insomnia, alcohol abuse, work related stress, general depression. One of the crucial aspects in advancement of secondary injury is the disturbance of calcium homeostasis. Surplus amounts of calcium cause disturbance in protein phosphorylation, microtubule composition, along with various other enzyme activities (Hayes and Dixon, 1994).

Intracellular and Interstitial are the two types of edema defined. They occur post TBI and are responsible in the development of secondary injury. It has been studied that 24 to 48 hours post injury, brain edema is at its worst. Vasogenic (Interstitial) edema occurs when the blood brain barrier dysfunctions. Levels of cellular osmolality when disturbed cause cytotoxic (Intracellular) edema (Crockard et al., 2000). In TBI cases where the TBI is nonpenetrating, the period post injury is outlined by extensive damage of the brain tissue but what remains intact is the organization of glial cells, neurons and axons (Gentry, 1994).

1.3 Outcomes of TBI

Behavioural deficits that occur due to TBI may persevere for ages without any improvement. The neurobehavioral hallmarks of TBI are Post traumatic amnesia and impaired consciousness (Lippert-Grüner et al., 2006). Post injury there are reports of around 65% patients complaining of various cognitive deficits. Planning, judgement, attention, learning and memory, emotional attachment, thought process, reasoning, motivation and impulsivity of the individual are deeply affected and the individual finds it difficult to perform daily tasks (Lippert-Grüner et al., 2006).

In cases of severe TBI, there is also observed motor impairment, psychological disturbance and various sensory deficits. Patients find it difficult to walk and also tend to lose their response to various sensory mechanisms. But these effects are directly proportional to the impact's severity (HAMM et al., 1992). The reason why processing speed gets impaired post injury is because alternate pathways take up the process due to which response time increases. Also reduced white matter in the TBI brain leads to less proficient neural transmission (Weiner and Lipton, 2009). Diffuse axonal Injury patients are at a higher probability of developing coma and there are few chances that they might avert back to their normal level (Povlishock, 1993). Studies are still being conducted to discover the possible chances of recovery (Hallbergson et al., 2003).

According to studies, the intra cranial injuries that patients suffer from are acute subdural haematoma (42%), acute epidural haematoma (22%), contusion (80%), traumatic subarachnoid haemorrhage (29%), skull-base fracture (34%) skull fractures (22%), and brain stem contusion(18%) (Williamson et al., 1996, Nolan, 2005). It is essential to

provide psychological therapies along with love, attention and care from family and friends to improve the life of such patients post injury.

1.4 Treatment and Management of TBI

In the emergency department, the first and foremost priority should be to maintain and manage the basic ABC's- Airway, Breathing and Circulation. There would be no point trying to address the patient with the latest technologies available if these guidelines are not implemented first. The basic goal of the management should be to eliminate all possible chances of the occurrence of secondary injury. This can be done by counteracting hypoxemia with a targeted PaO_2 >60 mmHg or O_2 saturation >93% also by recognizing the intracranial mass lesions and managing the systolic blood pressure >90mmHg. The first and foremost priority of the emergency department should be to assess and manage the airway. When the patient is unable to ventilate, or has distended and fixed pupils, is behaving affectedly, or shows signs of hemiparesis, he should be immediately subjected to intubation.

The intracranial pressure (ICP) increases as the cerebral edema worsens. Due to high levels of intracranial pressure, the levels of cerebral perfusion pressure (CPP), reduces. The auto regulatory mechanisms of the brain get disturbed and the brain becomes prone to disturbed blood pressure levels and ischemia. Report report that the cerebral perfusion pressure levels should be regulated between 60-80mmHg (Mangat, 2012). Lisa et al suggest etomidate for inducing general anaesthesia, rather than propofol and barbiturates as they have mild side effects such as hypotension. It has been reported to be rapid and stable with a brief half-life, along with effective neuroprotective effects (Escobedo et al.,

2013). They also recommend the use of fentanyl and benzodiazepines for effective sedation. Midazolam, Morphine, Dexmedetomidine, Ketamine are some of the other sedatives used to maintain the ICP levels post injury (El Fadl and O'Phelan). Mohammed et al summarize that despite various researches carried out to eliminate the technique of ICP monitoring to treat TBI patients, there is very little evidence to do so. Hence this method has been added to the guidelines of Brain Trauma Foundation (El Fadl and O'Phelan, Carney et al., 2017). The following guidelines have been outlined for managing severe TBI by the brain trauma foundation:

1. Managing completion of fluid resuscitation.
2. Evade hypotension and hypoxia by maintaining arterial pressure of >90mmHg
3. Patients with GCS score of 3-8 need to be monitored for ICP. Perform CT
4. In case of hyperventilation, avoid prophylactic hyperventilation during first 24h.($PaCO_2 \leq 35$ mmHg)
5. High doses of sedatives like Mannitol and barbituarates to be administered to patients with increased ICP levels.

Hypotension should be regulated at all costs. Patients with hypotension should be given isotonic levels to maintain their blood pressure levels as low levels increase the mortality rate by double (Carney et al., 2017). Hyperosmolar therapy uses hypertonic saline and mannitol treat TBI patients with high ICP (Wakai et al., 2013). Post early management that involves maintaining ICP and CPP levels and minor surgeries, the patient is moved to ICU in case of severe TBI. He is kept under constant monitoring to control ICP levels and manage any further secondary insults (Dutton and McCunn, 2003). In case of mild

and moderate TBI there are psychological techniques and therapies available that can be used to help the patients recover from cognitive deficits (McDonald et al., 2002).

Numerous studies have been conducted to establish a standard treatment for TBI. In September 2016 a study concluded that decompressive hemicraniectomy on patients with severe TBI yielded low mortality, decent recovery and normal disability. Roof et al, researched that Progesterone attenuated edema treatment and can prove to be a therapeutic treatment for treating edema (Roof et al., 1992). It is understood from various studies that administration of glucocorticoids is not a considerable option (Escobedo et al., 2013, Kochanek et al., 2013). Amyloid Precursor Protein (APP) secretases when blocked either b- or c-secretase, have been studied to improve the motor and cognitive deficits in post TBI patients. They also have the potential to reduce the cellular loss faced post TBI (Loane et al., 2009). The neuroprotective efficacy of Rapamycin, has been studied and Elrich and others propose that Rapamycin can be used as a novel treatment (Erlich et al., 2007). Excellent outcomes of managing TBI patients can only be attained by focusing on managing secondary injury and keeping in check the respiratory activities by mechanical ventilation.

1.5 TBI Model

Various methods are present in literature for trauma induction on rodents. The different models used are explained below in a flow chart.

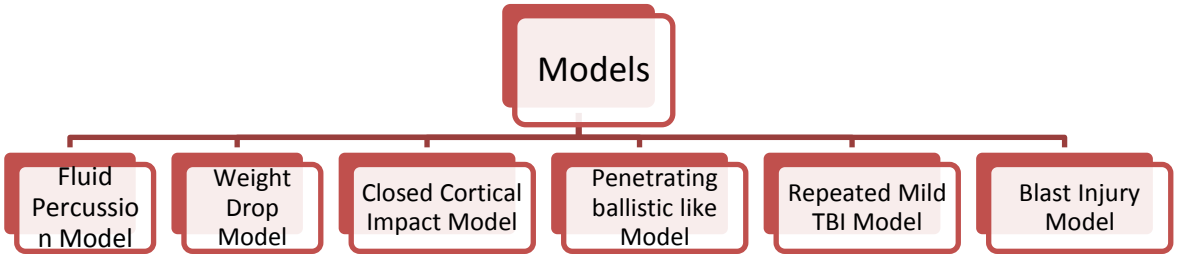


Figure1.1 Different models of TBI

These models can easily be modified to induce mild or severe TBI (Morales et al., 2005). Fluid percussion injury can be inducted by saline injection via a craniotomy into the epidural area causing the brain's movement inside (Morales et al., 2005). The craniotomy is either about the midline amidst the lambda and bregma, or across the parietal bone amidst the lambda and bregma (JOHNSON et al., 2015). This displaces and deforms the brain tissue and the severity of injury is evaluated by how strong the pressure pulse is (JOHNSON et al., 2015). Fluid percussion model is used when it is desired to replicate the pathophysiology of TBI involving intracranial haemorrhages, edema and damage of the grey matter. The height of the pendulum in fluid percussion model is the only parameter that can be adjusted here (Xiong et al., 2013).

By rapidly compressing the brain tissue, closed cortical impact (CCI) injury can be produced. This can be achieved through an air-driven piston by a craniotomy. This model is best when blood–brain barrier (BBB) dysfunction, chronic subdural haematoma,

cortical tissue damage, axonal injury, concussion and comas are to mimicked (Xiong et al., 2013). A contusion core is generated as a result of death of the destroyed cells in the compressed tissues. The interval, velocity and intensity of impact can be easily monitored in this model making it advantageous over other models. Post CCI injury, there have been reported cognitive deficits and emotional deficits (JOHNSON et al., 2015).

A weight from a calculated height is released directly on the revealed cranium to induce weight drop model. The height from which the weight falls can be adjusted to regulate different levels of severity (Morales et al., 2005). There are different weight drop models used these days. The weight-drop model devised by Feeney delivers the weight on the dura mater via a craniotomy. This leads to the formation of a cerebral contusion. Shohami's model delivers the weight drop laterally on the skull and the head is positioned on a firm place (Morales et al., 2005)The discussed brain injury models easily are modified to attain injuries of various severities with varying pathophysiology.

1.6 Therapeutic potential of Umbelliferone in TBI

Coumarins are organic compounds found in various plants. They belong to the benzopyrone class (1-benzopyran-2-one). Coumarins exhibit various antioxidant, antiviral, antidiabetic, antimicrobial, antiinflammatory, and enzyme inhibitory activity (Pillai et al., 1999). Coumarins are extensively distributed in nature. They were first isolated in 1820. Major herbs such as angelica, alexanders, parsley, celery, giant hogweed, and cumin come under the coumarin family. These coumarins are mostly oxygenated at C-7 and one of its first derivatives is Umbelliferone (Pan et al., 2015), extracted first from the Apiaceae family. It is also known as 7-hydroxycoumarin, and is

abundantly found in root, plants and fruits (Mazimba, 2017). One of the main features of the Umbelliferae family is its inflorescence. For various other highly oxygenated derivatives of coumarins, Umbelliferone is known to be the parent compound.

Umbelliferone is yellowish white in colour and is odorless. It is slightly soluble in water but dissolves well in ethanol, methanol and other oxygenated solvents. Its chemical formula is $C_9H_6O_3$ and the melting point is 224-227°C. The Pechmann condensation reaction is the most used method to synthesize Umbelliferone by using formyl acetic acid and resorcinol. It can also be biologically synthesized by the phenylpropanoid pathway (Oftense, 2017). It is difficult to isolate and purify Umbelliferone and its derivatives because of weak concentration levels and the dependence on season and region (Pan et al., 2015, Mazimba, 2017). In order to synthesize them chemically, it requires using dangerous agents, lengthy steps and severe reaction settings.

Studies prove that UMB is a pharmacologically active agent and it exhibits properties such as antioxidant (through consumption in food), antihyperglycaemic (Ramesh and Pugalendi, 2007) along with antitumor effects. According to Ramesh and Pugalendi ,UF reduces glucose levels and serves as an antioxidant (Ramesh and Pugalendi, 2007).It was studied that pure Umbelliferone exhibits antibacterial and antifungal properties. Kassim et al showed that Umbelliferone lowered the cellular and eosinophil numbers in asthmatic mice and hence had anti-asthmatic properties (Vasconcelos et al., 2009, Sim et al., 2015). Research has shown that Umbelliferone has neuroprotective, anti-nociceptive and anti-inflammatory effects (de Lima et al., 2011). According to a study conducted on animal models of pain by Flavia et al, Umbelliferone exhibited anti-nociceptive effect in correlation with inhibiting the migration of neutrophils, release of cytokines and

production of PGE2 (Leal et al., 2000). Xiangxing Wang et al reported that Umbelliferone improved cerebral ischemia reperfusion injury as Umbelliferone protects neuronal death by crossing the blood brain barrier (Wang et al., 2015). It also lessened the brain edema in injured mice models. They also reported oxidative stress inhibition and anti-inflammation by releasing cytokines (Wang et al., 2015). Subramaniam et al reported Umbelliferone's potential to prevent neuronal loss in Parkinson's disease. It successfully controlled neurotoxicity in their mice model of Parkinson's disease. The study concluded that Umbelliferone can maintain the Glutathione levels and control apoptosis (Subramaniam and Ellis, 2013). Umbelliferone is also known to have anti depression effects. It exhibited neuroprotective effects on a depression model by reversing the depressive behaviors and inhibited neuronal apoptosis (Qin et al., 2017). Umbelliferone has been known to be administered at various doses between 20-200mg/kg (Kanimozhi et al., 2012). The most frequent doses which have been proven to be effective are 30mg/kg, 60mg/kg and 90mg/kg (Vasconcelos et al., 2009, Kanimozhi et al., 2012, Subramaniam and Ellis, 2013).

Despite the fact that there is continuous research being carried out in search of better therapeutic strategies, evidently on the forefront, no therapy is currently available that benefits all TBI patients (Dutton and McCunn, 2003). Due to the shortcomings faced in therapeutic options of TBI and keeping in mind the various neuroprotective effects of Umbelliferone, it was hypothesized that Umbelliferone might have positive effects on the outcomes of TBI and can be a potential therapeutical option.

CHAPTER 2: MATERIALS AND METHODS

2.1 ETHICAL STATEMENT AND LETTER OF PERMISSION

The laboratory animal house of Atta-ur-Rahman School of Applied Biosciences, National University of Science and Technology (NUST) housed the animals under regulated environment. The Institutional Review Board at Atta-ur-Rahman School of Applied Biosciences, NUST approved this study (IRB-67). Each experiment was conducted in accordance with the principles established by the Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA (Council, 2010).

2.2 ANIMAL MODEL

40 male Balb/c mice provided by the Laboratory Animal House at ASAB, NUST were used. 8 plastic group cages (40 cm * 25 cm *15 cm), each cage containing 5 animals, were used to keep the animals under standard housing conditions with feed and water ad libitum and 12-hour light/dark cycle (Iqbal et al., 2016). The mice room had controlled temperature of 22 ± 2 C.

2.3 REAGENTS

Umbelliferone (UMB) was generously gifted by Dr Salman Khan (Department of Pharmacy, Quaid-i-Azam University, Islamabad, Pakistan) to be used for this current study. (Ketamine and Xylazine) anesthetics were purchased locally from the market.

Sodium Hydroxide, HCl, Sodium Acetate, DMSO, Hydroxylamine, Ferric Chloride (FeCl3), Acetylcholine (C3389) was purchased from Sigma Aldrich. Ethanol (Catalogue # 100983) was purchased from Merck.

2.4 STUDY DESIGN

2.4.1 Animal Groups

There were 5 groups and each group had total of 12 healthy animals of 3 to 5 months of age. Details of the groups are as follows (Figure 2.1 (a)):

a. **Sham group:** Only incision was given to this group.
b. **TBI group:** Trauma was given to this group.
c. **TBI + Ibuprofen (30 mg/kg/day):** Ibuprofen was given to animals orally. It was given in feed for 8 days to each group. 288 mg of ibuprofen (crushed form) was added to 576 g of feed. The feed was crushed and thorough mixing was ensured, sufficient amount of water was added to make medium size pellets of feed. Pellets were then air-dried, daily weighed amount of pellets were given to animals as feed.
d. **TBI + Umbelliferone (5mg/kg/day):** Stock solution of Umbelliferone was prepared in 1 ml of 100% DMSO which was further diluted with 1% DMSO. Daily 1µl stock was taken and diluted with 1881 µl of distilled water to make a total volume of 1900µl, out of which 142µl was injected in every mouse weighing (35g-40g) intraperitoneally.
e. **TBI + Umbelliferone (30mg/kg/day):** From already prepared stock solution 120 µl was taken and diluted with 2880µl of distilled water to make a total volume of

3000µl; further 214µl was injected in each mouse weighing(35g-40g) intraperitoneally.

2.4.2 Timeline

A 12 day protocol was planned for this study. The animals were subjected to trauma on Day 1 followed by Neurological Severity Score (NSS) after 4 hours. After 48 hours of trauma 3-4 mice were sacrificed to assess Edema levels. Ibuprofen and Umbelliferone were administered for 8 days. Behavioral studies were conducted from day 5. On the last day, Edema test was performed for the remaining animals as shown in Figure 2.1.

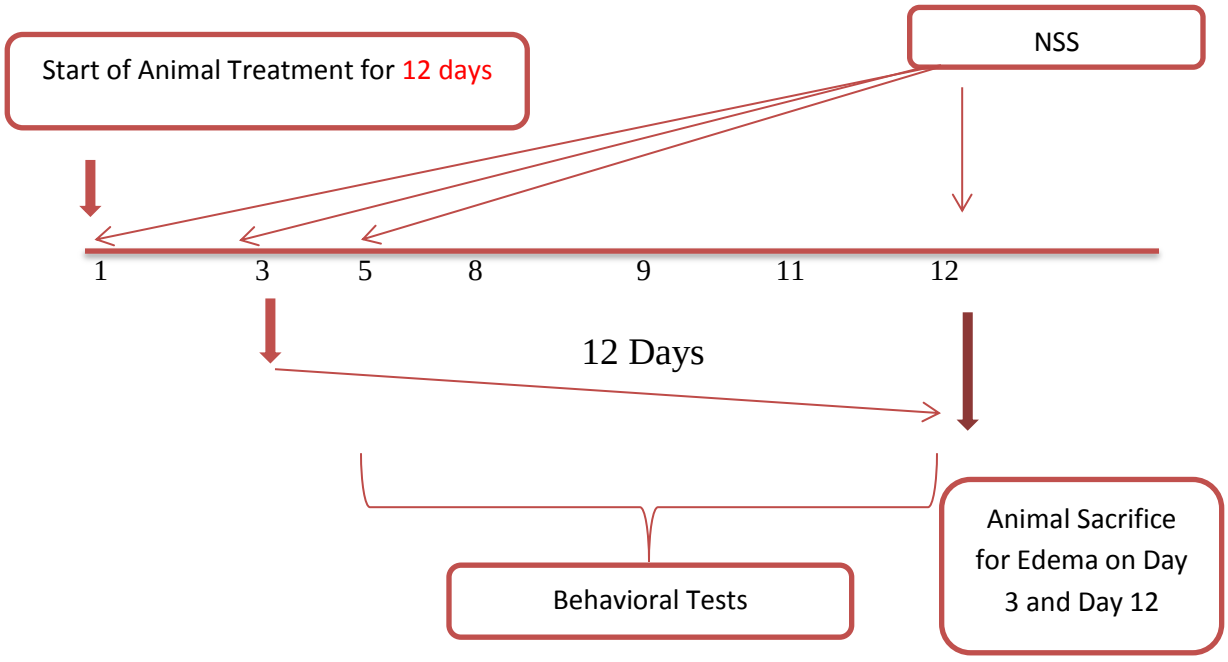


Figure 2.1: Study plan for the development of TBI model and to check the neuropharmacological activity of Umbelliferone.

NSS was performed from day 1st to 5th and on 12th day; while behavioral studies were conducted from day 8 to 12. On 3rd and 12th day, animals were decapitated for Edema and Acetylcholine Assay.

2.5 INDUCTION OF ANESTHESIA

Aesthesia was injected intraperitoneally via insulin syringes (30 gauge×0.3mm×8mm needle). The anaesthetics used were Xylazine (dose rate 5-10mg/kg) and Ketamine (dose rate 50-200mg/kg). Mouse to be injected was carefully restrained by holding its tail into fingers. Thumb and forefinger were used to make tent of skin over the scruff and then the needle was inserted at the anterior end. Material was gently injected. Multiple injections were given at alternate sites for 8 days.

2.6 INDUCTION OF TBI MODEL

After anaesthesizing the animals, an incision was executed through the scalp skin to reveal the cranium. Animals were directly positioned on a padded platform right below the weight drop apparatus. A rod of 100g was released from a height of 4 cm in a free fall directly on the open crania thrice to induce focal brain injury. The foci of injury were arbitrarily decided to be on the center. The incision was sutured by silk sutures and mice were allowed to recover**.**

2.7 BEHAVIORIAL TESTING

Behavioral tests were carried out during the light cycle of mice i.e. between 9am to 6pm, in order to avoid variability because of the circadian rhythm. They were habituated in a separate room. The room was regulated at 22± 2°C and was well lit. Environmental disturbance or human interference was kept to the minimum level. An interval of at least 30 minutes was kept between performing different behavior tests.

2.7.1 MORRIS WATER MAZE TEST

Richard G. M. Morris originally established the Morris water maze test (Morris, 1984). This test determines reference memory and spatial learning for rodents. It is based on navigating from start points of an exposed swimming tank in order to trace an immersed escape platform by using distal cues. The test was carried out in a circular tank, with a hidden platform. It was made sure that the temperature of the water was maintained at 23±2ºC.

2.7.1.1 TRAINING

Consecutively, the animals were trained for 5 days in the pool. They were put to 5 trials a day and in each trial they were put in the pool at different points. Each mouse was given an interval of 10 minutes between 2 trials. The animals were expected to locate the platform in 90secs and on failure to do so, they were placed at the platform for 20secs.

2.7.1.2 PROBE

On the last day of the test, the probe trial was conducted. The amount of time the mice took to reach the platform was noted without removing the probe.

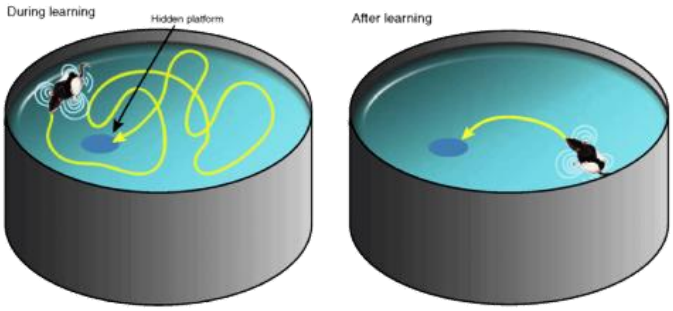


Figure2.2: Representation of Morris water maze test. The platform was hidden in one quadrant, while the mice were trained to locate it by releasing them into the tank from all quadrants. The platform was not removed in the probe trial.

2.7.2 SOCIAL PREFERENCE AND NOVELTY TEST

This test is studies the social memory and affiliation (Kaidanovich-Beilin et al., 2011). It is carried out in a three-chambered glass box. The animal can navigate easily through the three openings. There are two sessions in this test; Session 1 where the animal's social preference is assessed, and Session 2 where the animal's social novelty is assessed. Each session is of 10 minutes and between both sessions there is an interval of 20 minutes. Both the sessions were recorded using a camera.

2.7.2.1 HABITUATION

Mice were kept in the middle chamber of the apparatus for habituation. They explored the apparatus and acclimatized to the environment for 5 minutes.

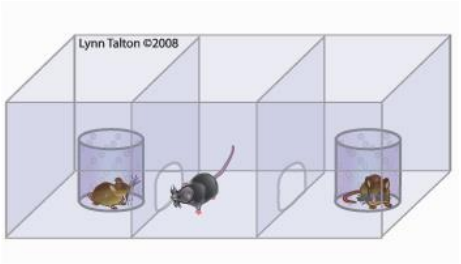


Figure 2.3: Representation of Social preference test. Mice habituated for 5 minutes in the absence of stranger mice; session 1 lasted for 10 minutes with stranger 1 and empty cage, and session 2 followed after a 20-minute interval, with stranger 1 and stranger 2 mice.

2.7.2.2 SOCIAL PREFERENCE TEST (SESSION 1)

The test mice were placed in the middle chamber, along with two small hollow wire cages in the other two chambers. In one cage the stranger mouse 1 was placed and the other cage was left empty. The test mice freely explored the chambers. The activities of the mice (movements, time duration in each chamber, interactions with the S1 mouse and the empty cages) were recorded through a camera. Sniffing and voluntarily touching the cage was considered as interaction of test mouse with S1 or empty cage.

2.7.2.3 SOCIAL NOVELTY TEST (SESSION 2)

Session 2 was carried out after an inter session period of 20 minutes. The test mice were put in middle chamber whereas the wire cages were put in their respective chambers. One cage contained S1, which was now a familiar mouse, while the other cage contained another stranger mouse (Stranger 2 or S2). S2 was of approximately the same sex, weight and age as the test mouse. All the interactions with S1 and S2, movements within the chambers and time spent were recorded by a camera. Sniffing and voluntarily touching the cage was considered as interaction of test mouse with S1 or S2. The test mouse in this session is free to choose between the first, now familiar S1 mouse, and a novel unfamiliar S2 mouse, and this way the social memory and social novelty of the mouse can be determined.

2.7.3 OPEN FIELD TEST

This test determines the locomotor and exploratory activity as well as anxiety levels in mice (Farhat et al., 2017). Exploratory behaviour can be observed when the time is spent inside the arena and there is rearing on the side, and grooming behaviour by the mice explains the stress and anxiety faced by them. The more anxious they are, the quicker they start grooming (Farhat et al., 2017).

A square shaped arena (40x40x40cm) was used in which the animal was kept for 20 minutes. Their activity was monitored using a camera. The following parameters were assessed.

1. The time duration the animal spends in the central and peripheral area.

2. The total number of rearings by the animal. An assessment of exploratory behaviour, rearing is the posture of the animal standing on its hind limbs

3. The amount of time the animal spend in anxious and relaxed grooming (Farhat et al., 2017).

2.7.4 FEAR CONDITIONING

An aversive stimulus (electric shock) is combined with a neutral stimulus (tone) (Iqbal et al., 2016). The animal expresses a fear response expression even in the absence of the aversive stimulus. The testing procedure was conducted in a plastic rectangular chamber, the floor of which was constructed of stainless steel rods (diameter 6mm and 5mm apart each other). An 80db tone was conveyed by a speaker connected and current of 0.5mA was conveyed by the amplifier attached with the shock grid. The camera was held above the chamber. The animal was subjected to habituation for 5 minutes. There were five tones (CS-conditioned stimulus) in the test session each paired with a foot shock (US-unconditioned stimulus) (Iqbal et al., 2016, Farhat et al., 2017). Freezing was measured as the reaction to aversive stimulus. The absence of all motor activities except respiration is defined as Freezing (Farhat et al., 2017). Freezing was recorded by automated video tracking software, ANY-maze. The values obtained were converted to percent freezing as follows: % freezing = Time of freezing (sec)/30 ×100

2.7.5 FEAR CONTEXT

The fear context test was carried out in a context (Iqbal et al., 2016). It was made sure that the equipment is completely clean. The mouse was given a time of 6 minutes to habituate. 20 CS trials 80dB of 30 sec each were given to the test animal, with the inter tone interval of 30 sec. No US were given to the animal (Iqbal et al., 2016). The ANY-maze recorded the freezing time which was plotted as percent freezing. The same formula as used for fear conditioning was used here.

2.7.6 NOVEL OBJECT RECOGNITION

A 5 minute habituation period was given for acclitimization with the box. They were subjected to familiarization session (Session 1) and test session (Session 2) for 10 minutes with an inter-time of 20 minutes. In the first session, two novel objects were kept opposite to each other in two corners allowing the animal for exploration. One object was swapped with a novel object and the animal explored in Session 2. Physical interactions (touching and sniffing) with the object and the time spent doing so was recorded as exploration time (Iqbal et al., 2016).

2.7.7 LIGHT DARK TEST

This test assesses the anxiety-like behaviour of animals (Takao and Miyakawa, 2006). Rodents prefer to be in the dark naturally but they also tend to exhibit exploratory behaviour in light areas. The light/dark box is composed of two compartments, dark and

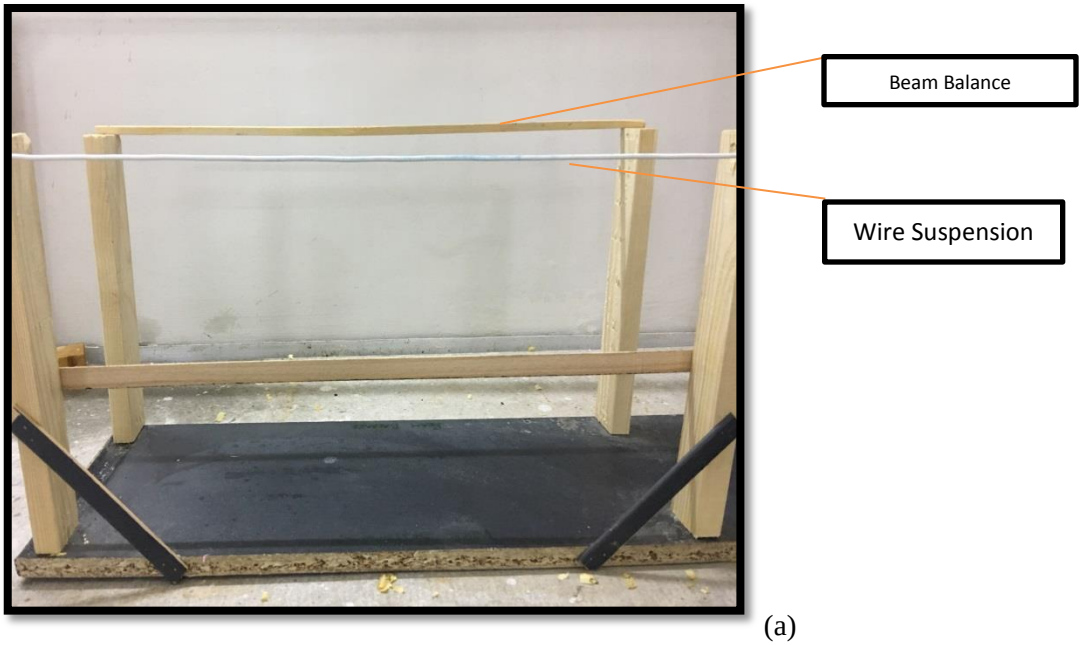
light. Mice are left to easily explore both chambers for 5 minutes. The number of times the animal entered into the light compartment and the duration of time it spent are recorded and evaluated for bright space anxiety.

2.7.8 ELEVATED PLUS MAZE

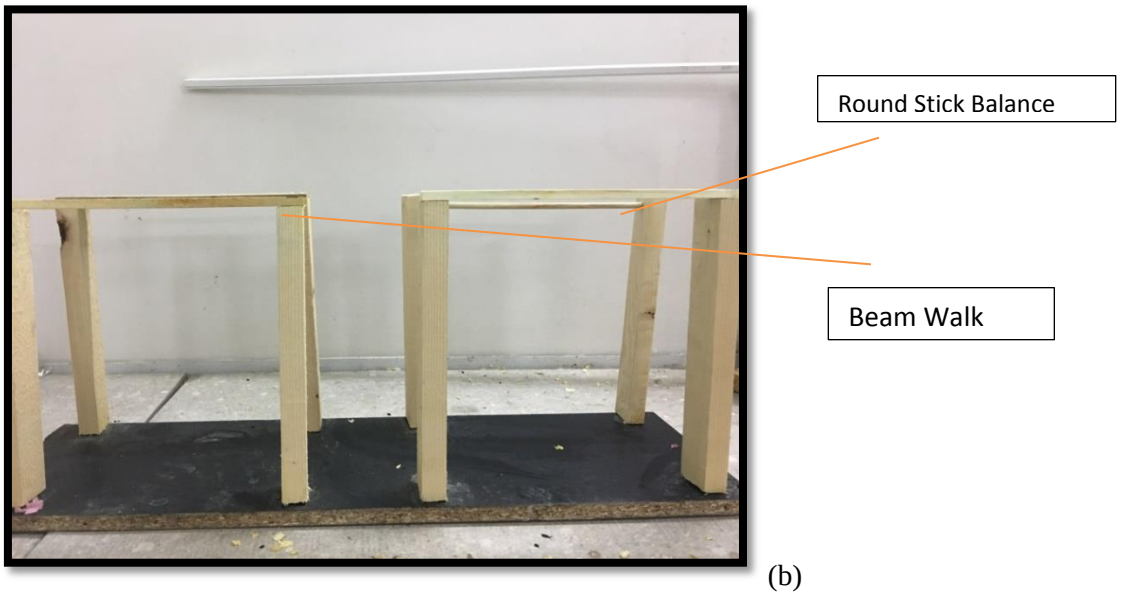
The anxiety of rodents can be assessed by the Elevated plus maze test (Arendash et al., 2004, Iqbal et al., 2016). A maze shaped as a plus raised 82cm above the ground was the apparatus used. There are two closed arms(30x5cm) with dark walls and two open arms without any wall (Iqbal et al., 2016). After keeping the animal in the middle, it was left to explore. The amount of time the animal spends in the open arm and its entries in that arm were noted and plotted.

2.8 NEUROLOGICAL SEVERITY SCORE

Neurological severity score (NSS) was recorded at 4h, 24 h, 72 h, 96 h and at 288h post injury. The injured mice were evaluated at the scheduled time points after TBI by evaluating NSS. The parameters and scoring paradigm of NSS is shown in **Table 2.2** and the set up for NSS is shown in **Figure 2.4**.



(a)



(b)

Figure 2.4: Apparatus for measuring the neurological severity score which includes **a).** Wire suspension and Beam balance while **b)**. Round Stick Balance and Beam walk.

Parameters and scoring paradigm of NSS (Table 2.2):

MOTOR COORDINATION		**Score (Max Sc. 28)**	
		Failure (Max Score)	**Success**
Seeking Behavior	Displaying interest in environment (time limit: 3mins)	**5**	**0**
Beam Balance	To be able to balance on 7mm beam for minimum 10s	**3**	
Round Stick Balance	To be able to balance on a 5mm round stick for minimum 10s	**1**	
Exit Circle	To be able to exit a circle within 2min (30cm)	**1**	
Straight Walk	Vigilance, initiative, and to be able to walk straight	**2**	
Beam Walk	To be able to cross 3cm, 2cm and 1cm beam	**3**	
NEUROLOGICAL REFLEX			
Mono-/Hemiparesis (Grasping)	Upper and/or lower limb paresis	**1**	**0**
Acoustic Startle Response	Bounces to a loud hand clap	**1**	
Wire Suspension	To demonstrate neuromuscular impairment and motor coordination	**6**	
Flexion Reflex	Alertness is detected	**1**	
Pinnae Reflex	To check auditory response	**1**	
Corneal Reflex	To check visual reflex	**1**	
SENSORIMOTOR			
Twisting	Wobbling in mice determined	**2**	**0**

2.9 DISSECTION FOR EDEMA MEASUREMENT

On the 3rd (48hr) and 12th day of the protocol, specific number of animals were decapitated for brain isolation. 200-500ul chloroform was used to anaesthetize the animals followed by quick decapitation. Decapitated brain was placed on a petri plate with a drop of PBS buffer. Cortex and hippocampus regions were separated which were further processed for edema.

2.10 EDEMA MEASUREMENT

The wet and dry weight method was used to determine cerebral edema levels (Lin et al., 1993). A tissue segment from the part adjoining the lesion was taken to weigh and determine wet weight (WW). It was dryed in a vacuum oven at 70°C, and reweighed (until constant weight was observed) to determine dry weight (DW). The water content percentage in the tissue was measured as% water content by using the formula:

[(WW-DW) ×100]/WW.

Edema levels were evaluated at 48 hours, and on day 12(288 h) post-injury. Eight animals were euthanized at each time point indicated to determine Edema.

2.11 STATISTICAL ANALYSIS

Statistical analysis was calculated by GraphPad Prism software (Version 5.03) The statistical tests applied to analyse the data were One-Way ANOVA and Two- Way

ANOVA and the Bonferroni multiple comparison test. The significant P value was noted to be less than 0.05. The data was shown as mean ± standard error of mean (SEM).

CHAPTER 3: RESULTS

3.1 BEHAVIOR ANALYSIS

3.1.1 MORRIS WATER MAZE TEST

This test determined the aftermath of TBI on spatial memory and learning. The effect of Umbelliferone dosage along with TBI was also analyzed. Repetitive training helps the animal obtain an escape plan to the hidden platform. How strong the memory has been developed over the days can be found out by calculating the average escape latency.

The average escape latency to reach the platform of each group is shown in **Figure 3.1(A)** shows. All groups exhibited improved learning throughout the training days. TBI+UMB (30mg) group showed slightly better learning than the rest of the groups and the animals were able to find the platform within 20s although no significant difference was observed.

Probe Trial

Probe trial was conducted on the 5^{th} day and the time the animal took to reach the platform was noted down. Figure 3.1(B)

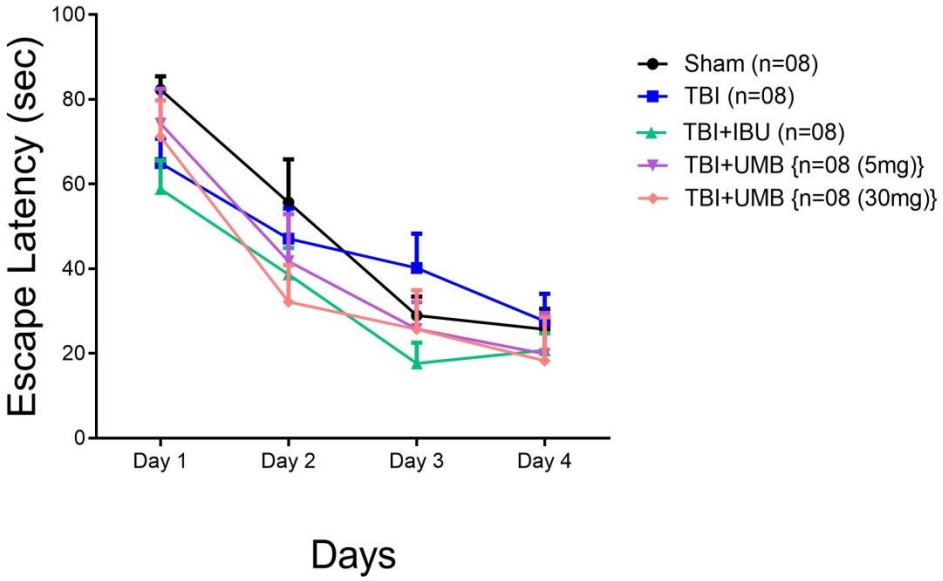


Figure 3.1(A): Effect of TBI and administration of UMB doses on learning and memory. The graph presents the pattern of acquiring spatial memory to locate the hidden platform across five training days among Sham, TBI, TBI+IBU, TBI+UMB (5mg), TBI+UMB(30mg). The error bars represent mean ± SEM.

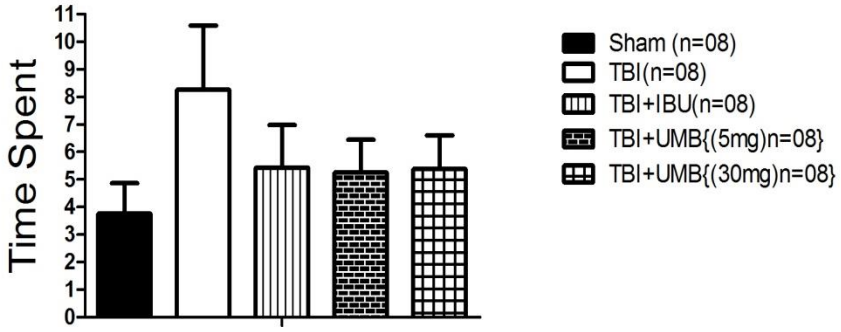


Figure 3.1(B). Probe trial performed on day 5

Although there was observed no significant difference, a mild trend was seen where TBI group reached the platform in maximum time **(Figure 3.1(A),(B))**. The Sham group performed the best. The three groups TBI+IBU, TBI+UMB(5mg), TBI+UMB(30mg) performed better than the TBI group.

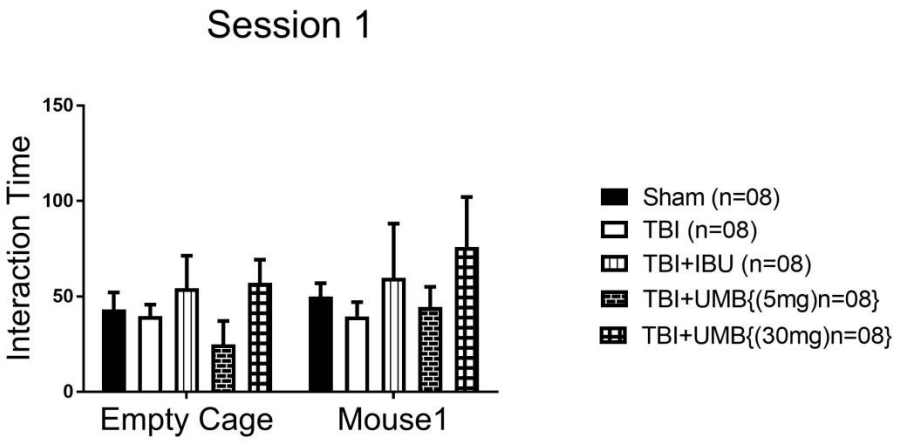
3.1.2 SOCIAL NOVELTY AND PREFERENCE TEST

Session I is performed to assess the social preference and sociability of the animals (**Figure 3.2(A)**), while Session II determines social novelty preference in them (**Figure 3.2(B)**). Time spent by test mouse with empty cage, Stranger 1 and Stranger 2 was analyzed.

3.1.3 OPEN FIELD TEST

To determine the anxiety, activity and exploratory behaviour of each mouse, this test was conducted. The parameters analyzed were the time animal spends in the center, number of rearings and grooming latency.

(A)



(B)

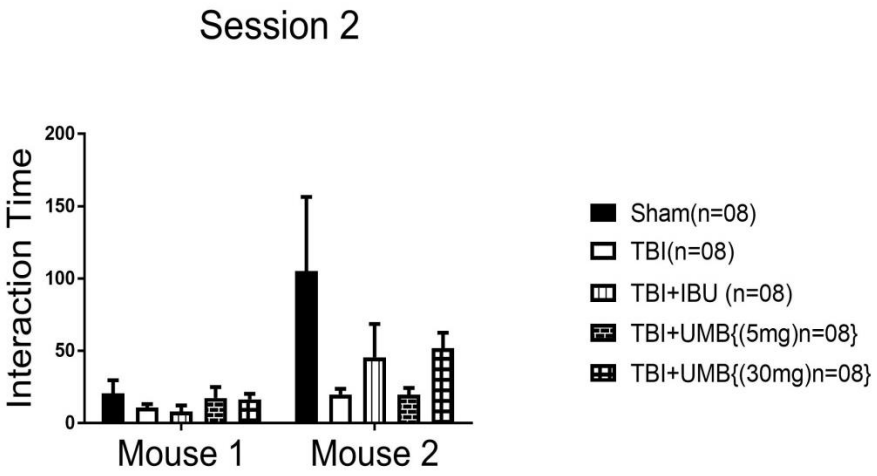
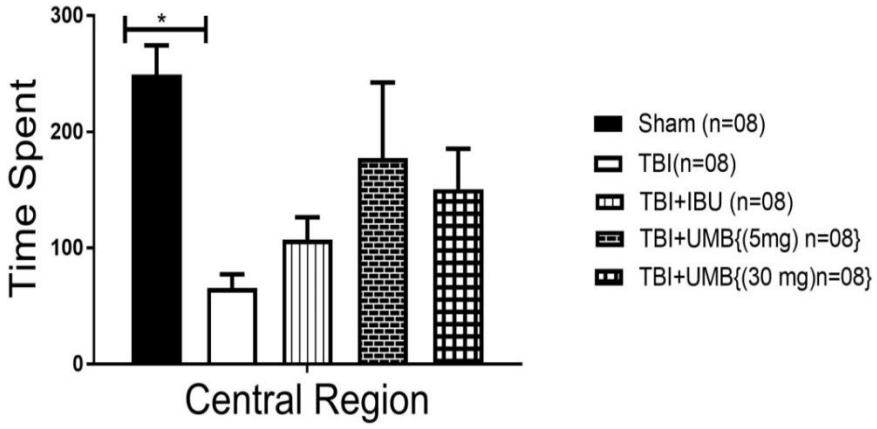


Figure 3.2. Effect of TBI and Umbelliferone dosage in Social Novelty Test.

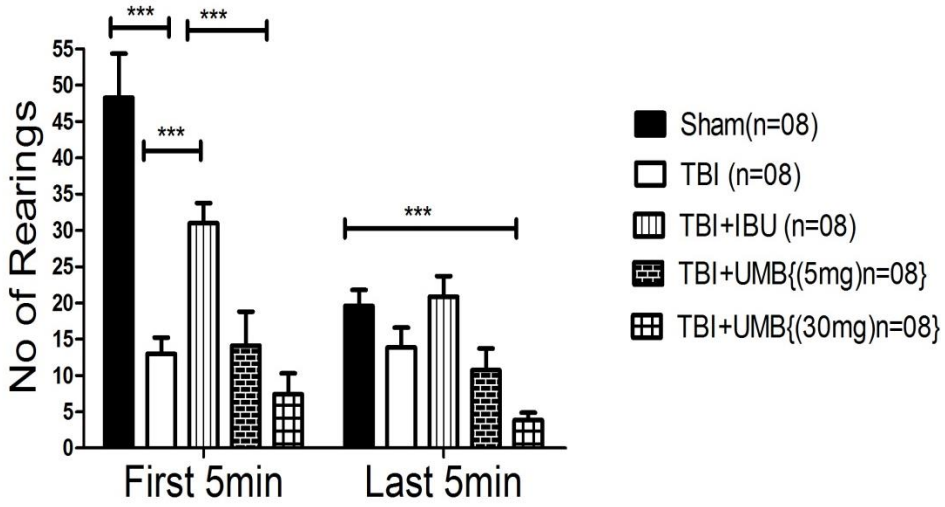
Mice sociability was exhibited by all the groups, Sham (46.63 ± 3.37), TBI (39.69 ± 0.06), TBI+IBU (57.00 ± 2.71), TBI+UMB(5mg) (34.69 ± 9.8) and TBI+UMB(30mg) (66.50 ± 9.44) by interacting more with the stranger mouse 1 **(Figure 3.2 (A))**. TBI+UMB (30mg) group showed improved activity as compared to the other groups interacting with the mouse 1 for 70seconds. Groups Sham, TBI+IBU, TBI+UMB (5mg) also showed improved activity while the group TBI showed the least activity, but it was observed that there is no significant difference.

Each group interacted more with the stranger mouse 2 rather than the mouse 1 which was now familiar to them **(Figure 3.2(B))**. Sham group (62.81 ± 42.31) showed normal response in the session II followed by the TBI+UMB (30mg) (26.79 ± 18.79) group and TBI+IBU (34.13 ± 17.63) group. The least activity was showed by the TBI group (15.25 ± 4.50). Overall no significant difference was observed.

(A)


300
200
100
0
Time Spent
*
Central Region
Sham (n=08)
TBI(n=08)
TBI+IBU (n=08)
TBI+UMB{(5mg) n=08}
TBI+UMB{(30 mg)n=08}

(B)


***
***
***
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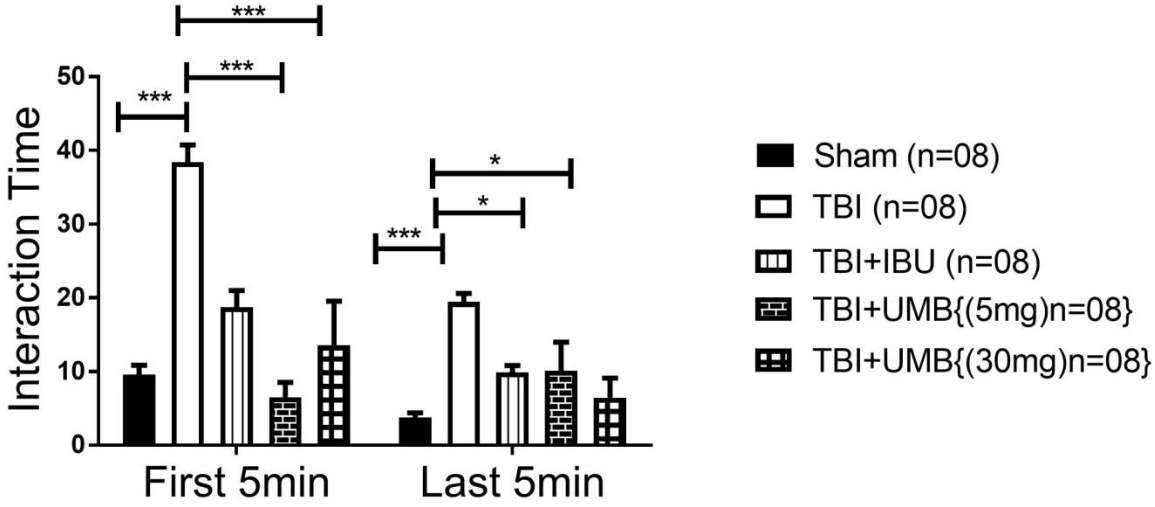
55
50
45
40
35
30
25
20
15
10
5
0
No of Rearings
First 5min
Last 5min
Sham(n=08)
TBI (n=08)
TBI+IBU (n=08)
TBI+UMB{(5mg)n=08}
TBI+UMB{(30mg)n=08}

(C)



(D)

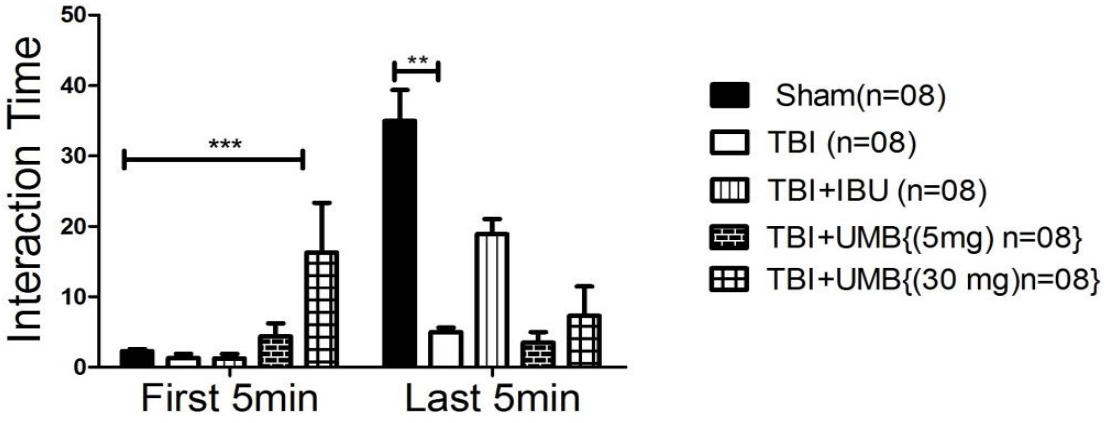


Figure 3.3 Effect of TBI and Umbelliferone dosage in Open field test

(A) shows the time spent in the center region, (B) rearing by hind paws, (C) anxious and (D) relaxed grooming by the groups Sham, TBI, TBI+IBU,TBI+UMB(5mg),TBI+ UMB (30mg). *= p< 0.05, **=p<0.01,***= p< 0.001 significance values. The error bars represent mean ± SEM,

Locomotor activity was exhibited by all groups except TBI group which did not spend much time in the center **(Figure 3.3(A))**. Sham and TBI group showed significant difference.

Exploratory behaviour was assessed by analyzing the number of rearings by hind paws in the first 5minutes and the last 5minutes **(Figure 3.3(B))**. Sham group (33.93 ± 14.36) showed the highest number of rearings as compared to the other groups. TBI+IBU (25.94 ± 5.06) showed improved results than the other groups. All groups showed significant difference (p value <0.001), TBI+UMB (5mg) (12.45 ± 1.70), TBI+UMB (30mg) (5.643 ± 1.78). In the last 5 minutes, there was not much difference among the various groups except for TBI+UMB (30mg) which performed weak. Significant difference was observed with p value <0.0001.

Anxious grooming and relaxed grooming were displayed by all the groups. **(Figure 3.3 (C))** and **(Figure 3.3(D))** respectively clearly depicts TBI group displaying highest levels of anxiety amongst all the groups. The Sham group (6.643 ± 2.92) and TBI group (28.93 ± 9.50) showed significant difference. Both the UMB treated groups showed considerably less levels of anxious grooming behaviour. TBI and TBI+UMB (5mg) (8.313 ± 1.81) (p<0.001) and TBI and TBI+UMB (30mg) (10.00 ± 3.57) in the last 5 minutes showed significant difference. Almost similar trend was seen in **Figure 3.3(D)** where TBI group showed weak performance in relaxed grooming. Both the UMB treated groups showed relaxed behaviour. Sham (18.64 ± 16.36) and TBI group (3.143 ± 1.85) showed significant difference (p<0.01).

3.1.4 FEAR CONDITIONING

Fear conditioning test determines the hippocampus dependent associative learning. Freezing response was calculated to measure condition-stimulus (CS) and context-dependent memory.

3.1.5 CONTEXTUAL FEAR

Contextual fear was measured by calculating the freezing memory in the same context and there was no conditioned stimulus.

3.1.6 NOVEL OBJECT RECOGNITION

The time duration in which the animal physically touched and sniffed the object was recorded as exploration time.

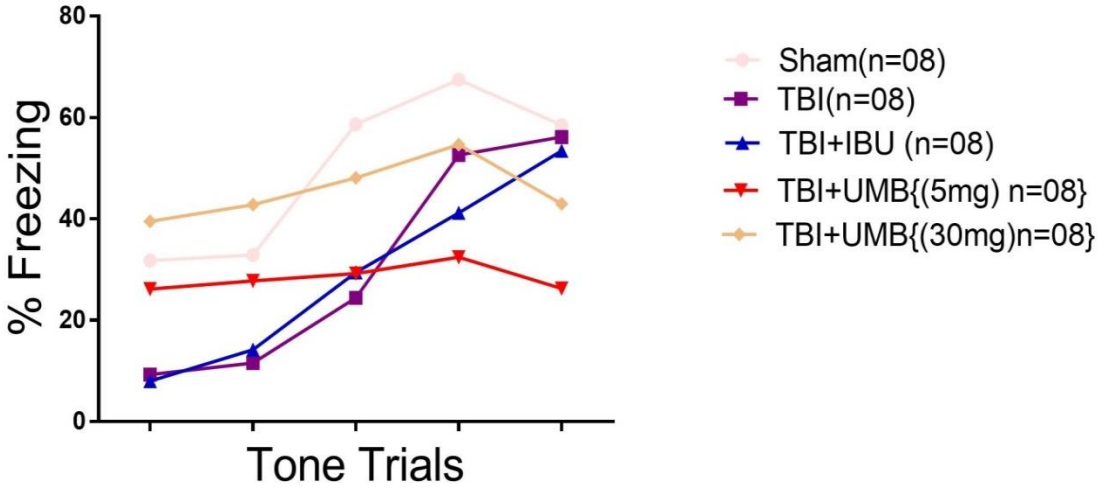


Figure 3.4 Effect of Umbellliferone treatment on fear conditioning in TBI induced mice.

Sham group (49.85 ± 7.34) showed the highest percentage of freezing response across all tone trials. It showed highest percentage at the fourth tone trial. TBI+UMB (5mg) (28.36 ± 1.16) group showed no development across the five tone trials. TBI+UMB (30mg) (45.59 ± 2.65) showed high freezing response at the fourth trial but it was lesser comparatively to the normal response. There was observed no significant difference.

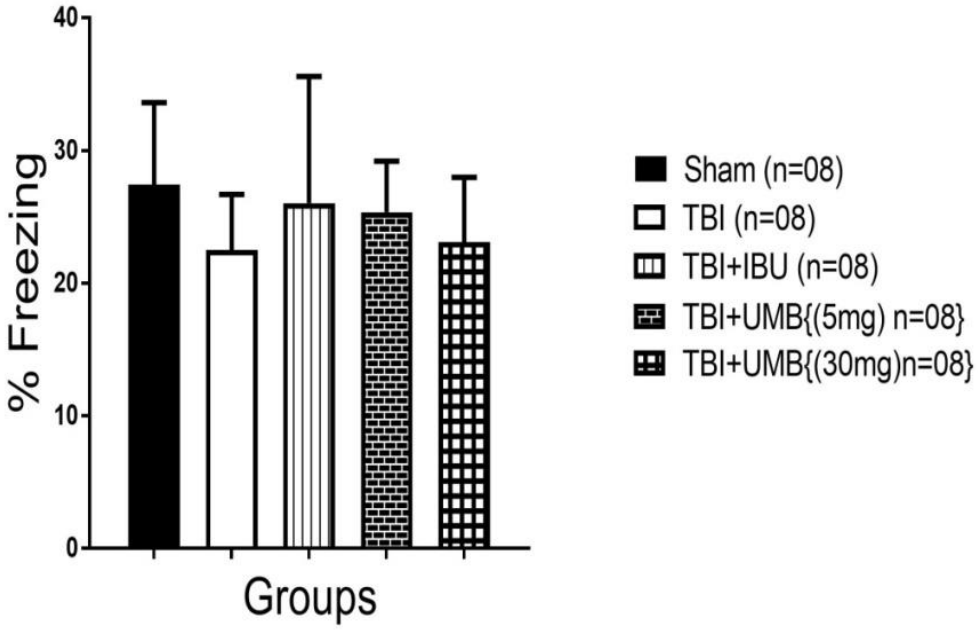
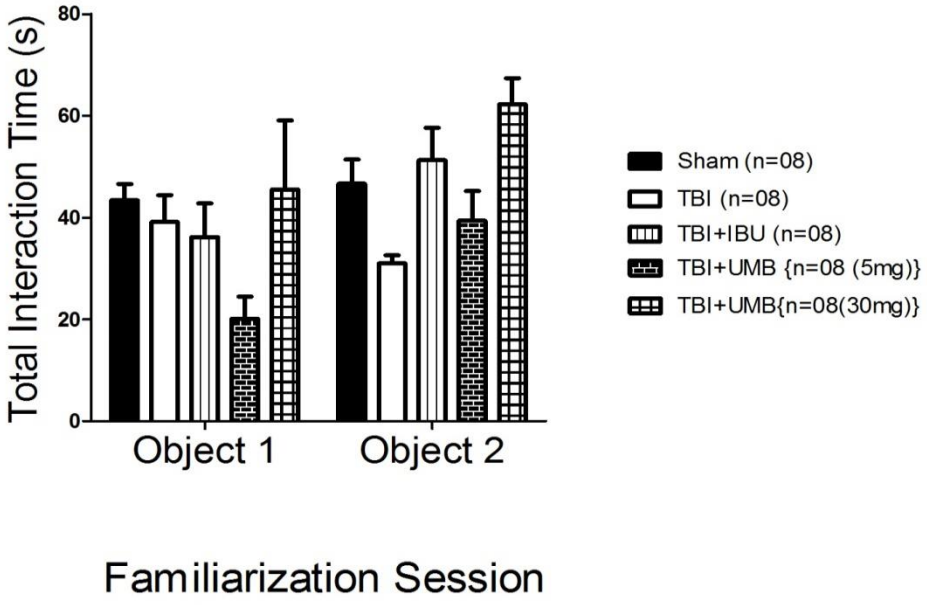


Figure 3.5 Effect of Umbelliferone treatment on the contextual fear memory in TBI induced mice.

Sham group showed the highest freezing response. TBI group showed the weakest response to freezing amongst the 5 groups followed by TBI+UMB (30mg). TBI+ IBU and TBI+UMB (5mg) showed better freezing responses comparatively. None of the groups showed any significant difference.

(A)



(B)

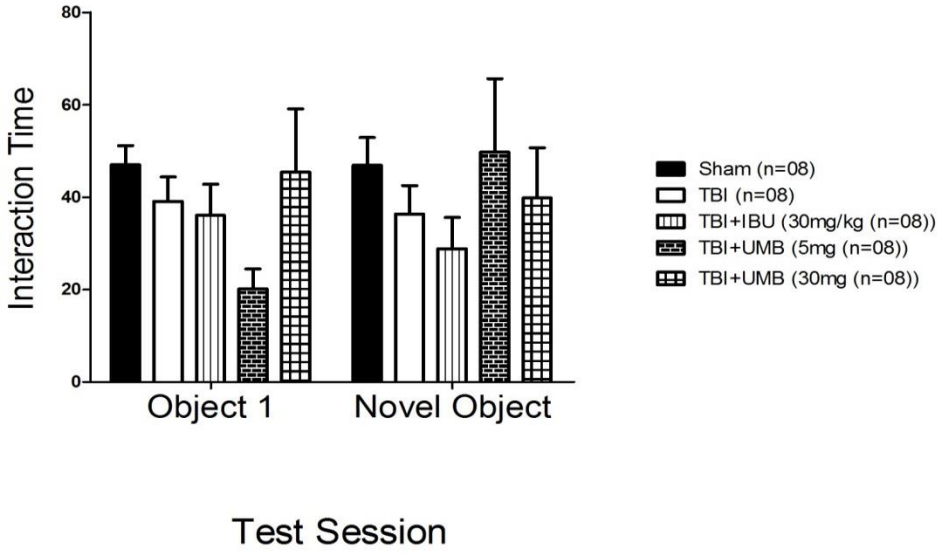


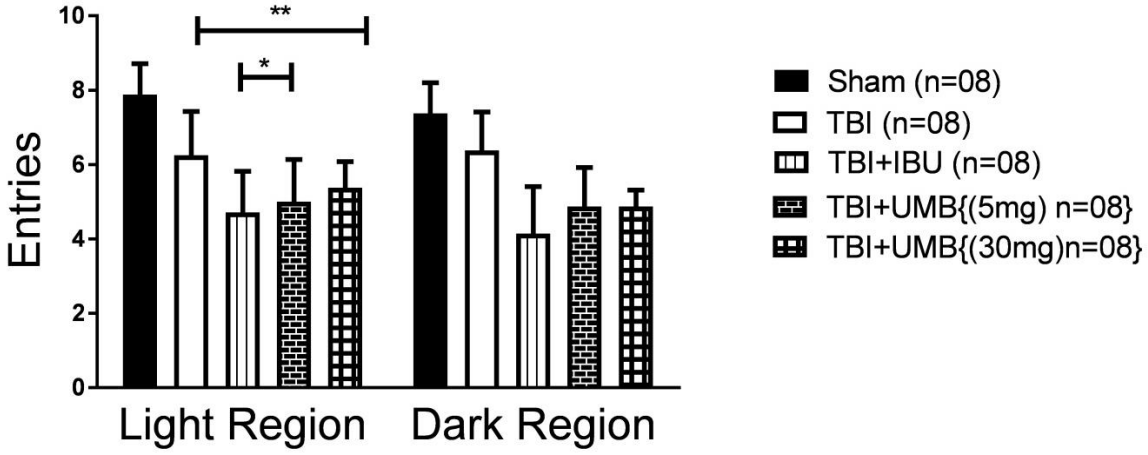
Figure 3.6 Effect of TBI and Umbelliferone dosage in Novel Object Test

In the Familiarization Session **(Figure 3.6(A))**, TBI group (35.06 ± 4.06) was seen spending time interacting with the objects lesser than the Sham group (45.00 ± 1.625). TBI+UMB (30mg) (53.88 ± 8.37) showed maximum interaction with both the objects. It showed no significance difference. Comparatively, in the Test Session **(Figure 3.6(B))**, TBI +UMB (5mg) (29.75 ± 9.62) spend the maximum time interacting with Novel Object followed by the Sham group and TBI+UMB (30mg) group. TBI group was seen showing less interaction with the novel object as compared to Object 1. Error bars represent mean + SEM.

3.1.7 LIGHT DARK TEST

The anxiety-like behaviour exhibited by the animal was evaluated by this test. The extent of time the animal spends and the number of entries in the illuminated chamber are recorded and evaluated for bright space anxiety amongst the animal.

(A)



(B)

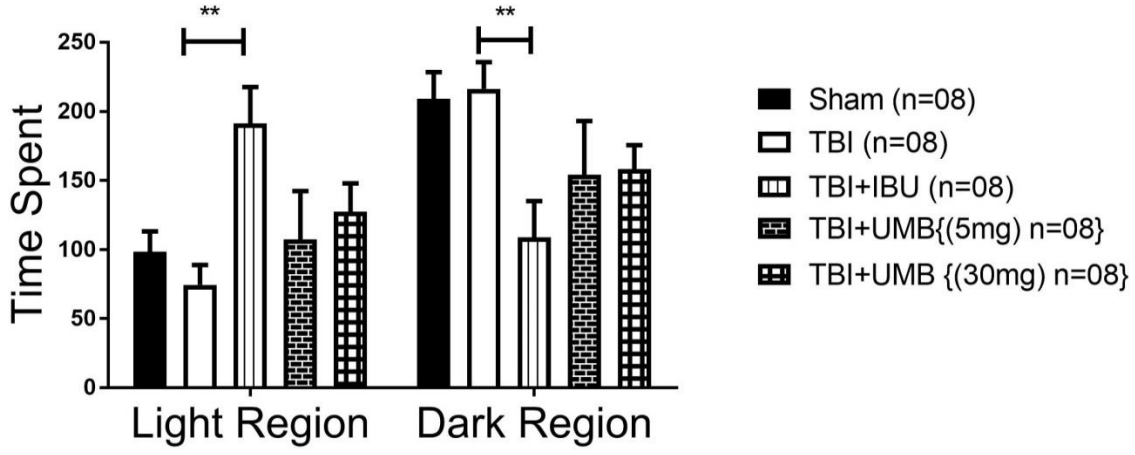


Figure 3.7 Effect of TBI and Umbelliferone dosage in Light/Dark Test

(A) The number of entries in Light and Dark region, (B) The time spent in the light region and dark region.

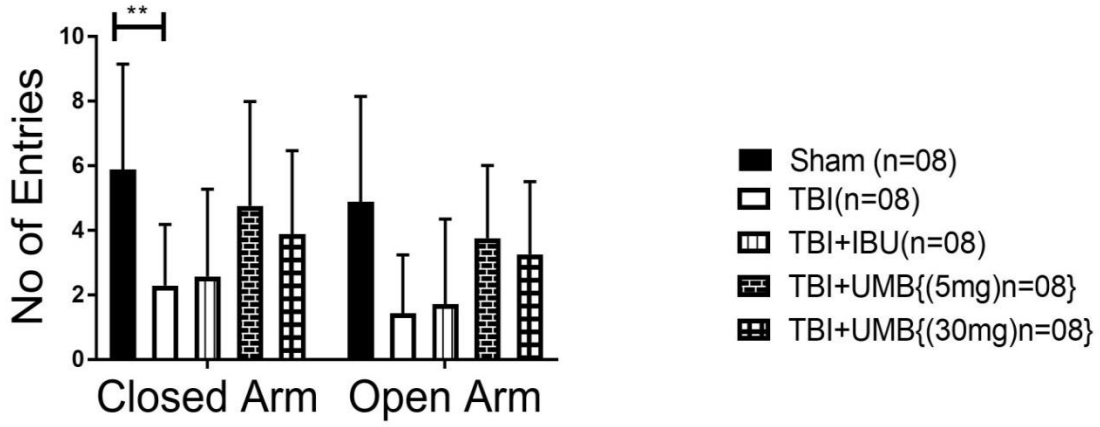
Bright space anxiety was assessed by determining the duration of time the animal spent in the Light region. TBI+IBU, TBI+UMB (5mg) (4.938 ± 0.0625), TBI+UMB (30mg) (5.125 ± 0.25) spend lesser time in the Light region in comparison to the Sham group (7.625 ± 0.25) **(Figure 3.7(A))**. There is observed a significance difference amongst the TBI group (6.313 ± 0.06) and TBI+UMB (30mg) group ($p<0.01$). It was also observed within TBI+IBU and TBI+UMB (5mg) group ($p<0.05$). TBI+IBU group (4.429 ± 0.26) showed less number of entries in the light region as compared to all the other groups.

A considerable difference was observed amongst the TBI (145.3 ± 70.94) and TBI+IBU group (150.0 ± 41.29) ($p<0.01$). The group which spends the least amount of time in the light region is the TBI group **(Figure 3.7(B))**. TBI+UMB (30mg) group (142.8 ± 15.31) spend slightly more time as compared to TBI+UMB (5mg) group (130.7 ± 23.44). In the dark region the group that spends the most time is the TBI group.

3.1.8 ELEVATED PLUS MAZE

This test assesses the anxiety caused due to height and open spaces. The duration of time the animal spend within the open arm and how many times the animal entered were noted and plotted.

(A)



(B)

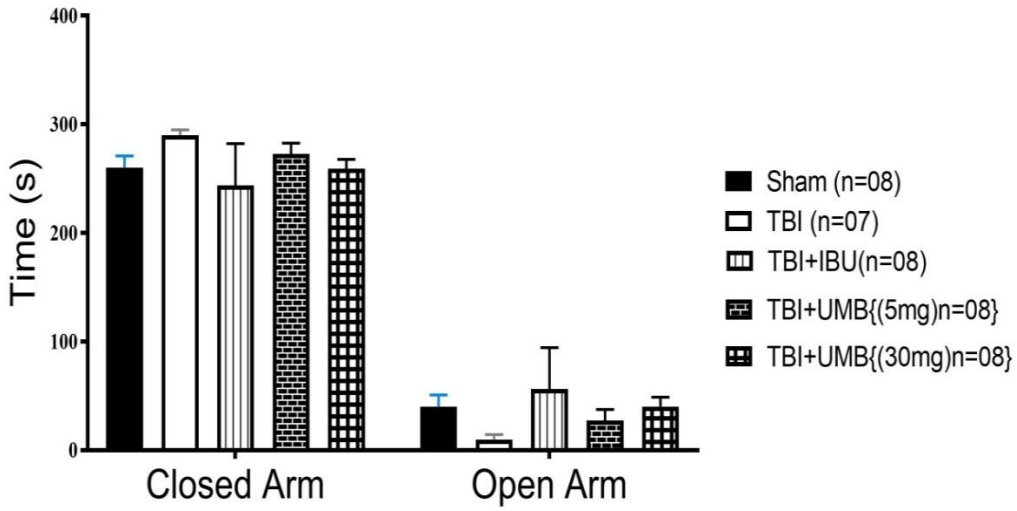


Figure 3.8 Effect of Umbelliferone dosage in Elevated Plus Maze Test

Anxiety levels were displayed by the TBI group (1.857 ± 0.42) with least number of entries in the open arm **(Figure 3.8 (A)).** TBI+IBU group (2.143 ± 0.43) performed slightly better than TBI. TBI+UMB (5mg) group (4.250 ± 0.50) and TBI+UMB (30mg) group (3.813 ± 0.44) performed comparatively better. The best activity was shown by the Sham group. The Sham group (5.375 ± 0.50) and TBI group showed significant difference between them ($p<0.01$). The TBI group (150.0 ± 140.3) spent more time amidst the closed arm as compared to the open arm **(Figure 3.8(B))**. The groups TBI+UMB (5mg) group (150.1 ± 122.7) and TBI+UMB (30mg) (149.7 ± 109.4) group performed slightly better. Overall no significant difference was observed.

3.2 NEUROLOGICAL SEVERITY SCORE

NSS was recorded at 4h, 24 h, 72 h, 96 h and lastly at 288h post injury. The injured mice were mice were evaluated at the scheduled time points after TBI by evaluating NSS to analyze the motor functions and reflexes.

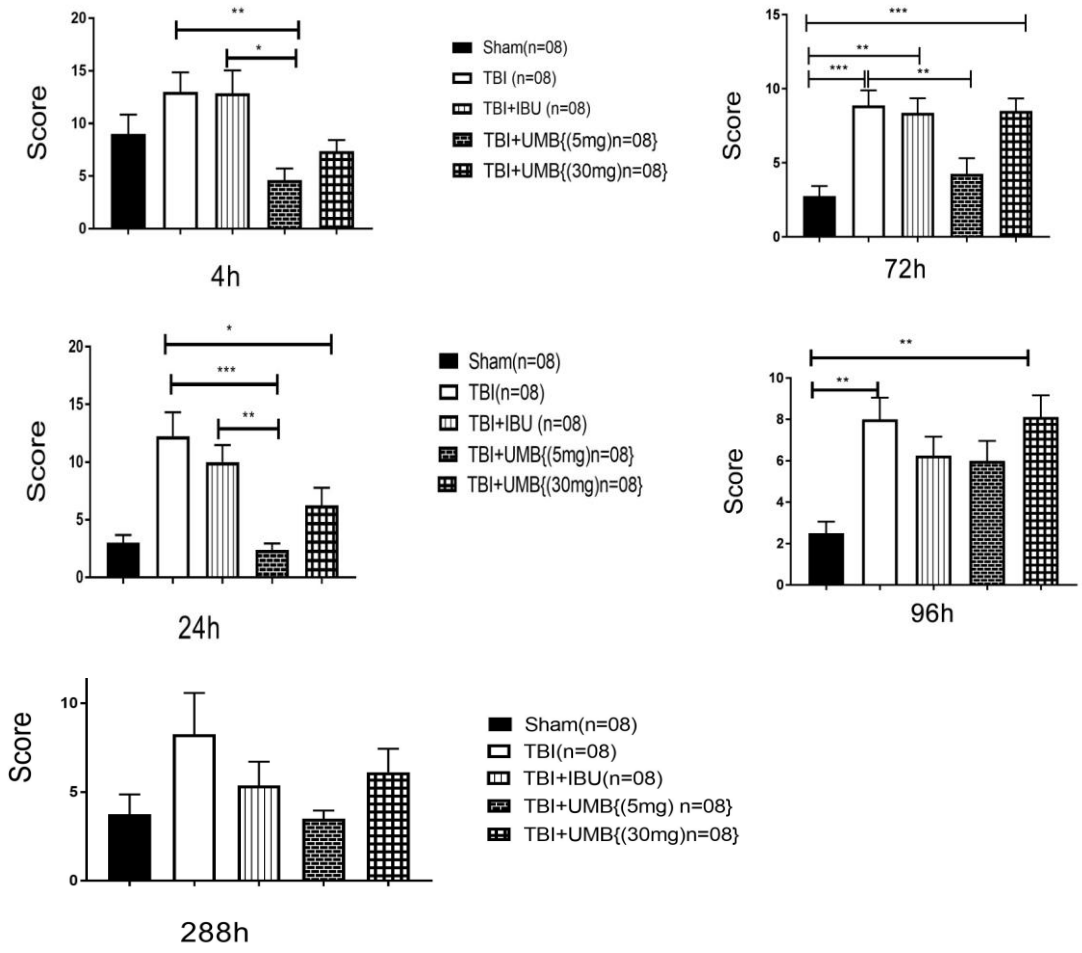


Figure 3.9 Effect of TBI and Umbelliferone dosage on Neurological Severity Score

Neurological Severity Score was calculated at 4h, 24h, 72h, 96h and 288h.

The TBI group performed poor and had a total score of 13 (13 ± 1.85) followed by TBI+IBU with a total score of 12 (12.88± 2.17) at the 4th hour i.e at day 1 **(Figure 3.9)**. TBI+UMB (5mg) performed the best at 4h with a total score of 5 (4.625±1.01) followed by TBI+UMB (30mg) with a total score of 8 (7.37±1.05). TBI and TBI+UMB (5mg) group ($p<0.01$) and TBI and TBI+UMB (30mg) group ($p<0.05$) showed significant difference among themselves.

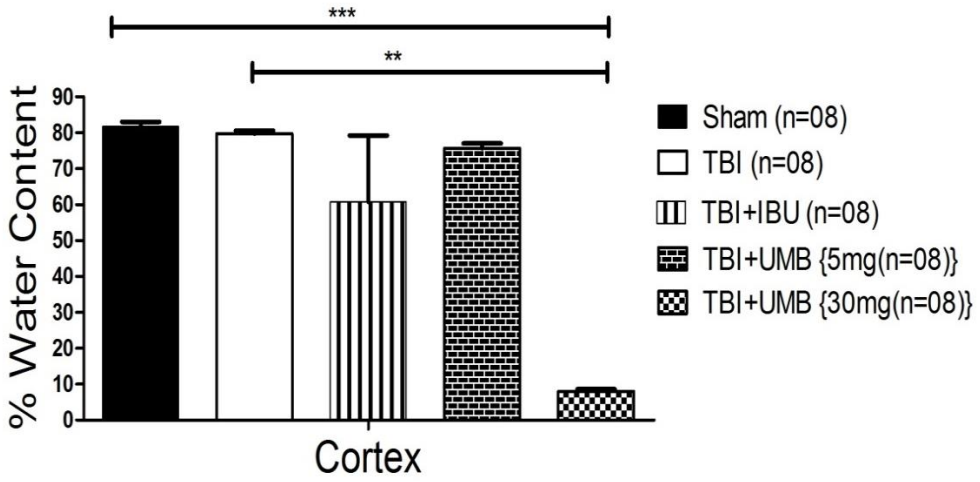
The TBI group performed poor than the other groups with a total score of 13 (12.25±2.07) at the 24th hour **(Figure 3.9)**.TBI+IBU group showed little improvement and had a total score of 8 (10±1.48). TBI+UMB (5mg) and Sham groups showed improvement and the score is seen to be reduced to 3 (2.375±0.57), 4 (3±0.68) respectively. Significant difference was witnessed amidst TBI and TBI + UMB (5mg) ($p<0.001$) and between TBI and TBI + UMB (30mg) group (6.25±1.52) ($p<0.05$).

TBI group showed no improvement and the score was at 9 (8±1.05) at the 96th hour. Sham showed normal response with a score of 3 (2.5±0.56). Sham and the other groups ($p<0.001$) and between TBI and TBI+UMB (5mg) group (6±0.96) ($p<0.01$), it was observed that there is a significant difference. At the 288h, TBI showed no improvement and the overall score of 8 (8.25±2.33). The scores were same as that of the previous hours. Overall there was no significance difference observed on the last day.

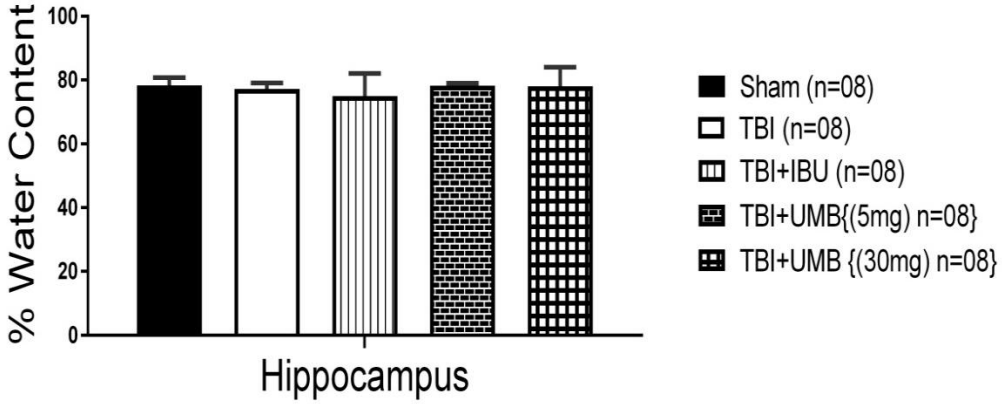
3.9 EDEMA CALCULATION

Edema levels were evaluated at 48h and at 228h for Cortex and Hippocampus.

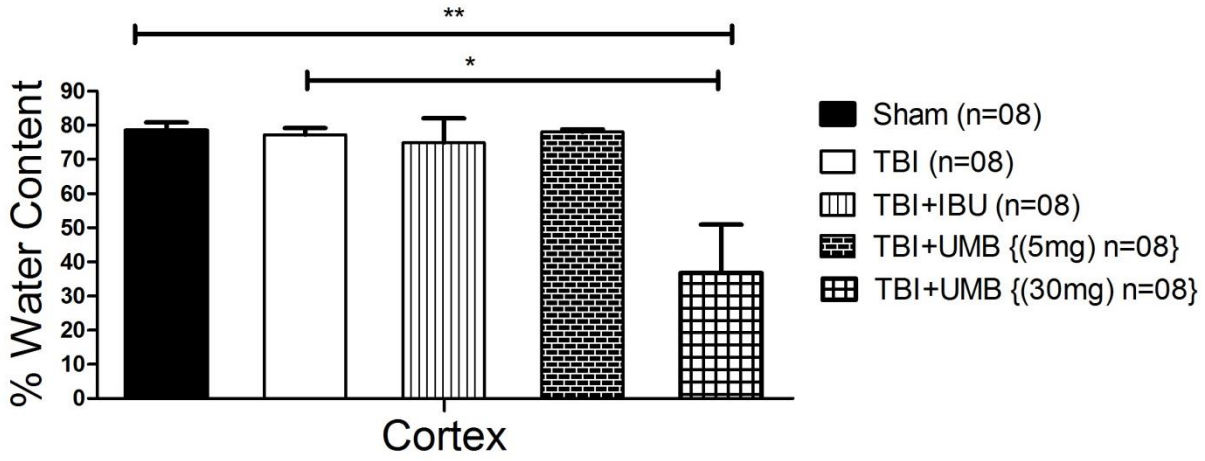
(A)


***
**
% Water Content
90
80
70
60
50
40
30
20
10
0
Cortex
Sham (n=08)
TBI (n=08)
TBI+IBU (n=08)
TBI+UMB {5mg(n=08)}
TBI+UMB {30mg(n=08)}

(B)


% Water Content
100
80
60
40
20
0
Hippocampus
Sham (n=08)
TBI (n=08)
TBI+IBU (n=08)
TBI+UMB{(5mg) n=08}
TBI+UMB {(30mg) n=08}

(C)



(D)

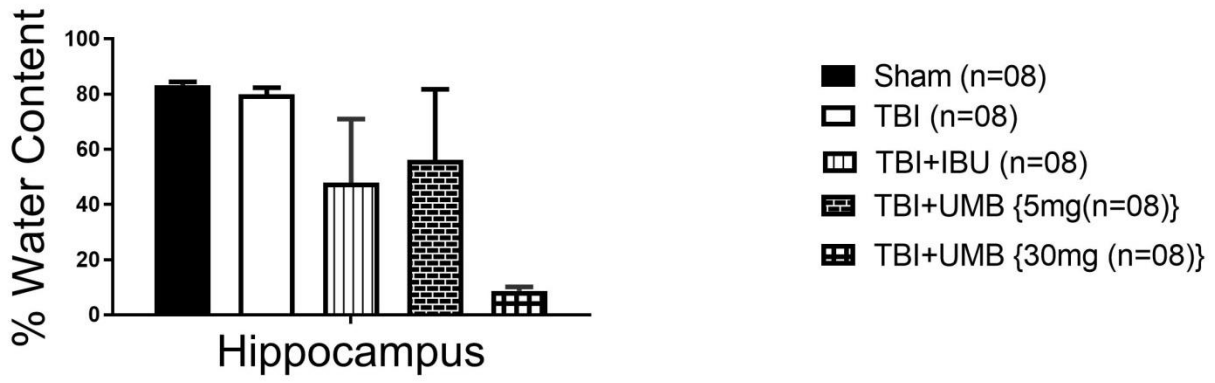


Figure 3.10 Edema levels at 48h and 288h of Cortex and Hippocampus

(A) Edema levels in Cortex at 48h, (B) Edema levels in Hippocampus at 48h, (C) Edema levels in Cortex at 228h, (D) Edema levels in Hippocampus at 288h.

At the 48h, Edema levels are considerably lesser in TBI+UMB (30mg)(8.000± 0.57)group in comparison to the rest of the groups **(Figure 3.10(A))**. All four groups showed almost the same levels of edema in the cortex. With p value<0.05, TBI and TBI+UMB (30mg) group, Sham group (81.73± 1.28) and TBI+UMB (30mg) ($p<0.01$) showed significant difference. At the hippocampal level, the edema levels were same **(Figure 3.10(B))**.Overall no significant difference was observed.

At the 288h, cortical edema levels were considerably reduced in the TBI+UMB (30mg) group(36.68± 14.20) followed by TBI +IBU group ((74.92 ± 7.10) **(Figure 3.10(C))**. The TBI and TBI+UMB (30mg) groups showed a significant difference. The p value was less than 0.05. TBI+UMB (30mg) showed considerably less edema levels in the hippocampus **(Figure 3.10(D))**. Overall in the hippocampus, edema levels did not exhibit any significance difference.

CHAPTER 4: DISCUSSION

The mortality rate of Traumatic brain injury is alarming globally and is a worldwide concern. Annually, millions die due to it or face various psychological, emotional and behavioural deficits along with motor impairment and sensory deficits. Patients suffering from TBI have been reported to have issues related to cognition, memory, attention, judgment, depression, anxiety, movement, comprehension among many others. A very important role in our lives is played by learning and memory. The brain regions most vital in these processes are the hippocampus and the frontal cortex (Xu and Südhof, 2013). Declarative memory, which records daily facts and events is regulated by these regions (Preston and Eichenbaum, 2013). The pathophysiology of TBI is very complex and the various underlying processes are yet to be studied in detail making it difficult to control the severe after effects of it.

Umbelliferone, a plant extract from the family of coumarins, has previously been reported to show neuroprotective properties. It has been previously reported to be possessing neuroprotective effects such as reducing depression (Qin et al., 2017), reducing pain and inflammation (Leal et al., 2000), enhancing learning and memory (Essa et al., 2016) along with other effects.

The current study studied the neuropharmacological effects of Umbelliferone in traumatic brain injury mouse model. TBI was induced by the weight drop method, followed by administration of Umbelliferone (5mg/kg and 30mg/kg) intraperitoneally. The changes in behaviour and structure of the brain were analyzed by performing various behavioural tests.

Morris water maze test showed that the group which developed the least memory was the TBI group. As previously reported, TBI patients face learning and memory issues. Comparatively the rest of the groups performed better than the TBI group. Over the days it was seen that the group administered with Ibuprofen, an anti-inflammatory agent, also acquired memory and found the platform quickly indicating improved memory. This can be validated as previously it has been reported that Ibuprofen reduces pain which indirectly enhances memory acquisition (Bradley et al., 1991)

Both the Umbelliferone groups (5mg/kg, 30mg/kg) showed learning throughout the days performing better than the Control group. The mice were able to find the platform in just 20seconds. It was reported that Umbelliferone is a strong inhibitor of the acetylcholinesterase activity and it has been observed to enhance the learning along with reversing amnesia effects (Essa et al., 2016). Although there was no significant difference observed it can be suggested that Umbelliferone administration might help in memory acquisition.

To measure the anxiety levels and exploratory behavior, the open field test is conducted. The mice which do not explore their surroundings and display grooming behaviour are anxious. Normal active mouse will explore the central arena, and display late grooming (Negishi et al., 2005).

TBI group spend the least time in the central region showing less activity in the arena. It displayed low rearing count and high levels of anxious grooming behavior in the first 5 minutes and last 5minutes of the test. Relaxed grooming was almost next to nothing. This clearly shows that the TBI group displayed high levels of anxiety. Comparatively, the

groups treated with Umbelliferone (5mg and 30mg) showed exploratory activity by spending time in the central region. The group treated with Umbelliferone (5mg) displayed rearing behaviour better than the TBI group. Both the groups treated with Umbelliferone (5mg and 30mg) displayed anxious grooming levels lesser than the TBI group and the group treated with Ibuprofen. Relaxed grooming was best observed in the Umbelliferone groups and the Sham group. There were no reports of relaxed grooming in the TBI group indicating high levels of anxiety. It has been previously reported that Umbelliferone, its analogues and derivatives have a positive effect on anxiety and can be used to treat anxiety (Mahendra and Bisht, 2011) and hence it can be proposed here that Umbelliferone dosage may have a positive effect in reducing anxiety.

In order to assess the social behaviour, the Social Novelty test was performed. Strong interaction with the stranger mouse explains the sociable nature of the mice. According to literature, interaction with the stranger mouse is seen to be more rather than with the empty cage (File and Seth, 2003). It was observed in this experiment that all groups spend time interacting with the Stranger Mouse 1 and not with the empty cage. Social novelty is a part of the normal behavior of mice as they are sociable animals. In Session 2, all groups favored interacting with the stranger mouse 2 rather than the stranger mouse 1. Comparatively it was observed that the TBI group interacted the least in both the sessions indicating that post TBI there is a decreased likeliness to be sociable as cited previously in literature that TBI patients suffer from social isolation (Schwarzbold et al., 2008). In comparison both the Umbelliferone groups showed better interaction than the TBI group although no significant difference was observed. It can be assumed that Umbelliferone administration might have a positive effect on the affected mice as

previously has been reported that Umbelliferone has proven to be helpful in reversing depression (Qin et al., 2017).

Fanselow and Gale state that fear memory is very quickly learned, is stable and can be studied finely (Fanselow and Gale, 2003). The two brain regions, amygdala and hippocampus, are involved in acclimatization of fear conditioning (Sehlmeyer et al., 2009) and in contextual memory (Sehlmeyer et al., 2009), respectively. In this study it was observed that there was no fear memory development across the five tones except the fourth tone trial. It may be possible that the mice did not develop memory may be due to attentional problems occurred due to stress (Park et al., 2001). It was observed that there is no significant difference in development of contextual fear but a very slight trend was observed of memory acquisition in the Umbelliferone treated groups.

Novel object test is performed to assess the learning and memory formation facilitated by the cortical and hippocampal region (Daenen et al., 2002). Rodents are prone to show more interaction towards the novel object rather than the known object. Time spent interacting and exploring the novel object distinguishes Object recognition (Bevins and Besheer, 2006). As already discussed in previous literature that TBI patients suffer from memory issues (Ghajar, 2000), our results showed TBI group had lesser interactions in comparison to other groups. Although there was no significant difference, it was observed that Umbelliferone treated groups exhibited interaction with the novel object rather than the familiar one indicating possible chances of improvement in recognition memory.

Elevated plus maze test involves cortex, hippocampus and amygdala dependent learning (Daenen et al., 2002). This study witnessed that the anxiety levels are slightly reduced post Umbelliferone administration. Previous literature cites that Umbelliferone and its derivatives reverse depression and also help in lowering anxiety levels (Mahendra and Bisht, 2011, Qin et al., 2017). This suggests that Umbelliferone and its derivatives might be involved in helping deal with depression and anxiety.

Light dark test is yet another test which assesses the anxiety in rodents. The TBI group displayed highest level of anxiety amongst the groups. The trends of TBI and Ibuprofen showed significant difference ($p<0.01$). As already discussed above, previous literature cites use of Umbelliferone in dealing with anxiety, we can say that even though there is no significant difference, a slight trend of improvement is evident post Umbelliferone treatment.

The neurological severity score assess various deficits encountered by the TBI model. In this study, post trauma, motor impairments along with sensory deficits were assessed along with the possibility of edema. It has been previously reported in literature that a NSS Score of 14-15 indicated injury and neurological impairment (Schaar et al., 2010, Kong et al., 2017). 4h post injury, the TBI group showed a score of 15 indicating neurological impairment. Gradually this score declined to 12 indicating that there is gradual improvement as discussed previously by Kong et al (Kong et al., 2017)

Umbelliferone has been reported to have neuroprotective effects (Subramaniam and Ellis, 2013). It acts as an anxiolytic as well as enhances the memory, controls neuronal apoptosis (Subramaniam and Ellis, 2013), reduces inflammation and has also been

reported to exhibit anti-nociceptive properties. The results of this study show that the groups treated with Umbelliferone, especially at 5mg dose, showed a NSS score of 6 at 4hours post injury. Gradually it reduced to a score of 5 indicating improvement by the end of 2 weeks. It can be concluded from here that Umbelliferone might have neuroprotective effects on TBI model.

Wang et al have previously reported that Umbelliferone improves brain edema (Wang et al., 2015). 48h post injury it was observed that TBI group displayed increased cortical and hippocampal levels. By the end of two weeks Umbelliferone group (30mg) showed considerably lower levels of edema in cortex ($p<0.05$) and also in hippocampus although significant difference was not witnessed in the hippocampal region.

CONCLUSION

Traumatic Brain Injury is a global concern worldwide with a very high mortality rate and leads to devastating cognitive, motor and psychological results. This study discussed the neuroprotective effects of Umbelliferone on Traumatic brain injury mouse model. Although there was hardly any significant difference observed, there were seen trends of improvement post Umbelliferone administration. In our estimation there needs to be conducted further future research to study the effectiveness of Umbelliferone in TBI and also the risks associated with it. It is imperative to know the negative aspects and effects of Umbelliferone as well to prevent its usage built upon inadequate information.

CHAPTER 5: REFERENCES

Alexander MP (1995) Mild traumatic brain injury: pathophysiology, natural history, and clinical management. Neurology 7.

Arendash GW, Lewis J, Leighty RE, McGowan E, Cracchiolo JR, Hutton M, Garcia MF (2004) Multi-metric behavioral comparison of APPsw and P301L models for Alzheimer's disease: linkage of poorer cognitive performance to tau pathology in forebrain. Brain research 1012:29-41.

Bevins RA, Besheer J (2006) Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study'recognition memory'. Nature protocols 1:1306-1311.

Bigler ED, Weiner M, Lipton A (2009) Traumatic brain injury. Textbook of Alzheimer Disease and Other Dementias 229-246.

Bradley JD, Brandt KD, Katz BP, Kalasinski LA, Ryan SI (1991) Comparison of an antiinflammatory dose of ibuprofen, an analgesic dose of ibuprofen, and acetaminophen in the treatment of patients with osteoarthritis of the knee. New England Journal of Medicine 325:87-91.

Carney N, Totten AM, O'reilly C, Ullman JS, Hawryluk GW, Bell MJ, Bratton SL, Chesnut R, Harris OA, Kissoon N (2017) Guidelines for the management of severe traumatic brain injury. Neurosurgery 80:6-15.

Clark RS, Bayir H, Chu CT, Alber SM, Kochanek PM, Watkins SC (2008) Autophagy is increased in mice after traumatic brain injury and is detectable in human brain after trauma and critical illness. Autophagy 4:88-90.

Council NR (2010) Guide for the care and use of laboratory animals: National Academies Press.

Crockard A, Hayward R, Hoff JT (2000) Neurosurgery, the Scientific Basis of Clinical Practice: Blackwell Scientific Publications.

Daenen EW, Wolterink G, Gerrits MA, Van Ree JM (2002) The effects of neonatal lesions in the amygdala or ventral hippocampus on social behaviour later in life. Behavioural brain research 136:571-582.

de Lima FO, Nonato FR, Couto RD, Barbosa Filho JM, Nunes XP, Ribeiro dos Santos R, Soares MBP, Villarreal CF (2011) Mechanisms involved in the antinociceptive effects of 7-hydroxycoumarin. Journal of natural products 74:596-602.

Dutton RP, McCunn M (2003) Traumatic brain injury. Current opinion in critical care 9:503-509.

El Fadl MHA, O'Phelan KH Management of Traumatic Brain Injury. Neurologic Clinics.

Erlich S, Alexandrovich A, Shohami E, Pinkas-Kramarski R (2007) Rapamycin is a neuroprotective treatment for traumatic brain injury. Neurobiology of disease 26:86-93.

Escobedo LVS, Habboushe J, Kaafarani H, Velmahos G, Shah K, Lee J (2013) Traumatic brain injury: A case-based review. World journal of emergency medicine 4:252.

Essa MM, Akbar M, Guillemin G (2016) The Benefits of Natural Products for Neurodegenerative Diseases: Springer.

Fanselow MS, Gale GD (2003) The amygdala, fear, and memory. Annals of the New York Academy of Sciences 985:125-134.

Farhat SM, Mahboob A, Ahmed T (2017) Cortex-and Amygdala-Dependent Learning and Nicotinic Acetylcholine Receptor Gene Expression is Severely Impaired in Mice Orally Treated with AlCl3. Biological trace element research 1-11.

File SE, Seth P (2003) A review of 25 years of the social interaction test. European journal of pharmacology 463:35-53.

Finnie J, Blumbergs P (2002) Traumatic brain injury. Veterinary pathology 39:679-689.

Gentry LR (1994) Imaging of closed head injury. Radiology 191:1-17.

Ghajar J (2000) Traumatic brain injury. The Lancet 356:923-929.

Greenaway MC, Lacritz LH, Binegar D, Weiner MF, Lipton A, Cullum CM (2006) Patterns of verbal memory performance in mild cognitive impairment, Alzheimer disease, and normal aging. Cognitive and Behavioral Neurology 19:79-84.

Greve MW, Zink BJ (2009) Pathophysiology of traumatic brain injury. Mount Sinai Journal of Medicine: A Journal of Translational and Personalized Medicine 76:97-104.

Hallbergson AF, Gnatenco C, Peterson DA (2003) Neurogenesis and brain injury: managing a renewable resource for repair. Journal of Clinical Investigation 112:1128.

HAMM RJ, DIXON CE, GBADEBO DM, SINGHA AK, JENKINS LW, LYETH BG, HAYES RL (1992) Cognitive deficits following traumatic brain injury produced by controlled cortical impact. Journal of neurotrauma 9:11-20.

Hawthorne C, Piper I (2014) Monitoring of intracranial pressure in patients with traumatic brain injury. Frontiers in neurology 5.

Hayes RL, Dixon CE (1994) Neurochemical changes in mild head injury. In: Seminars in Neurology, vol. 14, pp 25-31: © 1994 by Thieme Medical Publishers, Inc.

Heegaard W, Biros M (2007) Traumatic brain injury. Emergency medicine clinics of North America 25:655-678.

Hickey J (2013) Clinical practice of neurological & neurosurgical nursing: Lippincott Williams & Wilkins.

Iqbal G, Iqbal A, Mahboob A, M Farhat S, Ahmed T (2016) Memory enhancing effect of black pepper in the AlCl3 induced neurotoxicity mouse model is mediated through its active component chavicine. Current pharmaceutical biotechnology 17:962-973.

JOHNSON VE, MEANEY DF, CULLEN DK, SMITH DH (2015) Animal models of traumatic brain injury. Handbook of clinical neurology 127:115.

Kaidanovich-Beilin O, Lipina T, Vukobradovic I, Roder J, Woodgett JR (2011) Assessment of social interaction behaviors. Journal of visualized experiments: JoVE.

Kanimozhi G, Prasad NR, Ramachandran S, Pugalendi K (2012) Umbelliferone protects whole-body irradiated Swiss albino mice: Study on animal survival, tissue antioxidant status and DNA damage. Biomedicine & Preventive Nutrition 2:186-192.

Kochanek PM, Berger RP, Fink EL, Au AK, Bayır H, Bell MJ, Dixon CE, Clark RS (2013) The potential for bio-mediators and biomarkers in pediatric traumatic brain injury and neurocritical care. Frontiers in neurology 4.

Kong X, Guan J, Gong S, Wang R (2017) Neuroprotective Effects of Grape Seed Procyanidin Extract on Ischemia-Reperfusion Brain Injury. Chinese Medical Sciences Journal 32:92-99.

Leal L, Ferreira A, Bezerra G, Matos F, Viana G (2000) Antinociceptive, anti-inflammatory and bronchodilator activities of Brazilian medicinal plants containing coumarin: a comparative study. Journal of Ethnopharmacology 70:151-159.

Lin T-N, He YY, Wu G, Khan M, Hsu CY (1993) Effect of brain edema on infarct volume in a focal cerebral ischemia model in rats. Stroke 24:117-121.

Lippert-Grüner M, Kuchta J, Hellmich M, Klug N (2006) Neurobehavioural deficits after severe traumatic brain injury (TBI). Brain injury 20:569-574.

Loane DJ, Pocivavsek A, Moussa CE, Thompson R, Matsuoka Y, Faden AI, Rebeck GW, Burns MP (2009) Amyloid precursor protein secretases as therapeutic targets for traumatic brain injury. Nature medicine 15:377-379.

Maas AI, Stocchetti N, Bullock R (2008) Moderate and severe traumatic brain injury in adults. The Lancet Neurology 7:728-741.

Mahendra P, Bisht S (2011) Anti-anxiety activity of Coriandrum sativum assessed using different experimental anxiety models. Indian journal of pharmacology 43:574.

Malec JF, Brown AW, Leibson CL, Flaada JT, Mandrekar JN, Diehl NN, Perkins PK (2007) The Mayo classification system for traumatic brain injury severity. Journal of neurotrauma 24:1417-1424.

Mangat HS (2012) Severe traumatic brain injury. Continuum: lifelong learning in neurology 18:532-546.

Mazimba O (2017) Umbelliferone: Sources, chemistry and bioactivities review. Bulletin of Faculty of Pharmacy, Cairo University.

McDonald BC, Flashman LA, Saykin AJ (2002) Executive dysfunction following traumatic brain injury: neural substrates and treatment strategies. NeuroRehabilitation 17:333-344.

Morales D, Marklund N, Lebold D, Thompson H, Pitkanen A, Maxwell W, Longhi L, Laurer H, Maegele M, Neugebauer E (2005) Experimental models of traumatic brain injury: do we really need to build a better mousetrap? Neuroscience 136:971-989.

Mussack T, Biberthaler P, Kanz K-G, Wiedemann E, Gippner-Steppert C, Mutschler W, Jochum M (2002) Serum S-100B and interleukin-8 as predictive markers for comparative neurologic outcome analysis of patients after cardiac arrest and severe traumatic brain injury. Critical care medicine 30:2669-2674.

Negishi T, Kawasaki K, Sekiguchi S, Ishii Y, Kyuwa S, Kuroda Y, Yoshikawa Y (2005) Attention-deficit and hyperactive neurobehavioural characteristics induced by perinatal hypothyroidism in rats. Behavioural brain research 159:323-331.

Nolan S (2005) Traumatic brain injury: a review. Critical care nursing quarterly 28:188-194.

Pan L, Li X-z, Yan Z-q, Guo H-r, Qin B (2015) Phytotoxicity of umbelliferone and its analogs: Structure–activity relationships and action mechanisms. Plant Physiology and Biochemistry 97:272-277.

Pangilinan P Classification and Complications of Traumatic Brain Injury: Medscape: Drugs & Diseases [Internet];[updated Feb 13, 2017; cited Mar 06, 2017].

Park CR, Campbell AM, Diamond DM (2001) Chronic psychosocial stress impairs learning and memory and increases sensitivity to yohimbine in adult rats. Biological psychiatry 50:994-1004.

Pillai SP, Menon SR, Mitscher LA, Pillai CA, Shankel DM (1999) Umbelliferone analogues and their potential to inhibit Benzo (a) pyrene-and hydrogen peroxide-induced mutations. Journal of natural products 62:1358-1362.

Povlishock JT (1993) Pathobiology of traumatically induced axonal injury in animals and man. Annals of emergency medicine 22:980-986.

Preston AR, Eichenbaum H (2013) Interplay of hippocampus and prefrontal cortex in memory. Current Biology 23:R764-R773.

Qin T, Fang F, Song M, Li R, Ma Z, Ma S (2017) Umbelliferone reverses depression-like behavior in chronic unpredictable mild stress-induced rats by attenuating neuronal apoptosis via regulating ROCK/Akt pathway. Behavioural brain research 317:147-156.

Ramesh B, Pugalendi KV (2007) Influence of umbelliferone on membrane-bound ATPases in streptozotocin-induced diabetic rats. Pharmacological reports 59:339.

Roof RL, Duvdevani R, Stein DG (1992) Progesterone treatment attenuates brain edema following contusion injury in male and female rats. Restorative neurology and neuroscience 4:425-427.

Saatman KE, Duhaime A-C, Bullock R, Maas AI, Valadka A, Manley GT (2008) Classification of traumatic brain injury for targeted therapies. Journal of neurotrauma 25:719-738.

Schaar KL, Brenneman MM, Savitz SI (2010) Functional assessments in the rodent stroke model. Experimental & translational stroke medicine 2:13.

Schwarzbold M, Diaz A, Martins ET, Rufino A, Amante LN, Thais ME, Quevedo J, Hohl A, Linhares MN, Walz R (2008) Psychiatric disorders and traumatic brain injury. Neuropsychiatric disease and treatment 4:797.

Sehlmeyer C, Schöning S, Zwitserlood P, Pfleiderer B, Kircher T, Arolt V, Konrad C (2009) Human fear conditioning and extinction in neuroimaging: a systematic review. PloS one 4:e5865.

Sim M-O, Lee H-I, Ham JR, Seo K-I, Kim M-J, Lee M-K (2015) Anti-inflammatory and antioxidant effects of umbelliferone in chronic alcohol-fed rats. Nutrition research and practice 9:364-369.

Singhal A, Baker A, Hare G, Reinders F, Schlichter L, Moulton R (2002) Association between cerebrospinal fluid interleukin-6 concentrations and outcome after severe human traumatic brain injury. Journal of neurotrauma 19:929-937.

Subramaniam SR, Ellis EM (2013) Neuroprotective effects of umbelliferone and esculetin in a mouse model of Parkinson's disease. Journal of neuroscience research 91:453-461.

Takao K, Miyakawa T (2006) Light/dark transition test for mice. Journal of visualized experiments: JoVE.

Udekwu P, Kromhout-Schiro S, Vaslef S, Baker C, Oller D (2004) Glasgow Coma Scale score, mortality, and functional outcome in head-injured patients. Journal of Trauma and Acute Care Surgery 56:1084-1089.

Vasconcelos JF, Teixeira MM, Barbosa-Filho JM, Agra MF, Nunes XP, Giulietti AM, Ribeiro-dos-Santos R, Soares MB (2009) Effects of umbelliferone in a murine model of allergic airway inflammation. European journal of pharmacology 609:126-131.

Wakai A, McCabe A, Roberts I, Schierhout G (2013) Mannitol for acute traumatic brain injury. The Cochrane Library.

Wang X, Li R, Wang X, Fu Q, Ma S (2015) Umbelliferone ameliorates cerebral ischemia–reperfusion injury via upregulating the PPAR gamma expression and suppressing TXNIP/NLRP3 inflammasome. Neuroscience letters 600:182-187.

Weiner MF, Lipton AM (2009) The American Psychiatric Publishing textbook of Alzheimer disease and other dementias: American Psychiatric Pub.

Williamson DJ, Scott JG, Adams RL (1996) Traumatic brain injury; Neuropsycyhology for Clinical Practice: Etiology, Assessment and Treatment. Washington DC: American Psychological Association.

Xiong Y, Mahmood A, Chopp M (2013) Animal models of traumatic brain injury. Nature Reviews Neuroscience 14:128-142.

Xu W, Südhof TC (2013) A neural circuit for memory specificity and generalization. Science 339:1290-1295.