NEUROPHARMACOLOGICAL EVALUATION OF PAEONIFLORIN ON COGNITIVE FUNCTION 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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Dedicated to

My Parents

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All praise 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.

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LIST OF ACRONYMS

ACh	Acetylcholine
ANOVA	Analysis of Variance
CBF	Cerebral Blood Flow
ChAT	Choline Acetyltransferase
CNS	Central Nervous System
CPP	Cerebral Perfusion Pressure
DI	Discrimination Index
DW	Dry Weight
GCS	Glasgow Comma Scale
IBU	Ibuprofen
ICP	Intracranial Pressure
MWM	Morris Water Maze
NSS	Neurological Severity Score
PF	Paeoniflorin
SEM	Standard Error of the Mean
TBI	Traumatic Brain Injury
WW	Wet Weight

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ABSTRACT

Traumatic brain injury (TBI) is a major reason of mortality around the world. In this investigation the effect of Paeoniflorin (PF) was tested against TBI in mice. Two doses 5mg and 40mg of PF were used. The design of the experiment included five groups of Balb/c mice. Except the sham group the four groups (TBI, TBI+ IBU, TBI+PF 5mg and TBI+PF 40mg) were induced with the focal head injury using the weight-drop technique. Neurological severity score (NSS), measurement of edema and acetylcholine levels, behavioral tests like Morris water maze (MWM), Novel object recognition (NOR), social interaction test, Elevated plus maze (EPM), Light and dark box (LDB) were conducted. The result of each test was presented by a graph. The NSS results were recorded at intervals of 4h and 72h showing a significant difference in the severity score of sham group and TBI+PF (40mg) group. Edema was determined in the hippocampus and the cortex at 48h and 288h both revealing no significant difference. Acetylcholine levels were measured in hippocampus and cortex at 48h and 288h, depicting significantly more amount of acetylcholine levels in TBI+PF (40mg) group. The graph of Morris water maze test revealed an improved spatial learning in mice injected with 40mg of paeoniflorin while no improvement was recorded in the TBI group. The novel object recognition test revealed no significant difference. Social behavior test revealed an increase in social abilities of mice for both 5mg and 40mg dosage. In elevated plus maze test the paeoniflorin dosage reduced the anxiety levels compared to TBI group. Similarly, light and dark box test also revealed that 5mg dosage of paeoniflorin reduces anxiety in mice. Further studies on such anti-inflammatory drugs may result in reducing the mortality rate in TBI patients.

CHAPTER 1: INTRODUCTION

1.1 TRAUMATIC BRAIN INJURY

A leading cause of death and disability around the world is traumatic brain injury (TBI). The problem experienced by those suffering TBI, for example, impairment in memory or cognition, are visible, it has been eluded as the 'silent epidemic' (Langlois et al., 2006). TBI has been reported in over million people annually resulting in death and hospitalization. It has been reported that almost 60% of TBIs are because of the road traffic injuries (RTIs) in all parts of the world; around 20-30% are because of falls; 10% because of violence, and another 10% because of mix of work place and sports related wounds (Gururaj, 2002).TBI is a damage to brain tissue that causes retardation in function of brain (Parikh et al., 2007). The major reason may include harm to the head which affects the activity of the brain. The possible result in TBI patient after recovery is that the brain shows reduced and abiding thinking ability and dementia (Weiner and Lipton, 2009).TBI is multiplex, attributing injury to distinct brain areas caused by both the initial injury and ancillary events (Dombovy, 2011). The first injury is the mechanical or physical force exerted that causes the tearing of the vessels and neurons (Andriessen et al., 2010). It is followed by the secondary injury which is a result of molecular, cellular and metabolic activities that causes brain cell death (Marklund et al., 2006, Bramlett and Dietrich, 2007).

The medical conditions differ in different cases and different mechanisms which makes its treatment a difficult task. Even though the life expectancy of victims of TBI have increased over the years after a great deal of research but reports suggest that the patients

later in life suffer physically and mentally a lower quality of life. Depression is said to be the most common psychtriatic disorder (Raghavendra Rao et al., 2003).

1.2 CLASSIFICATION OF TRAUMATIC BRAIN INJURY

The possible categories of TBI are clinical, pathological and mechanistic. Mechanistic category of TBI includes impact, closed and penetrating brain injuries. Anatomical or pathological category describes focal or diffused injuries while pathophysiological injuries can be classified as primary and secondary injuries (as shown in Figure1.1) (Silver et al., 2011).

i. Mechanical Injury

Impact head injuries include coordinated head impacts utilizing a cylinder, pistons, pendulums and weight drop onto the skull. The focal mechanical stacking causes the twisting of brain that is practically incompressible, is especially vulnerable to strain damage (Finnie and Blumbergs, 2002).

In penetrating injuries the substance passes into the cranium but it doesn't exit; the pathology created is especially dictated by the nature of the object. Metals, knives and sharp objects harm the skull causing hemorrhage and necrosis. The pressure cavities are also produced when the object travels through the parenchyma leading to tissue damage. Studies conducted on animal model indicate that penetrating injuries initiates localized damages and inflammatory responses; cytokine expression and neutrophilic infiltration are seen in the acute phase; while white matter degeneration is seen in delayed responses developing after few days of injury (Williams et al., 2006).

ii. Focal and Diffused Brain Injury

It is caused by concussion which creates a pressure on the tissue beneath the skull at the specific area or on the opposite side of the effect (Pudenz and Shelden, 1946).

The area and the severity of effect to the skull decide the neurological inadequacy and cerebral pathology. Focal damage constitutes subdural and epidural hematomas, intraparenchymal hematomas and hemorrhagic injuries. Traumatic subarachnoid hemorrhage can be a consequence of focal injury but on the other hand it's often found in more diffuse vascular damage. Diffuse brain damage involves disseminated harm to axons, diffuse vascular damage, hypoxic-ischemia and edema (Gennarelli et al., 1982, Gennarelli, 1983).

iii. Pathophysiological Injury

A primary injury causes tissue deformation at that exact time. The impairments amend vessels, axons, neurons and glia focally or in a diffused pattern, initiating dynamic processes like inflammation, neurochemical and metabolic alterations.

A secondary injury occurs due to complications of primary brain damage; it involves ischemic and hypoxic damage and swellings, the repercussion of raised intracranial pressure, hydrocephalus, and infections (Finnie and Blumbergs, 2002).

Numerous clinical classifications have been developed over the years in terms of severity, but the most commonly and widely used is the Glasgow Coma Scale (GCS). TBI was categorized in mild, moderate and severe in terms of head injuries. GCS is based on following parts including eye opening, verbal response and motor response; summed to get total score. 13-15 scores for mild, 9-12 scores for moderate while, 8 or less scored for severe head injury (Teasdale and Jennett, 1974).

Important factors for treating TBI are classification through outcomes. Outcome can be assessed in wide ranges through **Glasgow Outcome Scale (GOS)**, **neuropsychological working, and mood**. Dimensions like behavior, neuropsychiatric issues can also be measured through different scales (Tate, 2010) Originally, Glasgow Outcome Scale has been classified in these parameters – dead, vegetative state, severe disability, moderate disability and good recovery (Jennett and Bond, 1975).

1.3 TBI PATHOPHYSIOLOGY

The principal phases of cerebral damage following TBI described by coordinate tissue harm and weakened direction of cerebral blood flow (CBF) and digestion. This 'ischemia-like' pattern prompts amassing of lactic acid because of glycolysis in the absence of oxygen, increased permeability of the brain followed by edema development.

The second phase of the pathophysiological course is portrayed by terminal layer depolarization with the excessive release of glutamate, aspartate, actuation of N-methyl-d-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazolpropionate and voltage-subordinate Ca^{2+}-and Na^{+}-channels.

From initial to final stages after TBI, CBF within a temporal range could be assessed by using different techniques (Inoue et al., 2005) like xenon scintillation detection (^{33}X), xenon computed tomography (^{133}Xe-CT), stable xenon CT (Bouma et al., 1992) or oxygen($^{15}O_{2)}$ positron emission CT (Coles et al., 2004b) revealing that focal or global cerebral ischemia occurs frequently.

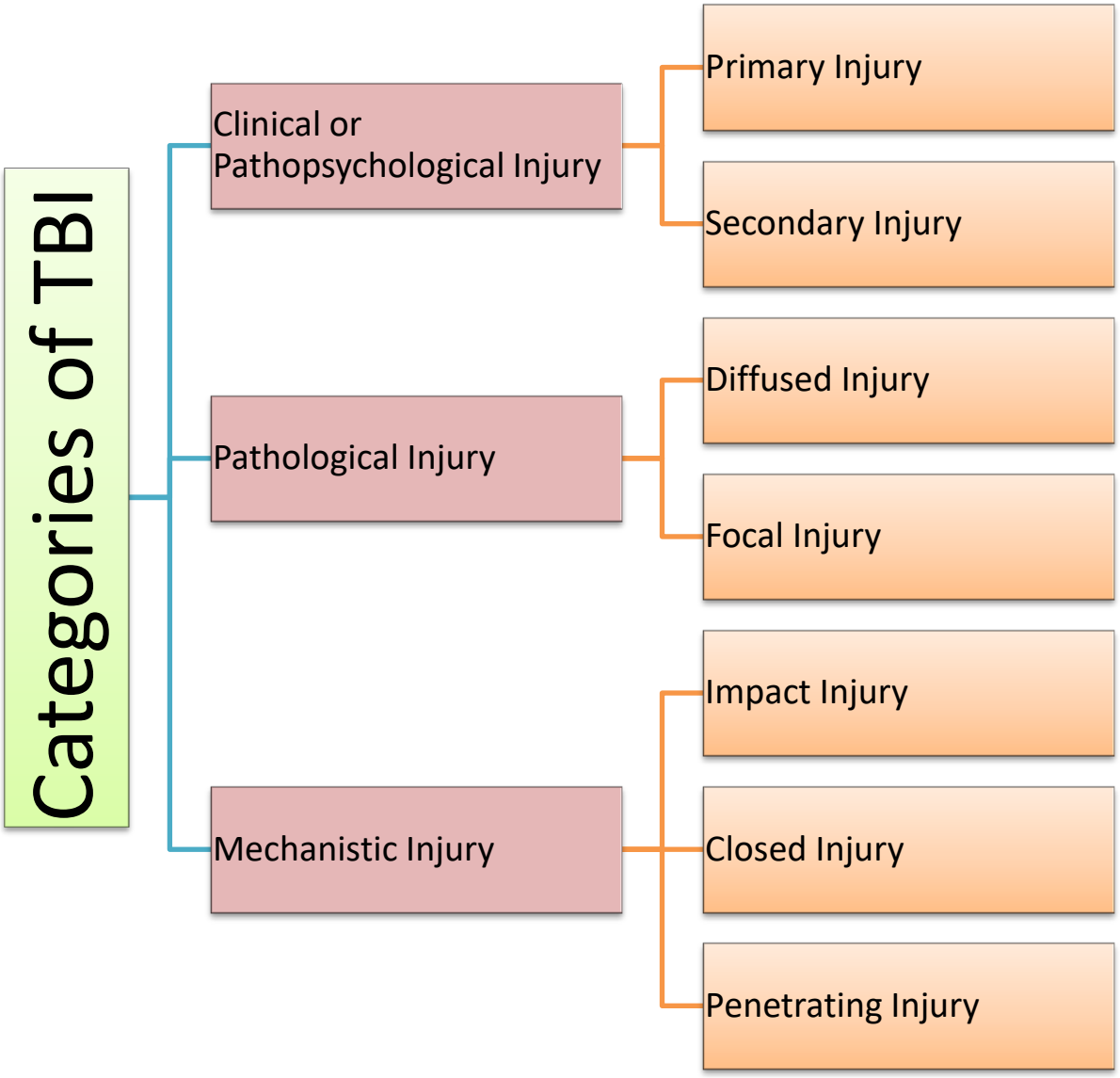


Figure 1.1 The three categories of TBI

Table 1.1 The three components of GCS

Eye Opening	Spontaneous	4
	Speech	3
	Pain	2
	None	1
Verbal Response	Oriented	5
	Confused Conversation	4
	Inappropriate Words	3
	Incomprehensible Sound	2
	None	1
Motor Response	Obey Command	6
	Localize Pain	5
	Normal Flexion	4
	Abnormal Flexion	3
	Extension	2
	None	1

1.3.1 Cerebral Ischemia

Ischemic brain accounts for an average of 10% by volume (Coles et al., 2004a). Cerebral ischaemia transcendently prompts metabolic anxiety and ionic irritations; head injury also uncovers the brain tissue causing a continuous damage to cell bodies, astrocytes, microglia, cerebral microvascular and endothelial cell (DeWitt and Prough, 2003, Bramlett and Dietrich, 2004).

The components that results in post-traumatic ischaemia incorporates morphological damage like vessel twisting due to mechanical relocation, hypotension within the sight of autoregulatory failure (McIntosh et al., 1996, Rodríguez-Baeza et al., 2003) and deficient accessibility of nitric oxide or cholinergic neurotransmitters (Scremin et al., 1997, DeWitt and Prough, 2003).

1.3.2 Caspase Activation

Caspases activation (ICE-like proteins), translocases, and endonucleases start dynamic auxiliary changes in biologic membranes and the nucleosomal DNA (DNA fracture and restraint of DNA repair) (Werner and Engelhard, 2007).

1.3.3 Hyperperfusion, Hyperaemia and Cerebral Ischemia:

The TBI patients may create cerebral hyperperfusion in the beginning times of damage (Kelly et al., 1996). Similarly, hyperaemia may take after instant post-traumatic ischaemia (Sakas et al., 1995). In this case there is an increase in Cerebral Blood Flow (CBF) resulting in vasoparalysis and an excessive accumulation of cerebral blood which causes an intracranial pressure (ICP) (Kelly et al., 1997). Cerebral ischemia and hyperaemia allude to confound amongst CBF and cerebral digestion; an increased

metabolic rate refers to ischaemic circumstance while decreased metabolic rate refers to cerebral hyperaemia (Bouma and Muizelaar, 1992, Martin et al., 1997). .

1.3.4 Cerebral Autoregulation and Carbon Dioxide Reactivity

It is an essential mechanism to give sufficient CBF whenever required. They administer cerebral perfusion pressure and ICP and debilitation of regulatory mechanism reflecting an increased risk for auxiliary brain harm (Hlatky et al., 2002). Autoregulation of CBF is weakened or annulled in many patients resulting in serious cerebrum damage and reactivity of carbon-dioxide is disabled (Enevoldsen and Jensen, 1978).

1.3.5 Edema

Edema pattern is frequently observed post-injury. In brain edema a prevailing division is identified with structural harm or an imbalance in the osmosis. Mechanical interruption and the malfunctioning of the endothelial layers cause Vasogenic brain edema. Breaking down of endothelial walls considers excessive ion and protein exchange between intravascular to the extracellular brain divisions guaranteeing water accumulation (Unterberg et al., 2004). Intracellular water accumulation in neurons, astrocytes and microglia occurs in cytotoxic brain edema. This occurs by expansion in the membrane of the cell, enhanced exchange of ions, exhaustion of ionic pumps and cell reabsorption of osmotically dynamic solutes (Stiefel et al., 2005).

1.3.6 Cerebral Vasospasm

Cerebral vasospasm after trauma is an essential secondary affront which decides ultimate final condition of a patient (Lee et al., 1997). Depolarization in the vascular smooth muscle is incorporated by vasospasm due to diminished K^+ ion movement (Sobey, 2001),

discharge of endothelin alongside decreased accessibility of nitric oxide,(Zuccarello et al., 1998) cyclic GMP exhaustion (Todo et al., 1998) and free radical development.

1.3.7 Cerebral Metabolism

The minimization in cerebral metabolism is identified with speedy affront prompting dysfunctioning in mitochondria accompanied by decreased respiratory rates, production of ATP, a diminished accessibility in the co-enzyme pool of nicotine, and intra-mitochondrial calcium over-burden (Tavazzi et al., 2005).

1.3.8 Immunological/ Inflammatory Response

A primary and secondary damage initiates release of cellular mediators like cytokines, prostaglandins, free radicals and supplements. Thus activating adhesion molecules and chemokines which further activates the immune and glial cells in a parallel and synergistic form (Potts et al., 2006). Proinflammatory enzymes are upregulated within few hours after damage. An advancement in damage is identified either by the direct or indirect release of neurotoxic mediators or cytokines and nitric oxides respectively. Discharge of vasoconstrictors, the blood–brain boundary injury and edema development additionally decrease tissue perfusion and subsequently results in secondary damage.

1.3.9 Excitotoxicity

The excessive release of excitatory neurotransmitters results in a series of pathological events called excitotoxicity (Bullock et al., 1998). Glutamate, a neurotransmitter, and its extracellular concentration turn out to be essentially expanded after damage. The level of glutamate expands almost fifty folds particularly in the parenchymal focal wounds (Bullock et al., 1995).

1.3.10 Glutamate as excitatory neurotransmitter

The pre-synaptic vesicles discharge glutamate after depolarization. Moreover, the typical glutamate re-take-up by astrocytes, by means of an ATP-subordinate Na^+-cotransport framework, diminishes because of annihilation and energy consumption of neighboring astrocytes. Exorbitant extracellular glutamate starts excessive influx of calcium and sodium ions into neurons and glial cells (Choi, 1987). It ties the N-methyl-D-aspartate aspartic corrosive and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic corrosive (AMPA) receptors bringing about enactment of ion channels in charge of calcium and sodium influx (Faden et al., 1989).

1.3.11 Necrosis and Apoptosis

Apoptosis and necrosis occurs simultaneously in traumatic brain tissues. Necrosis in brain cells does not need energy, at the sight of ATP cell death occurs. The event of cell death is based on the levels of calcium (Choi, 1996).

Generally, less calcium at the intracellular site may support death of the cell and more calcium advance necrosis. In tissue with broad mitochondrial decimation and energy exhaustion for the most part necrosis will be found in cells (Ankarcrona et al., 1995).

1.4 CHOLINERGIC SYSTEM AND ITS ROLE IN TBI AND LEARNING

Acetylcholine (ACh) is the essential neurotransmitter of the parasympathetic sensory system. Acetylcholine receptors are conspicuously expressed in immune cells and those cells from bone marrow (lymphoid and myeloid cells) (Jonge and Ulloa, 2007).

Acetylcholine is important to the CNS and it's synthesized by an enzyme Choline Acetyltransferase. Driven by the trans-vesicular proton gradient linked to an ATPase enzyme, acetylcholine is accumulated in the synaptic vesicles(Parsons, 2000). ACh is hydrolyzed in milliseconds by acetylcholinesterase or butyrylcholinesterase, producing acetic acid and choline (Mesulam et al., 1983).

1.4.1 Acetylcholine Receptors:

Two main classes of acetylcholine receptors include: muscarinic receptors and nicotinic receptors. The receptors are dispersed in the CNS and its periphery, exhibiting various functions in cholinergic transmission due to various synaptic locations (Pavlov and Tracey, 2006).

1.5 ANIMAL MODEL

Over the years animal model studies have been carried copying human TBI conditions to understand the human physiological disorders efficiently so that better treatments can be established (Kabadi et al., 2010). The model animals are necessary to study the molecular, cellular and biochemical processes occurring in TBI. No single animal can exactly copy the human TBI aspects, but under various controlled conditions different investigative studies can be carried out.

The large sized animals resemble humans in physiology and size. But in small sized animals (rodents) providing a good model for TBI study is a difficult challenge still they are preferred because they are more cost effective and smaller in size.

The preclinical trials for TBI are successful in animals but failing clinically (Kabadi et al., 2010) as it's difficult to produce brain injury in animals which will produce conditions similar to that in humans. One reason for this is the differences in the brain sizes of the research animals and the humans. Gyrencephalic animals (based on cortical folding includes primates and ferrets) for TBI modelling are more relevant than Lissencephalic species (mice- have smooth surface cortices) as they are possible to be used to produce traumatic axonal injury (TAI) which is important in human TBI (Denny-Brown and Russell, 1941, Gurdjian et al., 1954). .

A limitation in brain injury studies is that even though similarities do exists between rodent and human brain but there are various differences like brain functionality, size and amount of grey matter (Laurer and McIntosh, 1999, Morales et al., 2005).

Important factors like age and gender differences are commonly ignored in animal model studies. This leads to different responses by the brain to the trauma (POVLISHOCK et al., 1994). The behavioral responses also change among different mouse strains (Fox and Faden, 1998). In animals and humans the difference in genders also showcases different responses (ROOF and HALL, 2000).

1.6 IN VIVO METHODS OF TRAUMATIC BRAIN INJURY

The in-vivo methods of TBI includes,

1.6.1 Weight Drop Method

A pioneer and standard model for TBI, utilizes the gravitational powers of a free falling and guided weight to create a focal damage to brain. The anesthetized mice (Chen et al., 1996), or rat (Feeney et al., 1981), is appended to colliding objects or base of the injury

gadget and the skull is uncovered independent of craniotomy. The severeness of cerebral damage may effectively be controlled through the modification of the stature and weight utilized in damage. The weight drop exhibits fast, simple and advantageous method. One of the inconvenience includes the expanded likelihood of cranium cracks at larger magnitude of severity of the injury, and also the likelihood of a rebound injury (Feeney et al., 1981).

1.6.2 Closed Cortical Impact (CCI Model)

It utilizes a firm impactor to bestow mechanical energy conveyed to dura. This damage initially portrayed in ferrets. It was later adjusted for testing in rodent, mouse and sheep and the site of damage has been set both over the midline. The upsides of this damage display the simplicity of the control disfigurement parameters like time, speed, and profundity of effect, and along these line the capacity of damage severity to be controlled. Additionally, the comparison of this model to gravity-driven device is the absence from rebound injury (Dixon et al., 1991).

1.6.3 Midline Fluid Percussion Injury (mFLP)

The midline fluid percussion model of mind damage was at first created to test in cats and rabbit (Hayes et al., 1988). The skull exposure and trephination is performed over the midline. After arrival of a pendulum the damage is delivered, resulting in creating the effect of liquid bolus that quickly hits the in place dural surface. This is followed by stretching in epidural space from the place of infusion causing the diffused brain stacking (Sullivan et al., 1976). The injury creates a short relocation and twisting of tissues in the

brain, and the extremity of the damage relies upon the quality of pressure pulse and additionally the area of the craniotomy/damage.

1.6.4 Inertial (non-impact) Acceleration Models

It involves the acceleration of the head without an impact, unlike human vehicle accidents situations. Inertial damage models were first created in primates by (Ommaya and Gennarelli, 1974) and ensuing changes by Gennarelli and partners prevailing with regards to duplicating many components of human TBI (Gennarelli, 1983).

1.7 TREATMENT OF TRAUMATIC BRAIN INJURY

In the setting of intense head damage, quick appraisal and adjustment of the aviation route and course is organized after, consideration regarding prevention of secondary damage. Arterial vessel pressure over 90 mm Hg; arterial vessel saturation ought to be more noteworthy than 90%. Dire CT checking is a need.

Next, concentrate consideration is on diminishing the pressure on the cranium, as an increase in this is an autonomous indicator of poor result. If the pressure transcends 20-25 mm Hg intravenous mannitol, and the draining of CSF and hyperventilation needs to be utilizing (Berger-Pelleiter et al., 2016).

A recent report using the National Trauma Data Bank (2008) reflectively revealed a 45% increased mortality rate of people who suffered from intracranial pressure monitoring (Shafi et al., 2008).

Concussions are generally reported in patients with mellow head wounds. It is characterized as physical mind damage with no proof of structural adjustment.

Concussion is evaluated on a scale of I-V (as shown in Table 1.2), (Becker et al., 1977). After mild head damage, those displaying persevering emesis, extreme migraine, anterograde amnesia, unconsciousness, or indications of medicinal intoxication are said to have a moderate head injury (Jennett and Teasdale, 1981).

Treating moderate and extreme head injury starts with cardio-pulmonary adjustment initially, as guided by Advanced Trauma Life Support (ATLS) rules. The underlying revival of a person with head damage is important in avoiding hypoxia and hypotension. These bring about a death rate 2.5 times more noteworthy as compared to the absence of these elements. Later examinations of hyperoxia in patients with an extreme head injury revealed to be harmful. A Pa O_2 >300mmHg in TBI patients who are ventilated was related with greater number of hospital facility case casualty (Marshall et al., 1991).

1.7.1 Medications

The medication to control post-injury damage to brain includes:

- **Diuretics:** The tissue fluid is reduced while incrementing urine yields. This medicine is injected intravenously to individuals undergoing trauma to facilitate a pressure decrease in cerebrum.
- **Anti-seizure drugs:** After a week of extreme brain damage and individual may suffer from seizures. Medicines used against seizures are hence given after a week of damage to avoid extra cerebrum harm which a seizure may cause. Additional medications are utilized in case of an occurrence of seizure.
- **Coma-inducing drugs:** Specialists some of the time utilize medications to place individuals into transitory coma in light of the fact that such a brain requires lesser

oxygen. The importance of these medicines increase in conditions where the supply of oxygen and necessary food cannot reach the cells of brain due to an pressure increase in the brain vessels.

1.7.2 Surgery

To limit any further damage to the tissues of brain surgeries are performed. Emergency surgery may face the following issues:

- **Hematomas:** After a brain damage a pressure on brain tissues and bleeding results in blood to clot.
- **Repairing skull fractures:** It is to fix extreme skull fractures and removing the damaged skull pieces from the brain.

1.7.3 Rehabilitation

A great many people who have had noteworthy cerebrum damage will require recovery. They may require relearning the basic activities for instance, talking and walking. The purpose is to increase their day to day performance (Hemphill and Phan, 2013).

Table 1.2 Grades of concussion

Grade I Concussion	• No changes in memory • Confused Temporarily
Grade II Concussion	• A short disorientation • Anterograde amnesia
Grade III Concussion	• Retrograde amnesia • Unconsciousness
Grade IV Concussion	• Retrograde amnesia • Loss of consciousness for 5-10mins

1.8 PAEONIFLORIN (PF) – THE MAIN COMPOUND OF OUR STUDY

Paeoniflorin (PF) is hydrophilic, monoterpene glycoside segregated from the base of *Paeonia lactiflora Pall* which is utilized as a part of customary oriental prescriptions to mitigate different disorders.

It has numerous medicinal properties such as anti-inflammatory, antioxidant, anticonvulsant, anti-cancerous (Wu et al., 2010), intellectual enhancer or learning impairment attenuating agent, anti-hyperglycemic agent, and endothelium- dependent vasodilator, and neuromuscular blocker, antithrombotic and anti-hyperlipidemic agent.

PF could likewise avert calcium over-burdening damage in cultured primary cortex neurons, enhanced spatial cognitive disability, and enhances the learning impairment of matured rats (Xiao et al., 2005). It has been recommended to promptly cross the blood–brain hindrance under normal condition (Cao et al., 2006). It is beneficial in treating neuropathologies (Nam et al., 2013). Paeoniflorin has been accounted for having neuroprotective activities against cerebral ischemia, Alzheimer's and Parkinson's ailment in exploratory models (Liu et al., 2005, Guo et al., 2012).

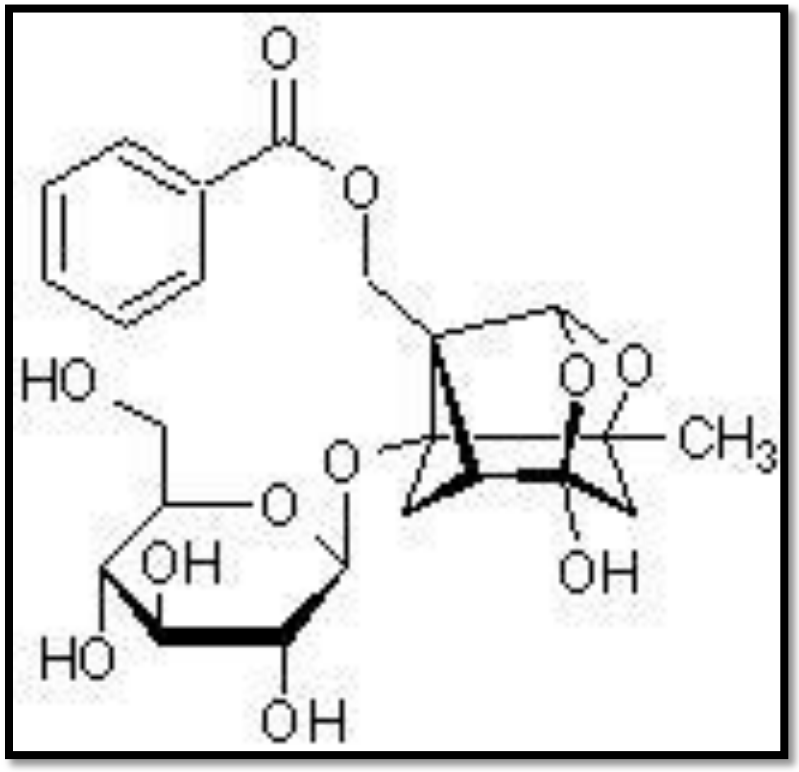


Figure 1.2 Chemical structure of paeoniflorin

Table 1.3 Properties of paeoniflorin

Molecular weight	480.46
Solubility	Soluble in water and ethanol
Molecular formula	$C_{23}H_{28}O_{11}$
Storage instructions	Store at -20°C.
Handling	The solution should be prepared and used on the same day. If the stock is prepared in advance its recommended to store the solution at -20°C, in tightly sealed vials.
Source	*Paeonia lactiflora*

1.9 RESEARCH OBJECTIVES:

The main objectives for this study are:

- Assessment of behavioral tests on TBI mice
- Pharmacological evaluation of paeoniflorin in learning, memory and cognitive functioning in TBI mouse model.
- Measurement of acetylcholine and edema levels in hippocampus and cortex.

The neuroprotective effect of PF against different neuropathologies determined us to design a study to find the effectiveness of paeoniflorin for traumatic brain injury; as none of the research work has been conducted in this area. If PF is found to be protective against traumatic brain injury, it could help us achieve a milestone and could be used to treat the traumatized individuals.

CHAPTER 2: MATERIALS AND METHODS

2.1 ETHICAL STATEMENT AND LETTER OF PERMISSION

Endorsement of the conventions was gotten from the Internal Review board (IRB), Atta-ur-Rahman School of Applied Biosciences, National University of Science and Technology (NUST). At Atta-ur-Rahman School of Applied Biosciences, National University of Science and Technology (NUST), the mice were kept under the controlled condition. All tests applied were permitted after the decisions of the Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA (Guide for the Care and Use of Laboratory Animals: Eighth Edition, 2011).

2.2 ANIMAL MODEL STRAIN

The investigation was led on Balb/c mice, provided by the Laboratory Animal House at our institute and random division of the 60 male mice into five groups was carried out. All the animals fall in the scope of 3-6 months of age and normal weight of 35-45g.

Husbandry

All animals were kept in plastic cages, under standard housing conditions with feed and water ad libitum and 12-hour light/dark cycle. The mice room had controlled temperature of 22 ± 2 C. around 5 mice were kept in a single confine of 40 cm × 25 cm ×15 cm measurements with wood shavings as bedding.

2.3 REAGENTS

Paeoniflorin (PF) was gifted to our laboratory to be used for current study. (Ketamine and Xylazine) anesthetics were purchased locally from the market. Sodium Hydroxide, HCL, Sodium Acetate, DMSO, Hydroxylamine, Ferric Chloride (FeCl3), Acetylcholine

(C3389) were purchased from Sigma Aldrich. Ethanol (Catalogue # 100983) was purchased from Merck.

2.4 STUDY DESIGN

2.4.1 Animal Groups

Animals are divided into 5 groups and each group had total of 12 healthy animals of 3 to 5 months of age. Details of the group 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. Pallets were then air-dried, daily weighed amount of pellets were given to animals as feed.
d. **TBI + Paeoniflorin (5mg/kg/day):** 1ml of Stock solution of Paeoniflorin was prepared; 100% DMSO contained 200mg/1ml of paeoniflorin which was further diluted to 1% DMSO containing 10µl of DMSO and 990µl of distilled water. Daily 14µl of stock was taken and diluted with 1290µl of distilled water to make a total volume of 1300µl, out of which 100µl was injected in every mice intraperitoneally.

e. **TBI + Paeoniflorin (40mg/kg/day):** From already prepared stock solution 120µl was taken and diluted with 3880µl of distilled water to make a total volume of 4000µl; then 270µl was injected in every mice intraperitoneally.

2.4.2 Timeline

12 days long study plan was formulated. 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 perform Acetylcholine assay and Edema tests. 8 days treatment was given in case of Ibuprofen and Paeoniflorin treated groups. From 5^{th} day and onwards behavioral studies were conducted. On the last day of the protocol Acetylcholine and Edema tests were performed for the remaining animals as shown in Figure 2.1(b).

2.5 INJECTIONS AND ANESTHESIA

Anesthesia was injected intraperitoneally via insulin syringes (30 gauge×0.3mm×8mm needle). Xylazine and ketamine were used as an anesthesia. 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. As the animals were given multiple injections for 8 days, injections were given at alternate sites.

2.6 WEIGHT DROP METHOD

Animals were anesthetized and a longitudinal incision was performed by scalp skin for exposer of cranium. Animals were placed on a platform directly under a weight drop

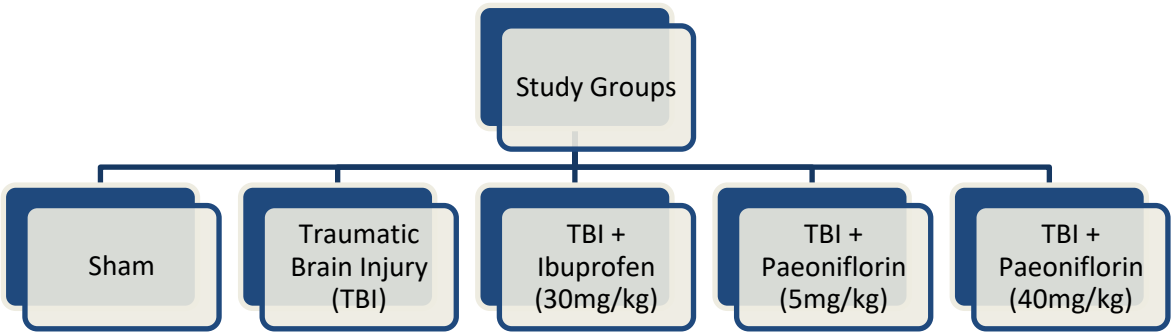
device. Focal brain injury was induced by allowing a rod of 100g to drop from a height of 4 cm in a free fall directly on exposed crania for 3 times maintaining energy of 0.03925J. The foci of injury were arbitrarily decided to be on the center. Post injury, silk suturing of mice were carried out and they were allowed to recover.

2.7 BEHAVIORIAL STUDY

Behavior tests were performed on mice at approximately 3-4 months of age. Behaviors were performed during the light cycle of mice i.e. between 9aam to 6pm, just to avoid the possible variability because of the circadian rhythm. In a separate behavior room mice were left for some time to familiarize with the environment before performing the test. The room was properly lit and the temperature was maintained at 22 ± 2°C. Any sort of 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 (MWM)

The test was originally developed by Richard G. M. Morris (Morris, 1984). It's performed to put spatial learning and reference memory to test in rodents. The protocol was elaborated by Bromley-Brits (Bromley-Brits et al., 2011), with a few modifications as described below. Morris water maze was performed in circular tank containing hidden platform. The water temperature was maintained at 23 ± 2ºC.



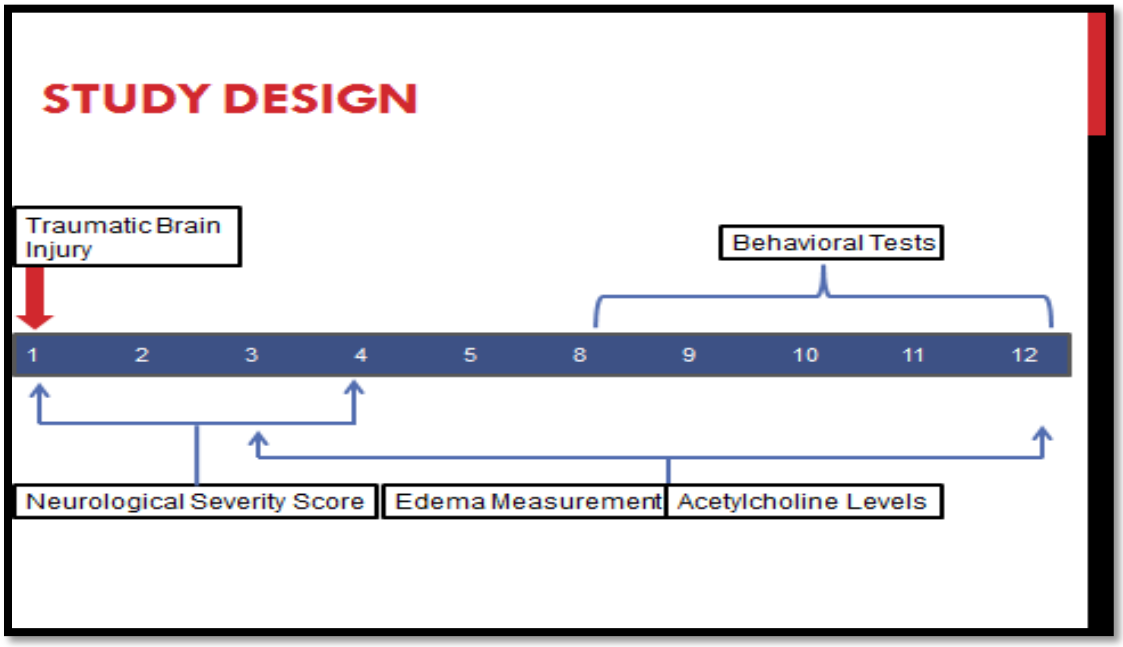


Figure 2.1 Animal groups and study design

(a) Animals, divided into five groups; Sham: with incision on their head, Traumatic Brain Injury: with trauma given through weight drop, TBI+ Ibuprofen: 30mg/kg/day dose is given for 8 days in feed, TBI+ Paeoniflorin I: 5mg/kg/day of dose was injected for 8 days intraperitoneally, TBI+ Paeoniflorin II: 40mg/kg/day of dose was injected intraperitoneally for 8 days.

(b) Study plan for the development of TBI model.

Figure 2.2 Intraperitoneal injections
Animal was carefully restrained by holding its tail into fingers. Thumb and forefinger were used to make a tent of skin over the scruff and then animal was injected with the drug

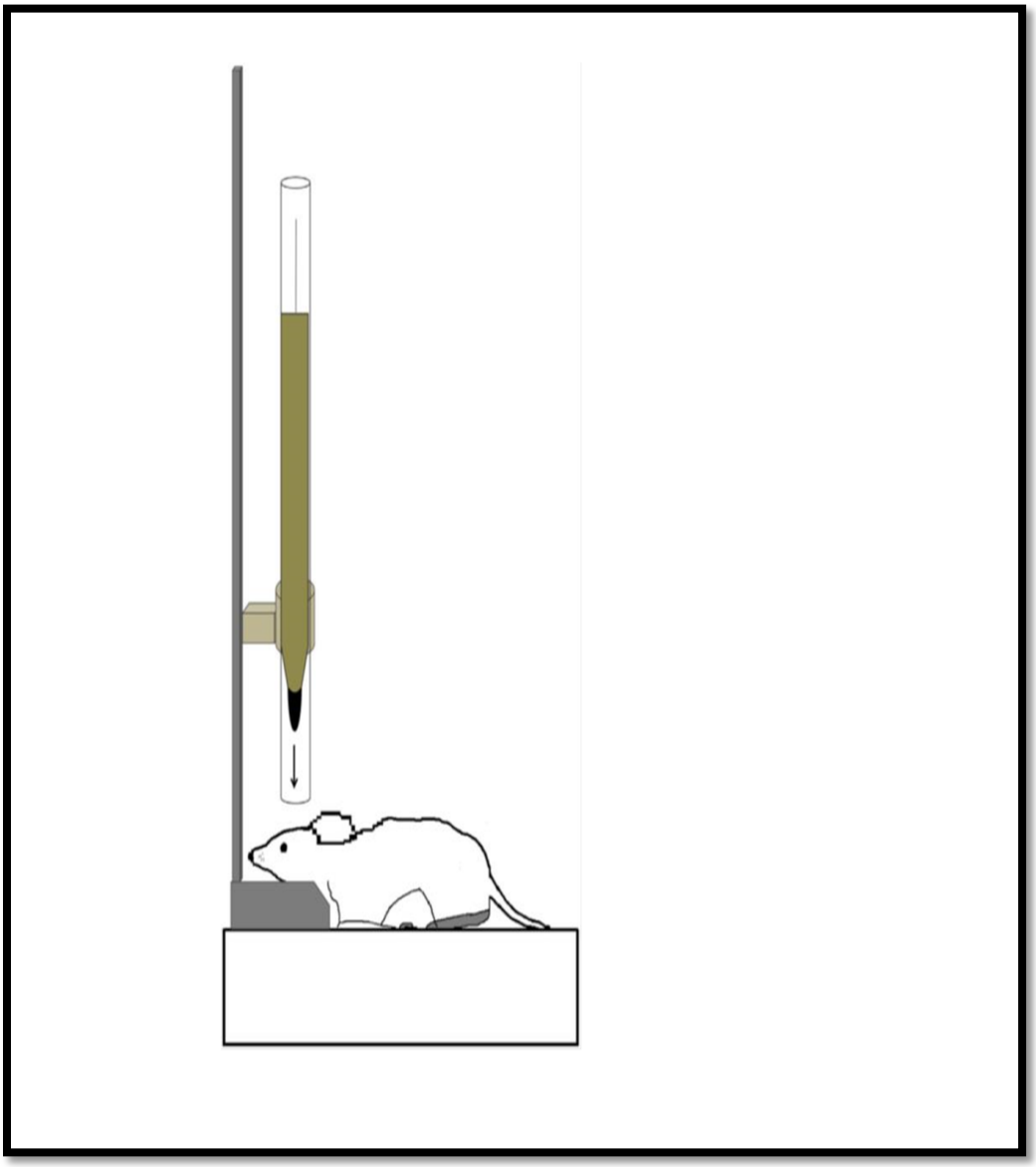


Figure 2.3 Schematic representation of the weight-drop model for inducing experimental traumatic brain injury.

TRAINING

For five consecutive days the mice were put to training to locate the hidden platform. The mice had to undergo five trials each day, by releasing them in the tank from different points in each trial, as shown in table 2.2. A gap of ten minutes was kept between each session. Each mouse was allowed to locate the platform for atleast 90 seconds in each trial. If the mice were unable to locate the platform for ninety seconds, they were then manually placed onto the platform for an additional twenty seconds. If they found the platform within 90 seconds and sat on it for at least five seconds, the time was recorded and trial was considered over.

PROBE

A single probe trial was performed on day 5 for all mice. The platform was not removed and the mice were released in the tank, trying to locate the platform. The trial was recorded with a camera, and was analyzed for time spent to search the platform.

2.7.2 SOCIAL PREFERENCE AND NOVELTY TEST

To contemplate social affiliation and social memory, Crawley's social preference and novelty test measures has been planned. The test is done in a three-chambered glass device in which mouse can without much of a stretch move between the three chambers through openings.

The test depends on two sessions; Session 1 surveys social inclination of mice, while session 2 is utilized to evaluate social novelty behavior of mice. The two sessions are recorded with a camera, while the between session interim is 20 minutes. After every session the glass mechanical assembly was completely cleaned.

Session 1 of the test decides social inclination, social connection and motivation of the test mouse. Session 2 is utilized to gauge social memory and social novelty. The test was executed as portrayed beforehand by Kaidanovich-Beilin (Kaidanovich-Beilin et al., 2011).

2.7.2.1 HABITUATION

The test mice were allowed to habituate by releasing them in the center of the apparatus. They were permitted to investigate the apparatus and adjust to nature for 5 minutes.

2.7.2.2 SOCIAL PREFERENCE TEST (SESSION I)

The test mice were put in the center chamber, while two little wire confines were set in the other two chambers, one in each chamber individually. One of the confines contained the more unusual mice (Stranger 1 or S1), while the other enclosure was kept purge. With Stranger 1 mouse the test mouse had never been in contact with, and it had around a similar weight, age and sex as the test mouse. The test mice could move openly among the three chambers, and their movements, time spent in each chamber and active interaction with S1 mouse and the empty cage was recorded through a camera. Sniffing and deliberately touching the confine was considered as cooperation of test mouse with S1 or empty cage.

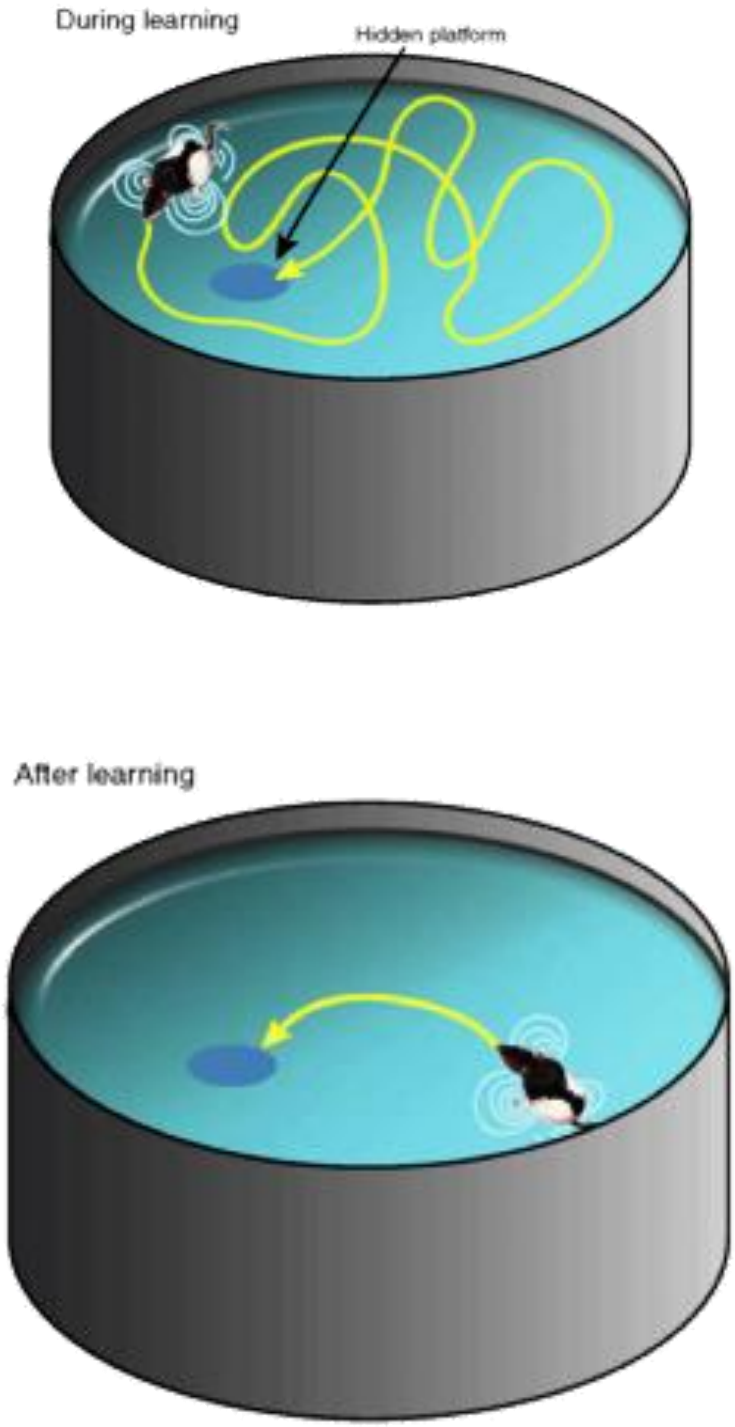


Figure 2.4 Representation of morris water maze test

Table 2.1 Direction of release in morris water maze test

No. of Day(s)	**DIRECTION OF RELEASE**				
	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**
Day 1	West	South	North	East	South
Day 2	North	West	East	West	South
Day 3	North	East	West	South	North
Day 4	East	South	West	East	North
Day 5	West	South	North	East	North
Probe (Day 5)	**SINGLE TRIAL WITH PLATORM. RELEASE DIRECTION, WEST.**				

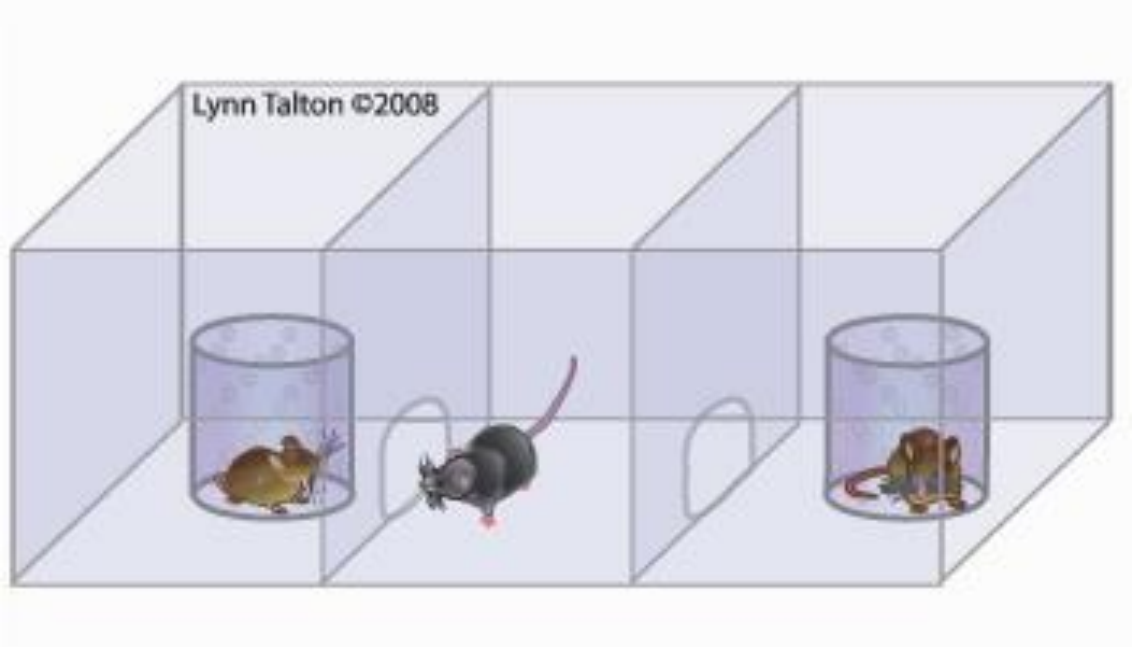


Figure 2.5 Representation of social novelty test

2.7.2.3 SOCIAL NOVELTY TEST (SESSION II)

Twenty minutes subsequent to completing Session I, Session II was performed. In this session, the test mice were put in the center chamber while the wire confines were again put in the other two chambers. One pen contained S1, which was presently a recognizable mouse, while the other pen contained another more unusual mouse (Stranger 2 or S2). S2 was of around a similar sex, weight and age as the test mouse. Movements of the test mice, time spent by them in each chamber and active interaction with S1 and S2 mice was recorded with a camera. Sniffing and voluntarily touching the confine was considered as connection of test mouse with S1 or S2. The test mouse in this session has a free decision between the primary, now recognizable S1 mouse, and a novel new S2 mouse, and therefore decides social memory and social novelty of the test mouse.

2.7.3 NOVEL OBJECT RECOGNITION (NOR)

The learning and memory was assessed. It measures the short term memory by assessing mice capability to memorize its encounter with the object. The test focuses on the idea that a mouse will spend extra time exploring and investigating the novel object which it has never come across, compared to familiar object. The NOR protocol earlier described by (Silvers et al., 2007) was adopted with few modifications. The test was carried out in a square box made-up of iron (40cm × 40cm × 40cm). Mice were familiarized to the box for five minutes, during Open Field Test to avoid bias based on intrinsic anxiety in new spaces. The test consisted of two trials. In the first trial, which is known as acquisition trial, after habituation two objects of approximately consistent height and volume but different shapes were placed equidistant to each other. Subject mice were placed in the

middle of box and permitted to explore and interact with the objects for ten minutes in acquisition period. Twenty minutes after acquisition period, Test trial was performed. The whole thing was identical as familiarization session except that one of the two objects was replaced with a novel object. In test session, mice were allowed to investigate both objects for ten minutes. This trial was videotaped to calculate the time the mouse spent exploring each object. Sniffing and voluntarily touching the object was considered interaction. The discrimination index was determined through:

$$[(\text{Time Spent on the Novel Object}/\text{Total Time}) \times 100]$$

2.7.4 ELEVATED PLUS MAZE (EPM)

It's used to evaluate an anxiety type, whether the anxiety was provoked by open places, in addition to height. The elevated apparatus (50 cm) consisted of 4 arms (30x5cm each). Two arms had 20 cm walls around them while other two arms projected without walls making a plus sign shape. Mice were left in the center of the maze, permitted to move freely around the maze for five minutes. Trial was documented by using camera to establish the total time the mouse spent in each arm along with the number of entries the mouse had made in each arm. The protocol was adopted from (Walf and Frye, 2007) with few modifications.

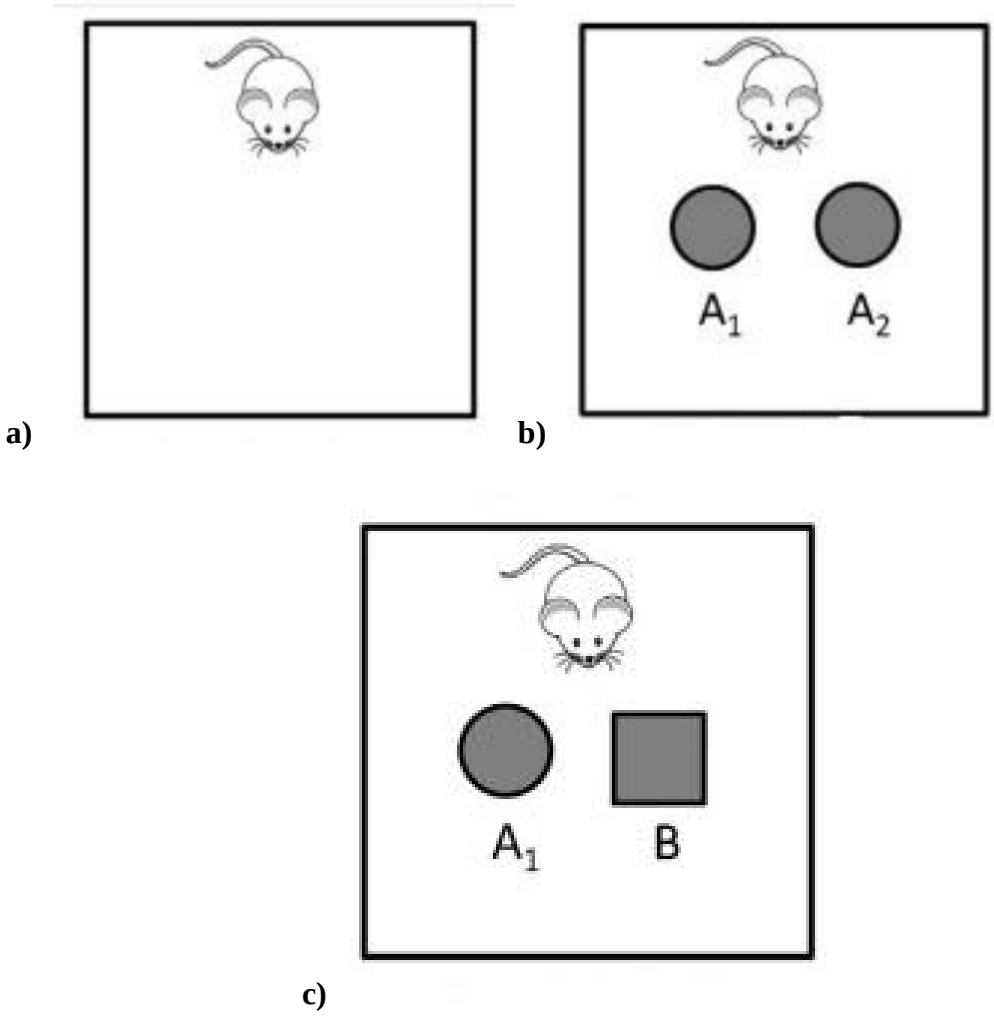


Figure 2.6 Three sessions of novel object recognition test

(a) Open field test. (b) Familiarization session. (c) Test session.

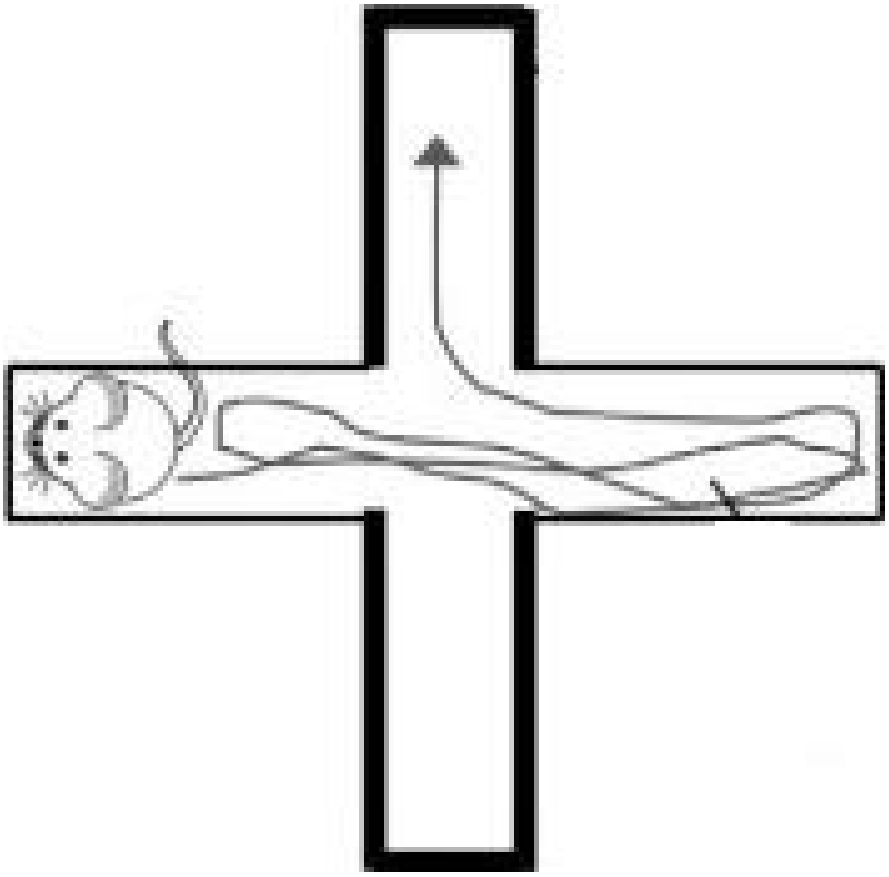


Figure 2.7 Elevated plus maze

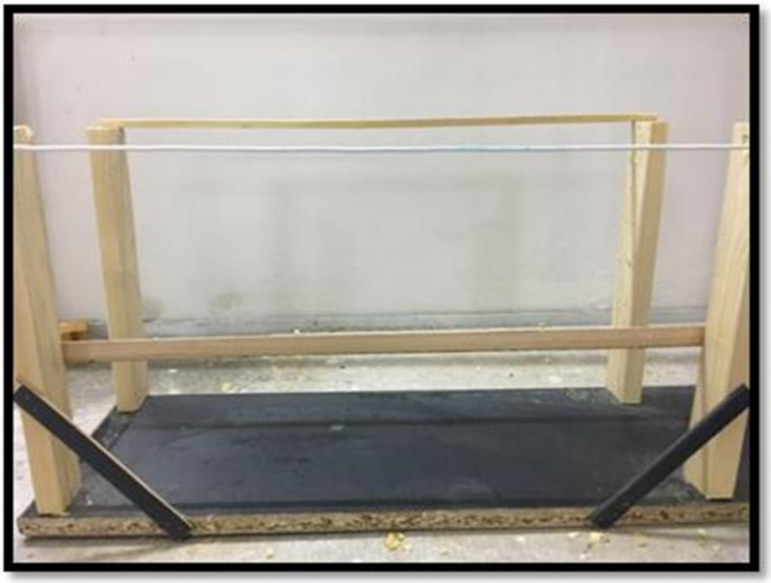
2.8 NEUROLOGICAL SEVERITY SCORE (NSS)

Neurological Severity Score (NSS) was recorded at 4h, 24 h, 72 h, 96 h and lastly at 288h post injury. Motor functions and reflexes of traumatically injured mice were examined at the scheduled time points 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**.

2.9 DISSECTION

On the 3rd and 12th day of the protocol specified numbers of animals were subjected to decapitation for brain isolation. Mice were put under deep anesthesia by using 200-500µl chloroform and then were quickly decapitated with the sharp scissors. Immediately whole brain was carefully harvested out of the skull and was placed in clean petri plate having ice cold Phosphate Buffer Saline (PBS). Dissection was performed to get cortex, hippocampus and amygdala of brain. The separated regions were further processed for edema and acetylcholine assay.

a)



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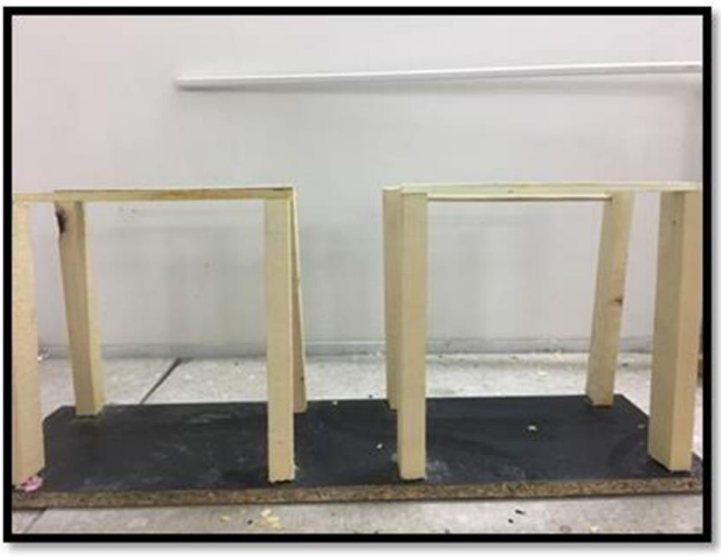


Figure 2.8 Apparatus for measuring neurological severity score
a). Wire suspension and Beam balance while b). Round Stick Balance and Beam walk.

Table 2.2 Parameters and scoring paradigm for NSS

MOTOR COORDINATION		**Score (Max Sc. 28)**	
		Failure (Max Score)	**Success**
Seeking Behavior	Physiological behavior as a sign of "interest' in the environment (time limit: 3mins)	**5**	**0**
Beam Balance	Ability to balance on beam of 7mm width for atleast 10s	**3**	
Round Stick Balance	Ability to balance on a round stick of 5 mm diameter for at least 10 s	**1**	
Exit Circle	Ability and initiative to exit a circle of 30 cm diameter (time limit: 2 min)	**1**	
Straight Walk	Alertness, initiative, and motor ability to walk straight, once the mouse is put on the floor	**2**	
Beam Walk	Ability to cross a 30 cm long beam of 3 cm, 2cm and 1cm width	**3**	
NEUROLOGICAL REFLEX			
Mono-/Hemiparesis (Grasping)	Paresis of upper and/or lower limb of the contralateral side	**1**	**0**
Acoustic Startle Response	The mouse will bounce in response 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**

2.10 NEUROCHEMICAL ANALYSIS: ACETYLCHOLINE MEASUREMENT

2.10.1 PREPARATION OF SOLUTIONS

A 0.04M Acetylcholine standard solution (pH 4.5) was prepared in Sodium Acetate and then the solution was kept at 4°C for up to 2 weeks. Following 2M of Hydroxylamine hydrochloride solution was prepared in Sodium Acetate and stored at 4°C. Similarly, 3.5N Alkali (Sodium hydroxide) solution and 0.1N of Hydrochloric acid solution was prepared and kept at room temperature. 0.37M Ferric chloride solution was prepared in Hydrochloric acid. Finally, Hydroxylamine solution was prepared freshly in Sodium hydroxide at a 1:1 ratio by volume and stored at room temperature, maximum for 3 hours.

2.10.2 ACETYLCHOLINE ASSAY

After separation of cortex from other brain regions following animal dissection, the individual cortices were weighed, and then boiled in water bath at 100ºC. The cortex tissue was then homogenized in 1ml double deionized water. The solution was centrifuged at 6000 rpm for around ten minutes at 4ºC to pellet down proteins. Supernatant was separated, and 300µl alkaline hydroxylamine was added to it. The mixture was incubated for 5 minutes at room temperature. An acid solution of 100µl and 100µl of Ferric chloride solution was then added.

Acetylcholine standard solutions (1nmol, 10nmol, 50nmol, 100nmol, 200nmol) were prepared by adding Sodium acetate solution to respective quantity of acetylcholine

solution followed by addition of 300µl hydroxylamine. Following 5-minute incubation, 100µl each of acid solution and ferric chloride were also added.

2.11 EDEMA

Cerebral oedema was examined through wet- and dry-weight method to determine the tissue water content in the injured brain. A tissue segment was taken from the area bordering the lesion, which was weighed to yield wet weight (WW). At 70°C, in a vacuum oven tissues were dried, reweighed again (until constant weight was observed) yielding dry weight (DW). The percentage of water in the tissue was calculated as% water content by using the formula:

[(WW-DW) ×100]/WW. Oedema was evaluated at 48 h, and on day 12(288 h) post-injury. Eight animals were euthanized at each time point indicated to determine oedema.

2.12 STATISTICAL ANALYSIS

GraphPad Prism Software (Version 5.03) was used for statistical analysis. One-Way ANOVA was applied followed by Bonferroni Multiple Comparison Test to the data for the analysis. P value less than 0.05 was considered significant. The data was shown as mean ± Standard Error of Mean (SEM).

CHAPTER 3: RESULTS

3.1 BEHAVIORIAL ANALYSIS

In order to assess the effect of paeoniflorin on learning and memory in traumatically injured mice Morris water maze, social and novel preference test, neurological severity score was performed. For the assessment of anxiety light and dark box and elevated plus maze was performed.

3.1.1 MORRIS WATER MAZE TEST:

Morris water maze was performed for cognitive and behavioral assessment. Effect of paeoniflorin on spatial learning and memory was checked. Repeated training enabled the mice to acquire an escape response to the hidden platform.

Average escape latency of mice to reach the hidden platform determined the development of a strong memory. During acquisition all the groups showed a little improvement, with sham (25.68±4.85) and TBI + PF (40mg) (25.4±16.19) groups consistently finding the platform at almost 25s at day 4^{th}.

The two groups of traumatic brain injury injected with two different doses of paeoniflorin TBI+PF (5mg and 40mg) respectively performed well and the animals were able to find the platform showing an improved spatial memory compared to the traumatically injured group (TBI).

Overall, sham and TBI + IBU (30mg/kg) (20.73±3.99) groups displayed lesser development of spatial learning and memory.

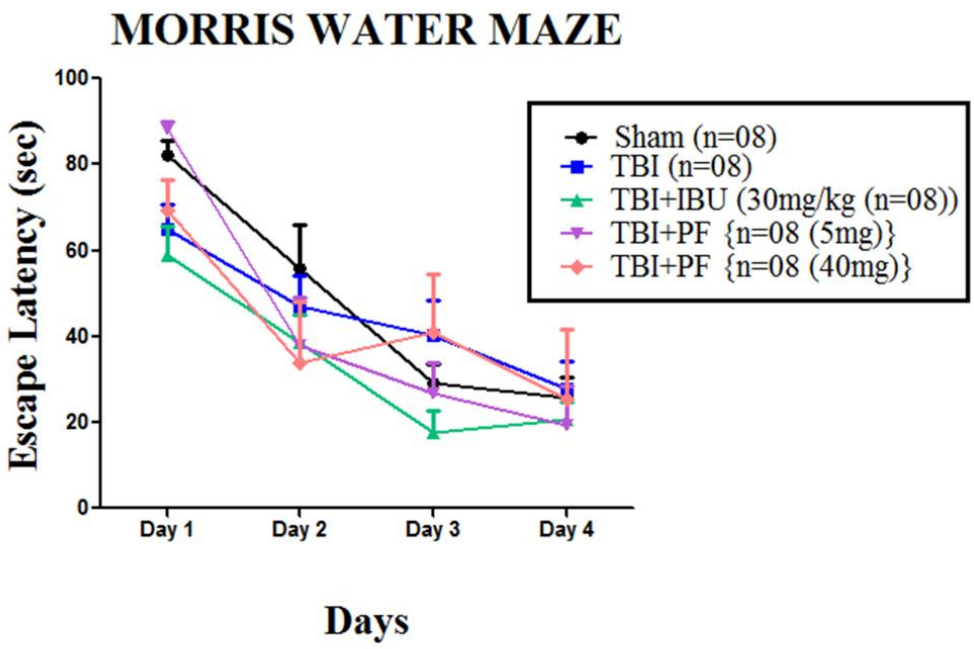


Figure 3.1 Effect of traumatic brain injury and administration of paeoniflorin on learning and memory:

The graph depicts escape latency (sec) of acquiring spatial memory and learning to locate the hidden platform across five training days in Morris Water Maze (MWM) test among Sham, TBI, TBI+IBU, TBI+ PF(5mg) and TBI+ PF (40mg). The error bars represents mean ± SEM.

PROBE TRIAL:

On day 5th probe trial was performed for assessment of reference memory, after the training was complete (**Figure 3.2**). The platform was not removed for the probe trial and the exploration time was recorded for the mice reaching the platform.TBI group (20.5±7.09) did not depict any improvement in developing spatial memory, even on day 5th of the trial. Sham (11.83±5.00) and TBI+PF (5mg) (11±3.68) found the platform at almost 11s on 5th day of the trial; while TBI+IBU (30mg/kg) took almost 9s.TBI+PF (40mg) group (8±2.68) showed a decrease in latency time which indicated an improved spatial learning.

Overall, no significant improvements were shown by the groups in developing the spatial memory throughout the MWM test.

3.1.2 NOVEL OBJECT RECOGNITION (NOR):

Spatial memory and exploratory behavior was determined using novel recognition test, by exposing the test animal to different objects i.e. familiarization session (identical objects) and test session (one familiar from session I and other novel) as depicted in **Figure 3.3**.

As compared to Sham group (39.88 ± 3.500) the time spent while interacting with the object by TBI+IBU group (32.50 ± 3.62) in session I and session II is significantly less ($p < 0.05$). Moreover TBI group (53.69 ± 2.56) alone showed significant interaction ($p < 0.05$) compared to the groups like TBI+PF (5mg) (46.93 ± 2.07) and TBI+PF (40mg) group (46.56 ± 0.18).

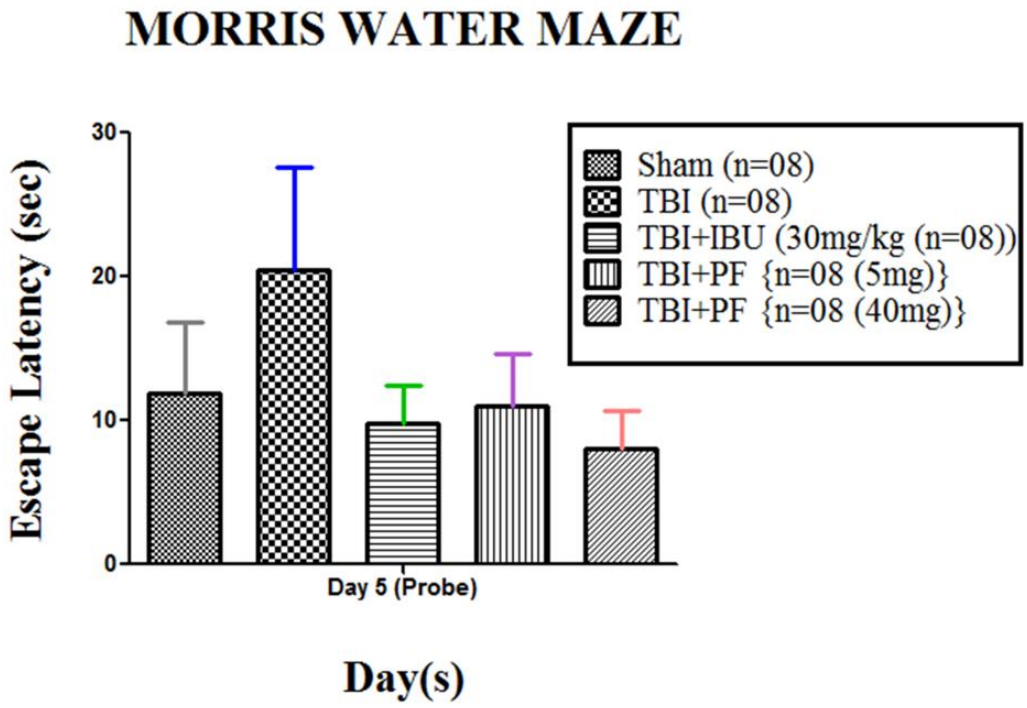


Figure 3.2 Morris water maze test, probe trial

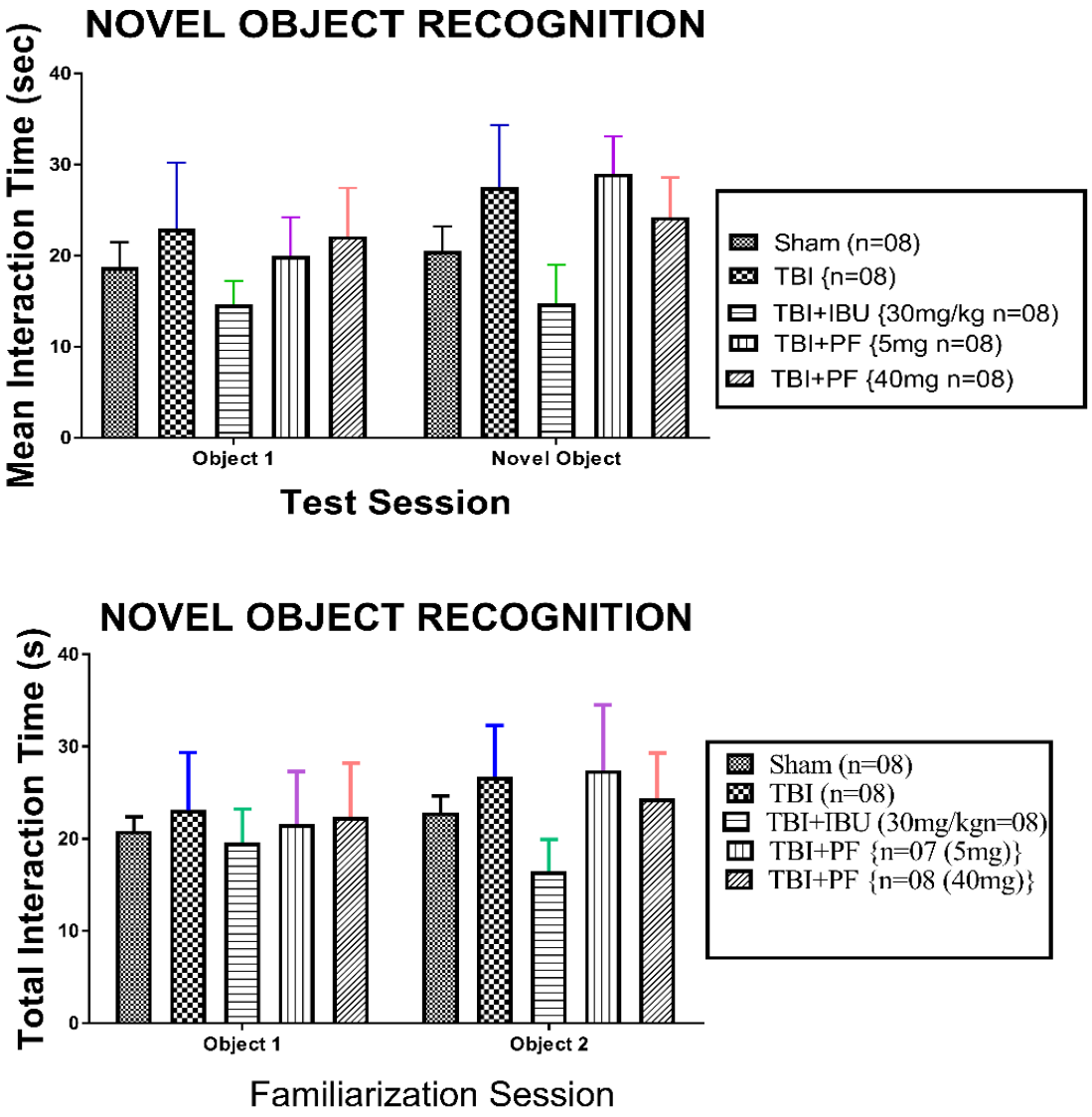


Figure 3.3 Novel object recognition test.

The figure depicts the **familiarization session (session I) and test session (session II)** among five experimental groups.

Discrimination Index (DI) was calculated as an index of discrimination ability and memory (**Fig.3.4**). Discrimination Index suggested that Sham group (6.639 ± 0.5787) spent less time with the object compared to TBI group (8.928 ± 0.43), TBI+PF (5mg) group (8.75 ± 0.045), TBI+IBU group (7.47 ± 2.65) and the TBI+PF (40mg) group (7.12 ± 0.60).

3.1.3 LIGHT AND DARK BOX:

Anxiety induced by light and dark spaces was assessed in light and dark box.

A. TIME SPENT

TBI group (145.3 ± 72.19) spent less time in the light region in comparison to the Sham (153.6 ± 55.38) group, representing high level of anxiety. TBI + IBU (150.0 ± 41.29), TBI + PF (5mg) group (152.6 ± 38.36) and TBI + PF (40mg) group (150.0 ± 73.88) groups spent significantly less time in light region compared to Sham group. There was no significant difference in time spent in light and dark box as depicted in **Fig 3.5.**

B. ENTRIES:

TBI+PF (5mg) group (9.21 ± 0.21) mice spent significantly more time ($p < 0.05$) in dark regions compared to Sham group (7.62 ± 0.25), representing high level of anxiety. A significant difference ($p < 0.05$) was seen with TBI+PF (40mg) (5.81 ± 0.06) and TBI+IBU (4.42 ± 0.28) group. TBI group (6.31 ± 0.06) significantly less time in dark regions compared to Sham group as depicted in **Fig** 3.6.

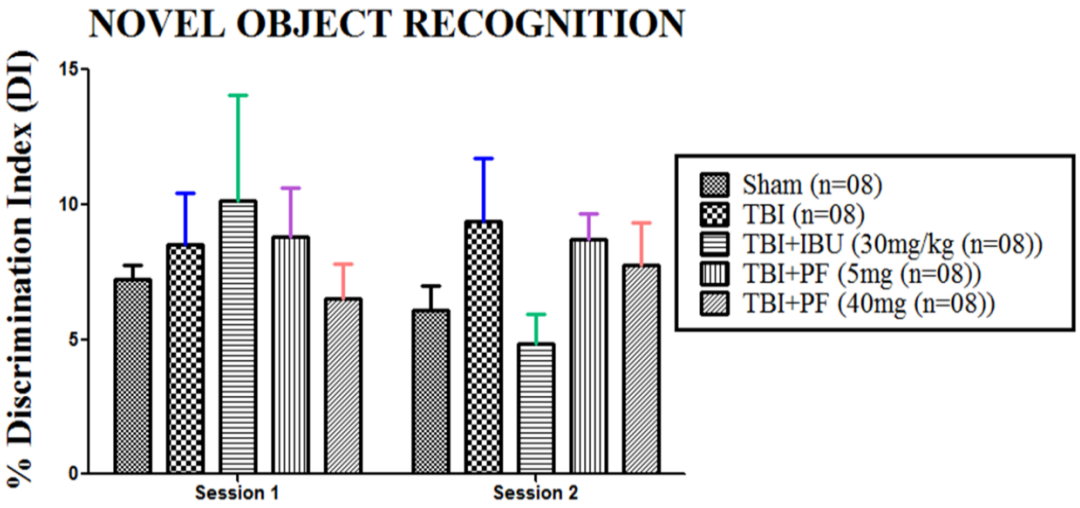


Figure 3.4 Discrimination index of novel object recognition test

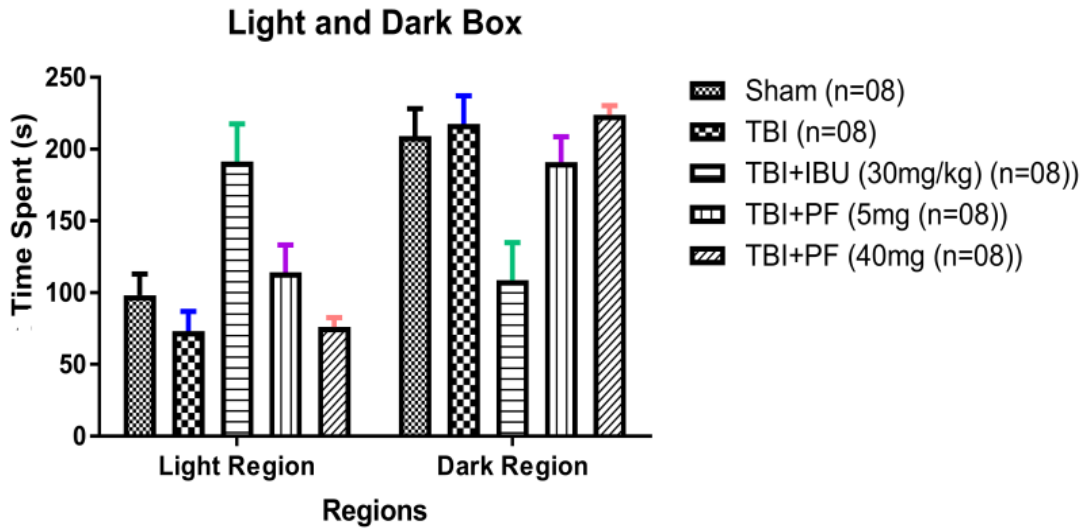


Figure 3.5 Bar graphs shows comparison of time spent in light and dark regions by mouse in Light and Dark Box

Comparison of time spent in light and dark regions by mouse in Light and Dark Box for Sham, TBI, TBI+IBU, TBI+ PF (5mg) and TBI+ PF (40mg) groups. The error bars represent Mean ± SEM for One-Way ANOVA, followed by Bonferroni's multiple comparison test.

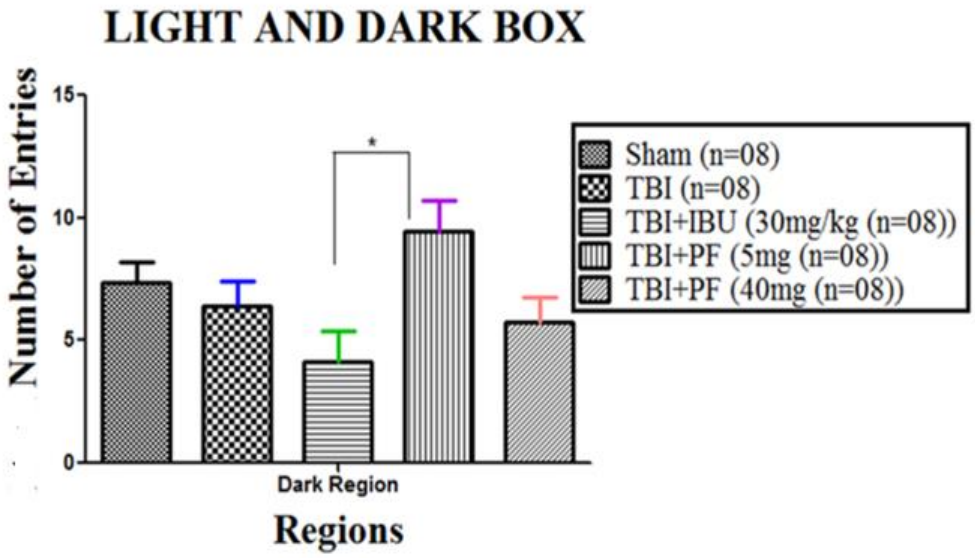


Figure 3.6 Bar graphs shows comparison of number of entries in dark regions by mouse in Light and Dark box

Comparison of number of entries in dark regions by mouse in Light and Dark box for Sham, TBI, TBI+IBU, TBI+ PF (5mg) and TBI+ PF (40mg) groups. The error bars represent Mean ± SEM for One-Way ANOVA, followed by Bonferroni's multiple comparison test.

3.1.4 ELEVATED PLUS MAZE

Anxiety induced by open spaces and height was assessed in elevated plus maze.

The number of entries in closed and open arms is shown in **Fig 3.7**. TBI+PF (5mg) mice spent significantly more time ($p < 0.05$) in closed arms compared to Sham group, representing high level of anxiety. No significant difference was seen with TBI+PF (40mg) group. TBI group and TBI+IBU groups alone spent significantly less time in closed arms as depicted in **Fig 3.8**.

3.1.5 SOCIAL PREFERENCE TEST

Mice being social species exhibit behavioral social interaction. Social preference test was carried out to check levels of sociability and preferences for the social novelty in traumatically injured mice and then to check the effect of paeoniflorin on them. As compared to Sham (46.63 ± 3.37), time spent by TBI group (39.69 ± 0.062) with Stranger A was quite less showing that traumatically injured animal lack innate social aptitude. On other hand when traumatically injured mice were treated with an anti-inflammatory drug the spent time Stranger A was increased TBI+IBU (57.00 ± 2.71). Moreover, paeoniflorin treated groups TBI+PF I (60.36 ± 36.79) and TBI+PF II (108.3 ± 36.19) showed an increase in sociability compared to TBI group indicating positive effect of paeoniflorin on social behavior (**Fig: 3.9.)**

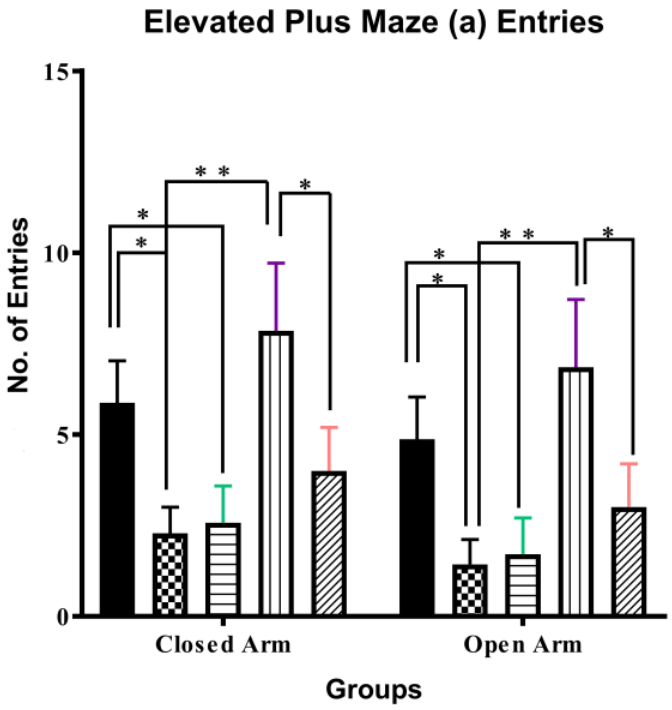


Figure 3.7 Effect of Paeoniflorin dosage in Elevated plus Maze.

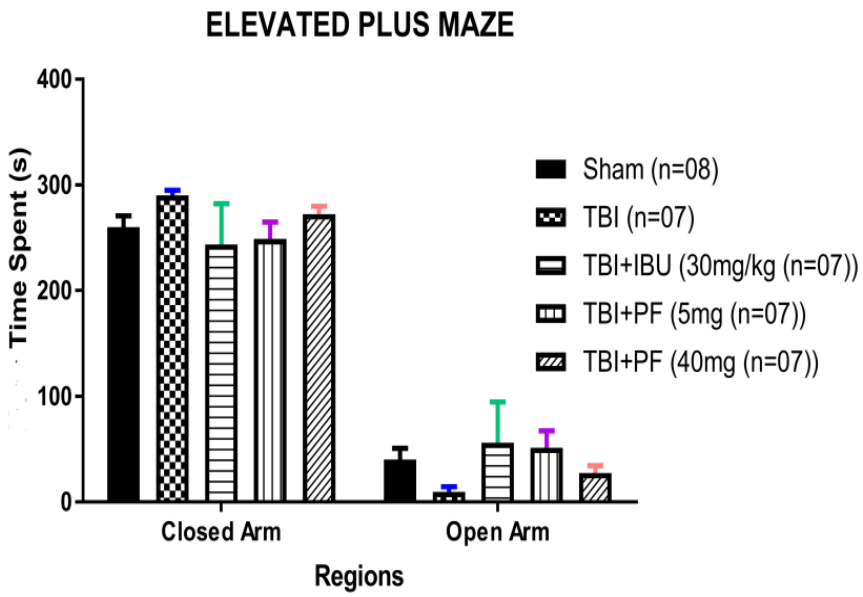


Figure 3.8 Effect of Paeoniflorin dosage in Elevated plus Maze.

Second session was performed to check animal's choice between a familiar mouse (Stranger A) or a novel mouse (Stranger B) in order to evaluate animal's preference for social novelty. Sham (62.81 ± 42.31) spent more time with unfamiliar Stranger B than with already investigated Stranger A mouse, as compared to TBI group (15.25 ± 4.50) and TBI+IBU group (26.79 ± 18.79). Positive effect of paeoniflorin on social novelty was shown by an increase in interaction time with unfamiliar Stranger B mouse in TBI+PF I (64.14 ± 8.57), TBI+PF II (74.87 ± 36.26) (**Fig: 3.10).**

3.2 NEUROLOGICAL SEVERITY SCORE (NSS):

Neurological Severity Scoring (NSS) at selected time points post-injury was measured. All injured mice showed marked impairments in neurological performance at post 4h post-injury which depicted the severity of injury. Gradual improvement in neurological performance was evident in all treated groups during observation period of 228h.

3.2.1 Neurological Severity Score (4h):

The NSS recorded 4h after the injury was considered as the baseline NSS following injury in all the groups. The neurological scoring on the same day of trauma marked impairment in the neurological performance at 4h post injury which depicts the severity of injury (**Figure 3.11**).

Sham group had reduced severity of injury compared to all other four groups which had experienced traumatic brain injury. TBI group and TBI+IBU (30mg/kg) had the maximum severity score at 4h. TBI + PF (5mg) and TBI+PF (40mg) showed almost the same severity score.

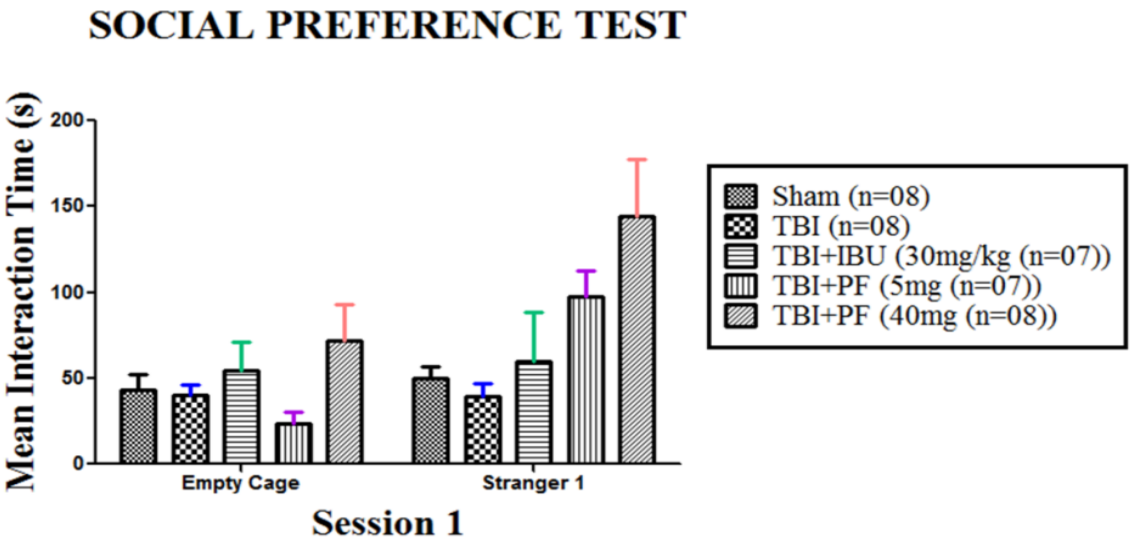


Figure 3.9 The bar chart shows Session I of Social Preference and Novelty Test.

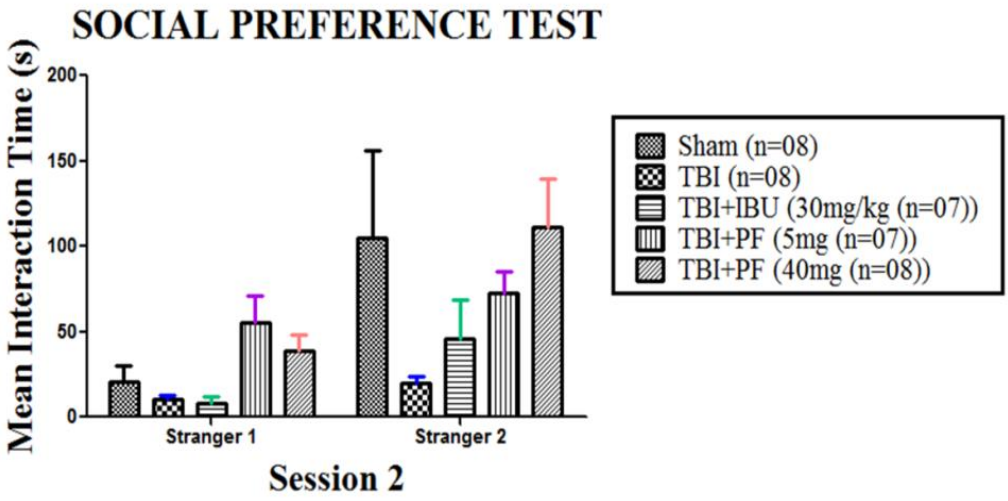


Figure 3.10 The bar charts shows Session II of Social Preference and Novelty Test.

3.2.2 Neurological Severity Score (72h):

Neurological performance is shown at 72h after the injury in (**Figure 3.11).**

A reduction in severity score is evident in Sham group compared to the severity score at 4h. A significant difference can be seen in severity score of Sham in comparison to the groups like TBI, TBI+IBU and TBI+PF (5mg). Overall severity score of TBI+PF (40mg) has reduced compared to severity score at 4h.

3.3 EDEMA:

Post injury the water content was calculated in different brain regions like Cortex and Hippocampus at 48h and 228h.

3.3.1 EDEMA (48H)

The % water content was calculated in Cortex and Hippocampus at 48h as depicted in **Fig.3.12** (a, b). Sham group and TBI group depicted almost the same %water content i.e. 80%. TBI + PF (5mg) group and TBI + PF (40mg) group had approximately 85% water content while in TBI+IBU (30mg/kg) group the % water content was up to 60% at 48h post neurological injury.

3.3.2 EDEMA (288H)

The % water content was calculated in Cortex and Hippocampus at 288h as depicted in **Fig.3.13** (a, b). Sham group, TBI group, TBI+IBU (30mg/kg) group, TBI + PF (5mg) group and TBI + PF (40mg) group depicted almost the same %water content i.e. approximately 80%. TBI + IBU treated group did show an increase in the brain water content compared to the edema test conducted at 48h.

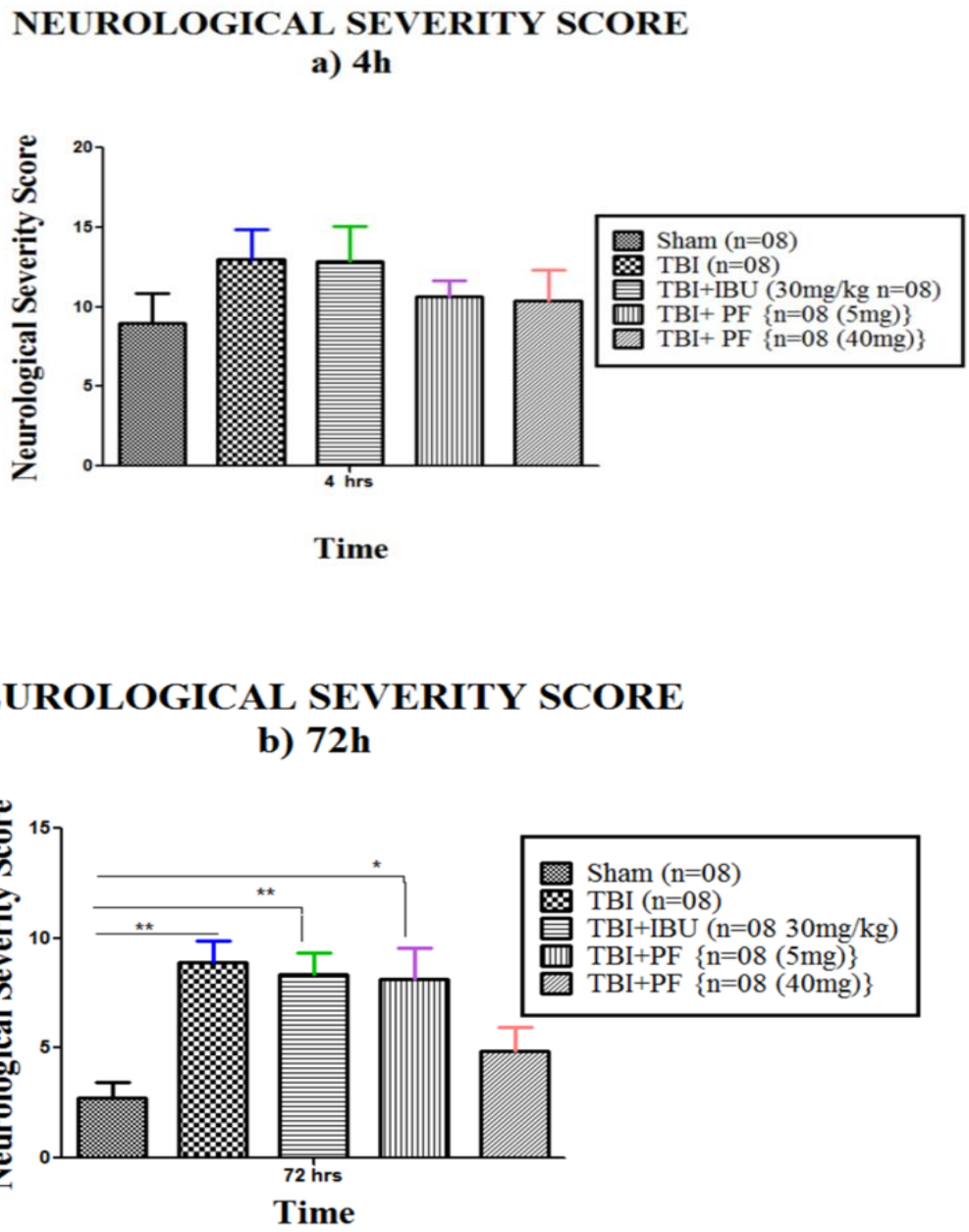
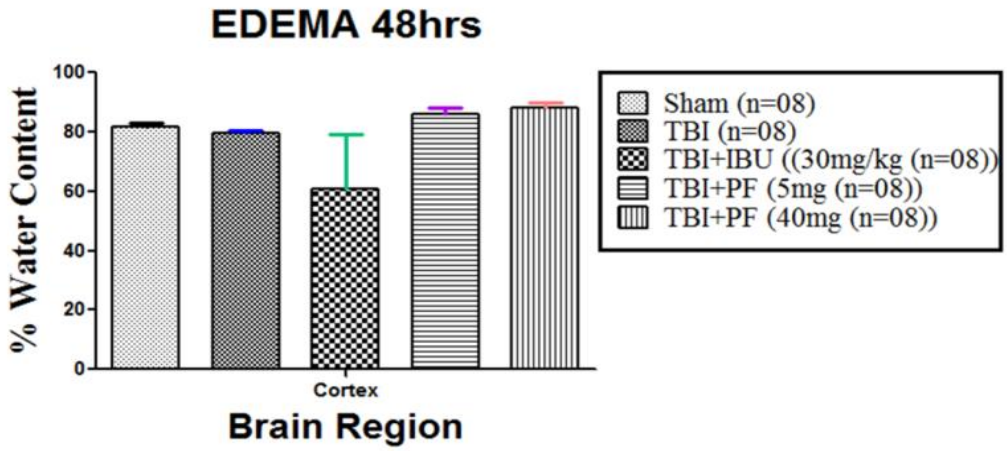
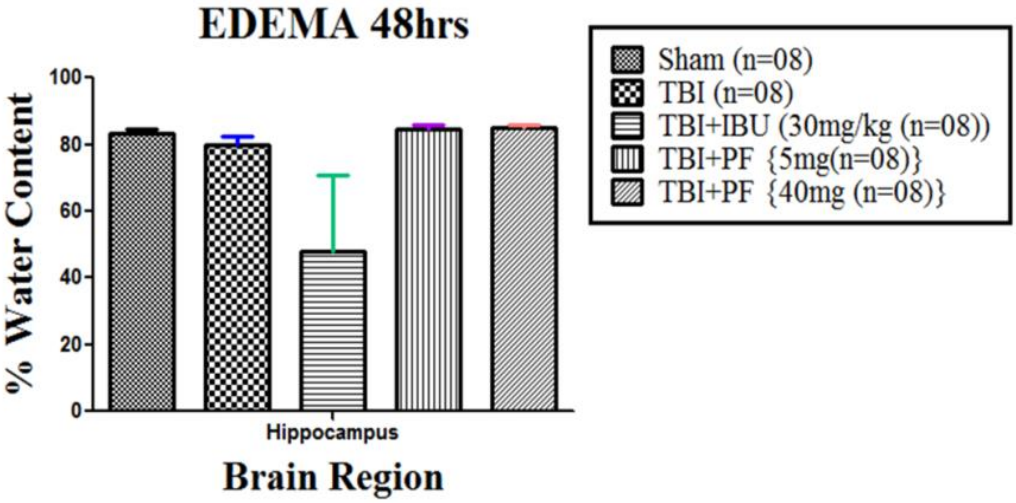


Figure 3.11 : Effect of TBI and Paeoniflorin dosage on Neurological Severity Score (NSS)



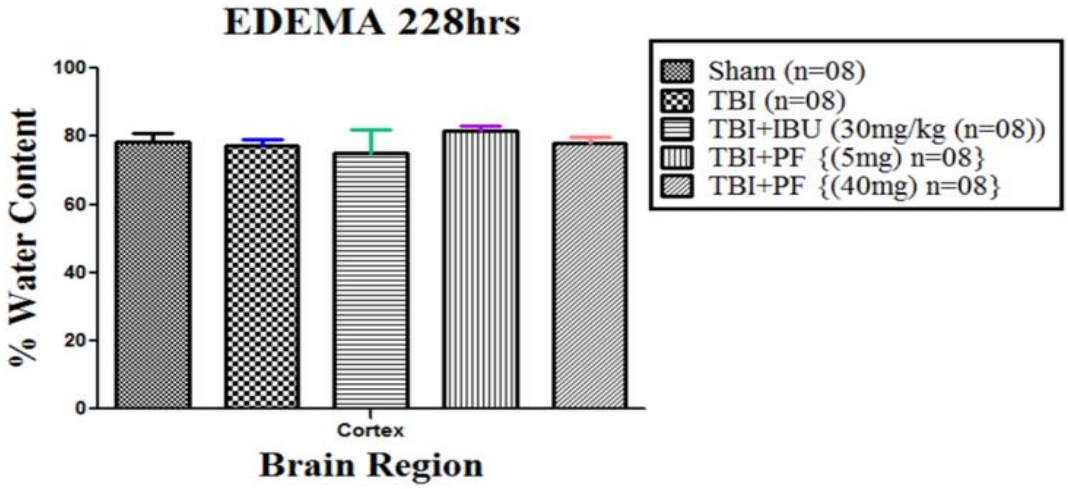
a)



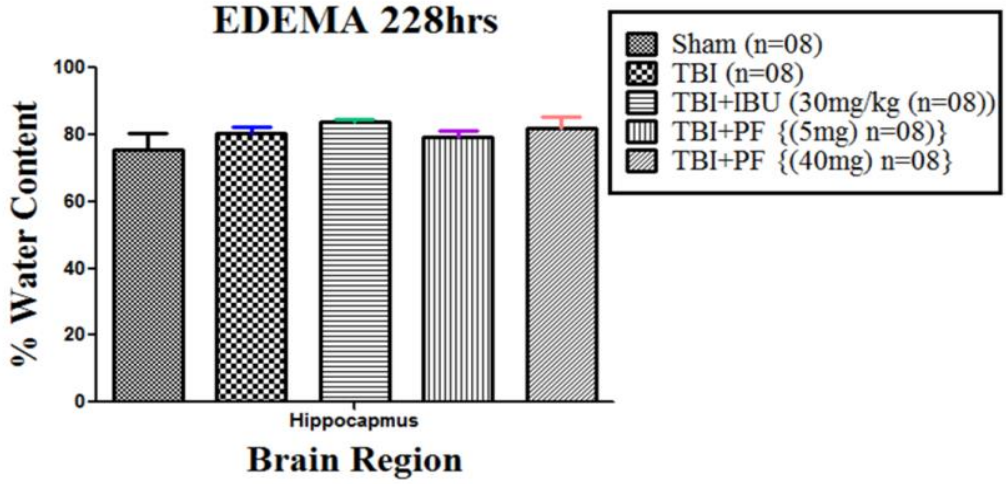
b)

Figure 3.12 Cortical and hippocampal edema measurement at 48h

% water content of cortex and hippocampus in left hemisphere is calculated at 48h for Sham, TBI, TBI+IBU, TBI+ PF (5mg) TBI+ PF (40mg) was analyzed. Data represents mean ± SEM.



a)



b)

Figure 3.13 Cortical and hippocampal edema measurement at 48h

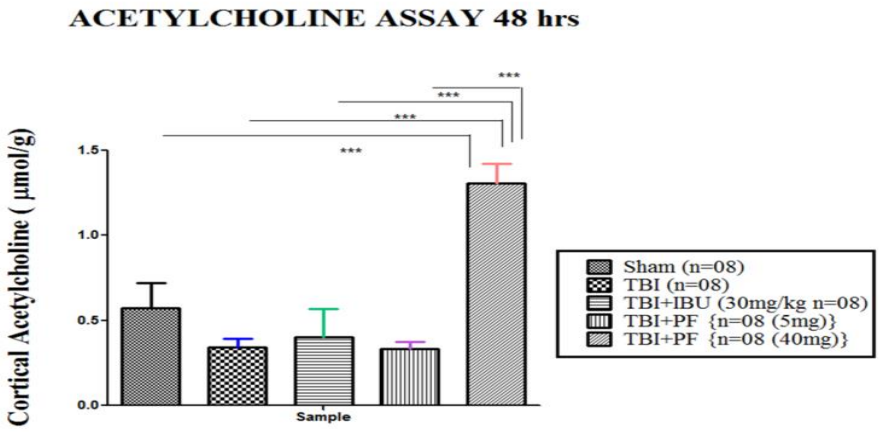
% water content of cortex and hippocampus in left hemisphere is calculated at 288h for Sham, TBI, TBI+IBU, TBI+ PF (5mg) and TBI+ PF (40mg) was analyzed using One-Way ANOVA. Data represents mean ± SEM.

3.4 ACETYLCHOLINE ASSAY (48h):

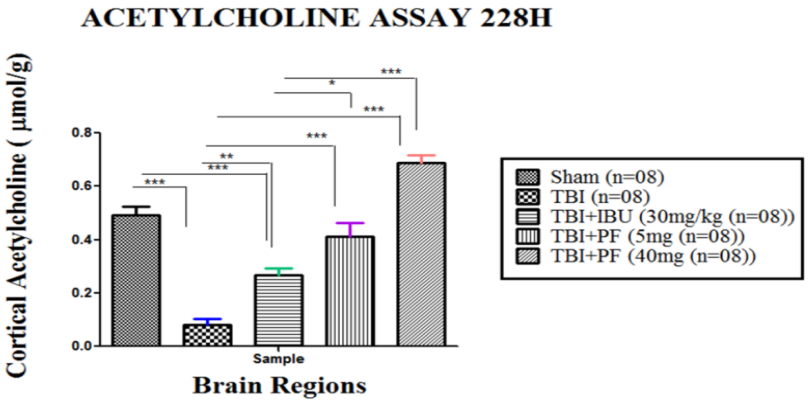
Levels of the neurotransmitter acetylcholine were also measured to determine the cholinergic activity in cortex and hippocampus as depicted in **Fig 3.14 and Fig 3.15**. Cortices of mice from the TBI+PF (40mg) group ($p < 0.001$) had significantly more amount of acetylcholine in µmol/g compared to Sham group, TBI group, TBI+IBU group and TBI+PF (5mg) group.

3.5 ACETYLCHOLINE ASSAY (288h):

Levels of the neurotransmitter acetylcholine were also measured to determine the cholinergic activity in cortex and hippocampus as depicted in **Fig 3.14 and Fig 3.15**. Cortices of mice from the TBI+PF (40mg) group ($p < 0.001$) had significantly more amount of acetylcholine in µmol/g compared to Sham group, TBI group, TBI+IBU group and TBI+PF (5mg) group

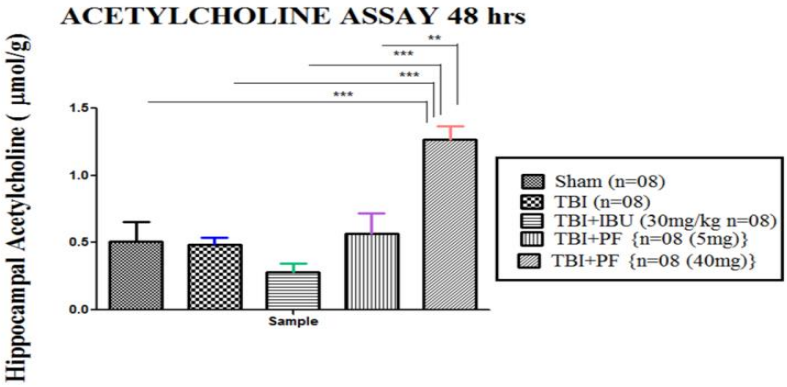


a)

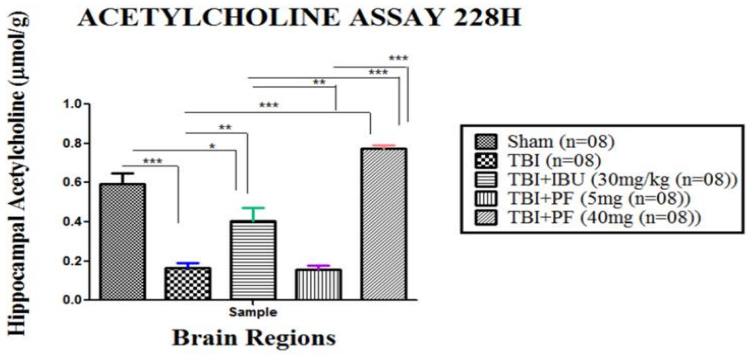


b)

Figure 3.14 Acetylcholine Concentration (µmol/g) in the cortex at 48h and 288h



a)



b)

Figure 3.15 Acetylcholine Concentration (µmol/g) in the hippocampus at 48h and 288h

CHAPTER 4: DISCUSSION

Learning and memory retention of Morris water maze were used to evaluate spatial memory in rats. Sham group acquired spatial memory across the training days, and their escape latency decreased on the fourth and final day to reach the platform as cited by (Morris, 1984). (SCHEFF et al., 1997) work resembles the work for our project showing that TBI group result in longer escape latencies and also significant deficits in search time. Similarly, for TBI+IBU group the escape latency decreased on the third and final day (Zhong et al., 2009), findings show that rats that were treated with PEF (15mg and 30mg/kg) dose did show decreased escape latency on 3rd and 4th day of training. No improvement was seen in learning in first two days of treatment with PEF. Similar results were observed in our experiment; as the mice injected with two different doses of PF (5mg and 40mg/kg) revealed no learning on first day but a gradual decrease in escape latency after Day 2 (Liu et al., 2006).

In the probe trials, the swimming time spent in the quadrant that had held the hidden platform was used to estimate performance. TBI group performed the worst among all groups; displaying an impaired spatial memory recall and impaired hippocampal activity compared to PF treated groups in probe trial. PF treated groups had decreased escape latency depicting an improved hippocampal activity.

NSS is the result of a battery of tests based on inquisitiveness, motor skill and reflexes. It has been commonly utilized in non-clinical studies to gauge neurological deficits following traumatic brain injury in rodents. NSS evaluation suggested that reduction in intracranial pressures and cerebral oedema, as a result of osmotic therapy, improved

motor coordination, response to auditory stimuli and alertness.(Khandelwal et al., 2017) works shows impairment in neurological performance; all five group showed marked impairments at 4h post injury which depicted the severity of injury.

Sham group severity score at 72h showed a decrease compared to the TBI group and TBI+IBU treated group showing no decrease. (Wang et al., 2012) and (Xiao et al., 2005) works also reveals the improvement in neurological scoring.(Xiao et al., 2005) their work shows that administration of PF (low dose) failed to produce significant decrease in neurological impairments but injecting (high dose) produced substantial decrease in neurological score, which was observed in our study that by injecting PF dose of 40mg/kg did show a decrease in the neurological impairment score compared to the 5mg/kg dose of PF which showed no decrease even at 72h.

Cerebral oedema is considered as a standout amongst the most serious complications following horrible cerebrum damage and is characterized as the overabundance collection of water in the intra-as well as extracellular spaces in the brain(Chen et al., 1996). Raised intracranial pressure due to oedema is known to induce deficits in working memory and motor coordination (Levin et al., 1991). In our experiment Sham group and TBI group had almost 80% water content in their hippocampus and cortex at 48h and 228h; while TBI+IBU treated showed an increase in water content of almost 20% at 48h in cortex and 40% increase at 228h in the hippocampus respectively.

For PF treated groups as cited in the literature no significant difference in the water content was observed, it remained the same at both the intervals (Wang et al., 2012) .

The novel object recognition (NOR) task has become a widely used model for the investigation into memory alterations. However, it can be configured to measure working

memory, attention, anxiety, and preference for novelty in rodents (Antunes and Biala, 2012). Mice and rats have tendency to interact less with the familiar object and more with the novel objects. Object recognition is distinguished by the amount of time spent interacting with the novel object (Bevins and Besheer, 2006). Sham group and TBI group didn't show any improved recognition memory and spent approximately same time with the object in familiarization as well as the test task. Our results are consistent with the previously reported findings in TBI that no statistical differences were observed in the object exploration index in the test sessions when compared with the training session, which suggests a deficit in the recognition memory (Moojen et al., 2012). In TBI+ IBU a decreased discrimination index was observed between familiar and new object in NOR task. Similarly, PF groups, PF (5mg) no significant difference was observed while in PF (40mg) shows an increase in discrimination between the familiar and novel object in NOR task was observed.

Light and dark box is a widely used behavioral test for finding anxiety in mice (Crawley, 2000), and the test is sensitive to anxiolytic drugs (Crawley, 1985). An individual can form emotional and behavioral disability after a TBI leading to chronic disorders.(Qu et al., 2016) used a SmartCage system to find the changes in the mice behavior during the early days of post brain traumatic injury that was induced. For behaviors like anxiety, the duration of the time in the light chamber and the number of movements between the light and dark chambers significantly reduced in the controlled cortical impact (CCI) group as compared to the sham group. Additionally, the CCI group mice spent more time in the dark chambers. In our experiment, Sham group was observed to spend more time in dark region. Also TBI group and PF treated groups preferred staying in dark regions and made

fewer entries in the light region contradicting the work cited in (Tanaka et al., 2013) showing that by administering different low doses of Shigyakusan extract SS (which has PF as one of its component) exhibits a tendency for increased length of stay in the light zone. TBI+IBU group in comparison to the other groups spent less time in the dark region and made more entries in illuminated chamber.

Elevated plus maze test involves cortical (locomotion), amygdalar (anxiety) and hippocampal (regarding depression) regions of the brain (Daenen et al., 2002). Traumatically injured group spend more time in the closed arms and made fewer entries in the open arms just like it is cited in the literature using CCI method of trauma (Washington et al., 2012).

The PF treated groups also preferred staying in the closed arms and made fewer entries in the open arms depicting an anxiolytic behavior which contradicts the work which showed that entry ratio in the open arm was increased and the anxiolytic-like effect was weaker in group that was treated with low doses of Shigyakusan extract SS (which has PF as one of its component) (Tanaka et al., 2013).

At molecular level, cholinergic system is involved in cognitive function such as attention, memory, learning and comprehension (Gallagher and Colombo, 1995, Everitt and Robbins, 1997). . The cholinergic system operates on the neurotransmitter acetylcholine (Ach), whose release in the synaptic terminals and binding to muscarinic or nicotinic cholinergic receptors facilitates cognitive functioning (Felder, 1995, Quirion et al., 1995). The concentration of acetylcholine is proportional to the activity along the cholinergic pathway, and the cognitive functioning (Sarter and Bruno, 1997). Acetylcholine levels were measured at 48h and 228h in the cortex and hippocampus for the treated groups.

The Ach levels in Sham were around 0.55µmol/g on average. TBI group, TBI+IBU and TBI+PF I had almost 0.3-0.35µmol/g on average showing a decrease in the Ach levels. In TBI+PF (40mg) group had an average of 1µmol/g of Ach level which shows an increase compared to the other treated groups; which seems a bit contradictory to what was cited in the literature which shows that PF did not affect the Ach contents in the hippocampus and cerebral cortex (Ohta et al., 1993).

In Session I, the social behavior of the experimental mice is measured. The more the interaction of experimental mice with the stranger mice, the more social it is; and more it prefers social interaction compared to its interaction with the inanimate objects. In our study, all groups interacted with the (S1) Stranger 1 in comparison to the empty cage; which is evident from the graphical presentation of Session 1; all the groups spent more than 50% of their time interacting with the Stranger 1 mice compared to the empty cage. This observation is in accordance with typical conduct of mice whose social working is intact (File and Seth, 2003).

Sham group spent about 33s with the empty cage, while the interaction with the S1 for 43.25s. TBI group spent almost the same time with the empty cage and the Stranger 1 i.e. 39s depicting that social behavior was affected; in line with the study shows that social behavior was affected by TBI which shows both anxiety-like and depression-like behavior (Pandey et al., 2009). TBI+IBU treated group spent 54.28s with the empty cage and 59.7s with the stranger mice. PF treated groups (PF I/ II) were observed to spend more time with the Stranger 1 mice i.e. 97.14s and 144.5s respectively compared to empty cage.

Session II determines social recognition; showing if the experimental mouse identifies and prefers a novel mouse (Stranger 2) over Stranger 1. Mice are sociable animals, and they prefer social novelty as a part of their normal social behavior. They spend more time with Stranger 2 compared to the Stranger 1 in Session II. This is according to the reported literature (File and Seth, 2003, Robinson et al., 2005). It was observed that TBI group spent the less time with the novel mice (Stranger 2) compared to the TBI+IBU treated group. PF treated groups and Sham group spent comparatively more time with Stranger 2 mice rather than the Stranger 1 mice from Session 1.

CHAPTER 5: CONCLUSION

The results of this study show that paeoniflorin doesn't have the ability of improving the cognitive functions, anxiety and learning and memory induced by traumatic brain injury. Administration of high dose of paeoniflorin did show promising result in enhancement of social interaction and reducing the inflammation. In addition, consumption of paeoniflorin doesn't offer improvement against the detrimental effects caused by trauma, and low dose paeoniflorin did not show any development in learning and memory and cortical morphology in traumatized mice.

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