Effects of Metals Exposure after Traumatic Brain Injury on Learning and Memory

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بسم الله الرحمن الرحيم

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This thesis is dedicated to

My Parents

&

Siblings

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Waqar Iqbal

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LIST OF ACRONYMS

Ach	Acetylcholine
nAChRs	Nicotinic acetylcholine receptors
mAChRs	Muscarinic acetylcholine receptors
ChAT	Choline acetyltransferase
AChE	Acetylcholinesterase
α7 nAChR	Alpha7 nicotinic acetylcholine receptor
α4β2 nAChR	Alpha4beta2 nicotinic acetylcholine receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Acetyl-CoA	Acetyl coenzyme A
CNS	Central nervous system
PNS	Peripheral nervous system
Al	Aluminium
As	Arsenic
Pb	Lead
RNA	Ribonucleic acid
cDNA	Complementary deoxyribonucleic acid
TBI	Traumatic brain injury
ANOVA	Analysis of variance

ABSTRACT

Traumatic brain injury (TBI) is a major reason of mortality around the world. TBI is linked with severe deficits in sensorimotor function, memory personality and cognitive behaviour. Heavy metals are known neurotoxicant, their chronic exposure cause cognitive and memory deficits. Aluminium, arsenic and lead are considered to be the most significant causes of neurological impairment. However, their combined effect is yet to be known. This study aims to evaluate the effects caused by the exposure of these metals on cognition, learning and memory. The design of the experiment included six groups of Balb/c mice. Except the control group the five group were induced with the focal head injury using the weight-drop technique. Control and Trauma were given normal saline water while PoTAl (aluminium 20 mg/kg), PoTPb (lead 20 mg/kg), PoTAs (arsenic 20 mg/kg) and PoTM (aluminium, lead and arsenic 20mg/kg each) were given metal doses in water. Neurological severity score (NSS) was measured at different time intervals to check severity of trauma. Effects of these metals exposure after trauma was assessed by behavioral tests like Morris water maze (MWM) , Novel object recognition (NOR), Elevated plus maze (EPM), Fear conditioning and contextual fear and open field test were conducted. The result of each test was presented by a graph. The graph of Morris water maze test, Fear conditioning and contextual fear and novel object recognition test revealed an aggravated level of learning and memory in metals treated mice. Elevated plus maze test and the open field test showed high level of anxiety and depression in metals treated mice. Further studies for in-vitro verification of these results and evaluation of neuronal effects of metals in combination after trauma is required to develop better regime of drug

INTRODUCTION

Traumatic brain injury (TBI) and its consequences lead mortality among people of 45 years old (Marshall, 2000). Brain damage occurring after severe head trauma is considered to be a foremost causing of poor result (W. Finnie, 2001). In order to understand the pathophysiology of this condition many researches have been done on experimental models on development of brain injury in the past decade. (Dixon et al., 1988, Faden et al., 1989, Mattson and Scheff, 1994, OMMAYA, 1995, Chimakurthy and Murthy, 2010). The Design of novel therapeutic strategies should be based on the identification of bio-chemical pathways that are activated in the injured brain and the inhibition of harmful processes at various levels (transcription, translation, receptor activation or intracellular transduction mechanisms), or the activation of protecting mechanisms (Laurer and McIntosh, 1999).

Arsenic and lead are neurotoxic and distributed naturally in the atmosphere. All individuals are evident to trace amount of these metals; yet, if the amount of exposure to arsenic and copper exceeds above trace levels are related to the founding of copper (Diazbarriga et al., 1993). According to evidence, in the childhood if the exposure to lead is relatively low (10 mg/dl of blood) it leads to intellectual insufficiencies (Control, 1991, Christoforidou et al., 2013). Studies on animals have added awareness into the effect of lead contact on the neural system. The frequent studies in the animal showed that the metabolic rate of the bio-genic amines is affected by lead (Dubas et al., 1978, Meredith et al., 1988, Cory-Slechta, 1995). The effects of lead studied in animals at different levels of neurochemistry, specifically, metabolism, endorsement, production, storage, and release, due to the role of these systems in the mechanism of main behavioural purposes.

Arsenic disturb the central and peripheral nervous systems together. An acute exposure of 1 mg/kg/day, in persons central nervous system may cause encephalopathy (Armstrong et al., 1984). An exposure of chronic and intermediate duration to lower levels of 0.02– 0.6 mg/kg/day are considered to cause peripheral neuropathy (Franzblau and Lilis, 1989) of both sensory and motor pathways, which causes deterioration and demyelination in axon distally (Goebel et al., 1990). In San Luis Potosí it was described that children in contact with arsenic of about 0.600 mg/kg of soil and 0.1001 mg/kg of dust and other metalloids alters the central nervous system, causing sleep disturbances, inability to perform activities, and auditory problems (Diazbarriga et al., 1993, Olivo et al., 1995).

Arsenic is considered to be the most neurotoxic metal polluting the environment. Humans are exposed to arsenic through contaminated drinking water. Exposure of arsenic causes lung, skin and bladder cancers, diseases of vascular system, hypertension, cellular intrusion and diabetic diseases (ATSDR, 2007). Although Arsenic has carcinogenic potential shown by most of the studies, it also affect the cognitive development studied in rodents by Rodriguez et al. (Rodrı´ guez et al., 2001), but, its effects on nervous system received less attention. Although studies relating to the exposure of arsenic on human population effecting cognition is scarce, but it have been considered, based on research through epidemiological studies from countries which have considerably high concentration of arsenic than the US (Calderón et al., 2003, Wasserman and Sandelin, 2004, von Ehrenstein et al., 2007). Major part of the studies conducted so far have focused on progenies; and discrepancies in erudition and cognition, mainly in hippocampal reliant activities, consequent critical and prolonged exposures of arsenic have been studied. Whereas, on the cellular level its mechanisms of action are still to be reported.

The most commonly used nonferrous metal Aluminium, is stated to be a neurotoxicity causing agent that can biochemically persuade deficits in brains by disturbing level of neurotransmitter and producing ROS species causing in stress oxidation. Reports for the

assessment of neuroprotective consequence of Ginkgo bilobas extracts (210 mg/kg for 25 days) in provoking aluminium-induced neurotoxic effects by examining definite factors like blood aluminium level, brain aluminium concentration, brain oxidative stress biomarkers' concentration, and brain acetyl cholinesterase activity.

Popular aluminium uses via oral and inhalation routes have reached a high percentage of intake. Aluminium amalgams are mostly practiced in the production of various pharmaceutical merchandises such as antacid, antiperspirant, abrasive agent and food flavourings. The prevalence of Alzheimer's disease is epidemiologically associated with persistent aluminium toxicity (Bondy, 2010). Recent study shows the presence of additional aluminium associated with Alzheimer's patients in the brains (Andrási et al., 2005). Furthermore, reports about workers who have exposure to aluminium dust particles working in industries had the suggestion of damaged cognitive function (Meyer-Baron et al., 2007). Furthermore, a failure in pictorial retention was practically detected in haemodialytic subjects who presented higher concentration of aluminium levels in serum (Bolla et al., 1992).

Neurotoxicity occur by aluminium is mainly facilitated by its pro-oxidant characteristics as well as its ability to persuade/aggravate stress oxidation and improved fats peroxidation (Members et al., 2012). A promising actions related to aluminium inducing neurotoxic effects suggesting that it can be the cause produced from its pro-oxidant mechanism, it endorses natural oxidation in vitro and in vivo, aiding: (1) peroxidation of iron inducing fats accumulation, (2) peroxidation of non-iron inducing fats , (3) oxidation of non-iron mediating NADH, and (4) corrosion of non-iron mediating hydroxyl radical (Exley, 2004). Various studies emphasized that aluminium accretion is associated in the tissue occurring due to the site of accumulation causing protruding aggravation in oxidative stress and apoptosis. The existence of aluminium indicates an upsurge in OH^- and this one predecessors

(peroxides and O2−) which could be merely be nullified by straight permitted radical vultures, which alters the redox reactions in the cell (Sharma and Sharma, 2012).

Furthermore , aluminium has been revealed to be the reason causing significant malfunction in numerous neurotransmitter relating activity (Abu-Taweel et al., 2012), augmented deposition of amyloid, changed energy breakdown, compromised homeostasis of calcium and worsen provocative reaction. Hence, aluminium brought brain malfunction could be the reason to cause these types of processes or the relations among them. The described neurotoxic effects and malfunction activity of brain by aluminium revealed as substantial diminishing of memory and notable worsening in erudition ability of aluminium-treated subjects.

Aluminium brought neurotoxic effects is the greatest vital feature of aluminium poisonousness due to the neurons seem to be predominantly susceptible to be confronted by open radicals for these possible causes: (a) low content of the glutathione concentration (an imperative normal antioxidant) , (b) high percentage of poly unsaturated lipid concentration in their membranes , and (c) considerable amounts of oxygen is required for brain metabolism (Christen, 2000). This study was conducted to check the exposure of these metals after inducing trauma in mice. To assess how the behaviour of mice especially learning and memory is effected after the exposure of metals.

LITERATURE REVIEW

2.1 Traumatic Brain Injury (TBI):

TBI is an outcome of peripheral force producing instant mechanical disturbance of the tissue of brain and hindered pathogenicity which mutually interpose extensive neurodegeneration (Ghajar, 2000, Marshall, 2000). Its diversity can be depend upon brain injury type, brain damage distribution and damage mechanism. The damage it cause to the brain nerve could be diffused or central whereas the conditions of wound govern the virtual degree causing focal and diffused traumatic progresses. Focal brain damage is triggered by straight influence to the head region, and results in vascular injury, cortical contusion, and haemorrhages complemented by ischemia. While diffused brain damage categorised by diffused axonal damage is triggered by mechanical navies. Depending upon the nature by which crucial injury is caused, several cell produce a provoke response which can deteriorate the damage.

TBI is a prominent source resulting in demise and debility in the industrialized countries, (Ghajar, 2000, Peden, 2005) also epitomises an increasing health problem in under developed regions (Kaufmann and Cardoso, 1992, Peden, 2005) and so even a modest result enhancement can be the reason to have foremost public health consequences. Treatments mainly focus on disturbance or inhibitory effect of the subordinate injury cascades although the instant death of cell caused due to the preliminary impact produced on the brain tissue is irretrievable. Nevertheless, no operational neuroprotective behaviour is been studied so far (Kofke, 1993, Yilmaz et al., 2007). For improved sympathetic of the subordinate injury courses and on behalf of the improvement on unique therapeutic mechanism, use of rodent prototypes is critical. While large animals may be required to examine particular features of Traumatic brain injury, rodents have appeared as the most usually used type, since they are simply accessible to many

research workers, normative documents for an extensive variety of functional and behavioural variances in rats and mice are highly recognized and the use of transgenic technologies permit to generate rodents line by precise heritable modifications. A large quantity of mice and rat prototypes of traumatic brain injury are being established to assess neurotoxicity. For this process some of the commonly used models of rodents are weight drop injuries model, fluids percussions injuries model, and cortical contusions injuries model.

2.2 Effect of TBI on neurobehavioral sequelae

There are a few high-chance areas defenseless with the impacts of neurotrauma, yet note that these cerebrum locales are critical nodal focuses in frontal and subcortical circuits that sub serve insight and social conduct. In particular, three significant frontal-sub-cortical circuits have significant jobs in non-engine types of conduct. A track emerging in the dorso-parallel prefrontal cortex tweaks basic leadership capacities, for example, working memory, execution, critical thinking, and mental adaptability. One more, emerging from cells in the orbitofrontal cortex, assumes a basic job in instinctive reflexive social practices and the ability to self-correct and self-monitering in genuine time inside a social setting.

A third course fundamental in the front cingulated modulate the behaviors associated with reward and motivation. In spite of the fact that not a frontal subcortical circuit, in essence, tracks crossing average transient districts assume significant jobs in intermittent memorial and novel learnings, just as the flat reconciliation of enthusiastic reminiscence with existing practice and continuous appraisal of motivation striking nature. Subsequently, the run of the mill locales powerless to harm related with TBI cover impressively with crucial districts and nodals focuses in these types of frontals subcortically activated circuit, constructing it promptly beguiling that issues with discernment, social comportment, and official capacity, just as an expanded relative danger of explicit mental issue could be regular subsequently TBI.

2.2.1 Effect on Cognitive Behaviour

Acute and chronic cognitive complexities are the most widely recognized grievances after TBI (Silver et al., 1994, Whyte et al., 1996) and be able to display substantial difficulties to free livings, communal readjustment, families lives, and come back to works (Ben-Yishay and Diller, 1993, Cicerone et al., 2000). Frontal basic leadership capacities (critical thinking, set moving, motivation control, self-observing), consideration, momentary memories and learnings, swiftness of data handling, and discourse and linguistic capacities can be intellectual fields regularly weakened (Mattson and Levin, 1990, Lehtonen et al., 2005, O'Jile et al., 2006, Rassovsky et al., 2006, Mathias and Wheaton, 2007). Damage to average fleeting districts, the dorsolaterally prefrontal's cortex, and subcortically white issue linking these areas promptly represent these troubles (Vakil, 2005).

2.2.2 Effect on personalities

The word character alteration is normally utilized by survivor and families to depict alteration in the regulation of behavioural and emotional conduct after cerebrum damage. In certain people, this presents as embellishment of pre-damage qualities. It is significant in this setting to get some information about varieties in the recurrence or potentially quality of practices or attributes that may have been available before the damage occurred. (McMillan and Glucksman, 1987). On the other hand, these practices can present as fundamental variety accordingly examples. A few normal gatherings of side effects that depict the "personality changes" are identifiable.

2.2.3 Impulsivities

This might be clear in oral articulations, corporeal activities, on the spot judgment calls, and misguided thinking spilling out of the inability to completely think about the ramifications of a assumed activity. This can be intently interrelated to the idea of improvement boundednesses, in which the person reacts to the furthermost notable sign in nature or joins extreme remarkable

quality to a specific sign, regardless of recently decided foci of consideration or needs, a disorder regularly found in people with frontals cortically harm or deterioration from a decent diversity of clutters (Mathias and Wheaton, 2007).

2.2.4 Irritability

Survivors are characterized as increasingly terrible tempered or all the more effectively irritated. Reactions can go from verbal out-blasts to vicious and assaultive conduct. Albeit a specific sign may be seen as an authentic exacerbation, the reaction is normally out of extent to the hastening motivating force. This modulatory inadequacy varies in power, beginning, and length from the pre-damage design for some people. This conduct dis-restraint is in all likelihood owing to harm to orbitally frontals locales and grey issue associations beside the orbitofrontals subcortically hardware of community demeanour (Arciniegas et al., 2005).

2.2.5 Disturbing instability

Survivors and family regularly depict luxurious showcases of emotional articulation, out of extent to the hastening improvement and the pre-damage scope of reactions. Extra highlights incorporate a paroxysmal beginning, brief term, and consequent regret. This marvel happens in other focal sensory system issue and has been called neurotic effect, full of feeling obligation, pseudobulbar influence, and emotional incontinence, (Lehtonen et al., 2005)(61) and is in all likelihood associated to disturbance of "top-down" variety of limbi's reactions to enthusiastic upgrades by frontals cortex (McMillan and Glucksman, 1987).

2.2.6 Apathy

Protests of spurred conduct could be of worry to relatives and could be a boundary to advancement in convalescence agendas. It is regularly mixed up as lethargy or discouragement and might be connected to hostility when endeavors to include the person in exercises resulting in little intrigue could hasten assaultivity conduct. (Kant et al., 1998, McAllister, 2011)

originate that disregard happened in 62% of their example. (Andersson et al., 1999) establish that practically 50% of the people with TBI resulting in noteworthy degree of detachment. Shortages in persuaded conduct could happen in relationship with damage to the hardware of "remunerate" (Chau et al., 2004) Significant models focuses in these types of hardware incorporate the amygdalae, hippocampus activities, caudated, entorhinal and cingulated cortex, the ventrally tormented territory, and the average forebrains group. Catechol-aminergic frameworks, especially the mesolimbically dopaminergics framework, seem to assume basic jobs in the regulation of the remuneration framework (Ruff et al., 1989).

2.2.7 Deficits in awareness

The variation in personality as depicted above are every now and again increasingly hard to address in light of the fact that the harmed individual might be not able value that their conduct is diverse after the damage (Freedman et al., 1987). Of intrigue is that people with Traumatic Brain Injury can be least inclined to know about variations in conduct and official capacity than fluctuations in increasingly solid fields, for example, motion function. Additionally, the level of mindfulness has been found to connect with practical and professional outcome in many (Binder et al., 1997), in spite of the fact that not everyone thinks about.

2.3 Weight-drop models

A pioneer and standard model for TBI, uses only gravitational force of freely falling and guided load to make a central harm to mind. The anesthetized mice (Chen et al., 1996), or rodent (Feeney et al., 1981), is added to impacting items or base of the damage device and the skull is revealed free of craniotomy. The seriousness of cerebral harm may viably be controlled through the alteration of the stature and weight used in harm. The weight drop displays quick, basic and profitable strategy. One of the bother incorporates the extended probability of skull splits at bigger extent of seriousness of the damage, and furthermore the probability of a bounce back damage (Feeney et al., 1981). The seriousness of head injury can be fluctuated by utilizing

various loads as well as statures of the weight-drops. The great passing degree is because of apnoea which could be decreased by initial respirational help and by using creatures with a specific age and proper weight (Shapira et al., 1988, RAGHUPATHI et al., 2000).

2.3.1 Feeney's weight-drops models

Commonly this type of rodent models in which an effect is shipped to the unblemished dura (Dail et al., 1981, Feeney et al., 1981) outcomes in a cortical injury with discharge (Morales et al., 2005) and harm of the blood-mind boundary (Bellander et al., 1996, Mikawa et al., 1996). Fiery procedures leading to the cause of incitement of microglial cells and astrocyte, actuation of the supplement framework and attack of neutrophilic cells and macrophage (UHL et al., 1994, Bellander et al., 1996, Mikawa et al., 1996, Holmin et al., 1997, Allen et al., 2000, Morales et al., 2005). Conceded microcirculatory aggravations and cortical spreading discouragement (Holmin et al., 1997) have additionally been accounted for in these models. The example of post-horrible cell demise relies upon the seriousness of effect (Lindh et al., 2008). In spite of the fact that the essential damage is generally central, diffusely scattered axonal damage has been seen in the neutrophil of the cortical sore (Shapira et al., 1988, Chen et al., 1996, Morales et al., 2005).

2.3.2 Shohami's weight-drops models

It's a modified weight drop model which was later exhibited for shut head damage utilizing a weight-drop effecting other portion of the unprotected skull in mouse and rat (Chen et al., 1996, Morales et al., 2005) The damage seriousness in these models could be reliant on the bulk quantity and dropping stature of the weights utilized. In this way, heavier loads or potentially expanded falling tallness delivers an ipsilateral cortical cerebrum wound and blood-mind hindrance unsettling influence pursued by mind oedema (Chen et al., 1996, Flierl et al., 2009), actuation of the supplement framework, cell passing developing after some time from the injury site and attack of fiery cells (Shohami et al., 1988, Leinhase et al., 2006, Flierl et al., 2009).

Another model utilizing lighter loads and additionally shorter fall statures brought about a concussive-like cerebrum damage, two-sided cell misfortune, brief span of mind oedema and durable psychological deficiencies (Leinhase et al., 2006). Besides, reciprocal diffuse mind harm, cell demise (respective and underneath the effect site), and provocative reactions were accounted for (Zohar et al., 2003, Tashlykov et al., 2007, Tweedie et al., 2007).

When all is said in done mellow weight-drop wounds are related with diffuse damage design anyway progressively serious weight-drop wounds generate a central injury. This drawback of the weight drop model was the highly changeability cause due to the damage seriousness. A significant improvement in these models can be that it tends to be immediately accomplished in the presence of gas anaesthesia and subsequently permits neurologically severity scores following damage (Chen et al., 1996, Flierl et al., 2009). In this way clinically critical randomizations of creatures into the different treatments gatherings is conceivable.

2.3.3 Marmarou's weight-drops models

To display "entire head" movement bringing about diffuse mind damage,(Marmarou et al., 1994) enables the head to quicken at effect. Contingent upon the seriousness of damage, the actuated mind damage brings about haemorrhages, neural cells passing, astrogliotic cells, diffused axially damage, and cytotoxicity caused to cerebrum oedema (Marmarou et al., 1994, Nawashiro et al., 1998, Cernak, 2005, Morales et al., 2005, Ding et al., 2009). This impact increasing speed model utilizing weight-drops can be valuable models for examining diffused cerebrum wounds going from mellow to unembellished. Full composed, weight-drop model convey a clear method to evaluate mind wounds near the clinical situations going from central to diffused cerebrum wounds.

2.4 **Fluids percussions injury model**

Fluids percussions injury (FPI) models are used to induce traumatic brain injury by quickly infusing liquid bulks onto the unblemished dural surface of the brain. The craniotomy can be made by halfway (CFP, MFP), over the sagittally sutures halfway among bregmas and lambdas, or horizontally (LFP), on the parietals cortices. Evaluated intensities of damage seriousness could be accomplished by changing the power of the liquid weight beat. Similar in different other Traumatic Brain Injury model, a high demise degree because of apnoea is apparent (Levasseur et al., 1989, Yamaki et al., 1998).

The focal and horizontal liquid percussion damage models were adjusted to rodents in 1987 and in 1989 separately (Dixon et al., 1987, McINTOSH et al., 1987). These models produce a blended sort of cerebrum damage. Horrible pathology incorporates cortical wound, discharge and a cytotoxic as well as cacogenic mind oedema either normally reciprocal for CFP damage or ipsilateral for LFP damage (Bellander et al., 1996, Cernak, 2005, Morales et al., 2005).

The postponed advancement of cerebrum harm is joined by astrogliosis, diffuse axonal damage, fiery occasions, cortical spreading sadness and neuro-degeneration (Morales et al., 2005). Independent of damage area, FPI prompts intellectual issue (Morales et al., 2005) (Hoshino et al., 1998, Yamaki et al., 1998, Thompson et al., 2005) and consequently it very well may be a valuable model for post-horrible dementia. In addition, FPI brought horizontally is a proper model for post-awful epilepsy (Pitkänen et al., 2009).

The FPI model has been the most generally utilized model for TBI in the rodents. In any case, for both CFP and LFP variabiity's in damage parameters concerning research facilities are show. For example, starting examinations utilizing LFP saw an ipsilateral mind damage (Smith et al., 1991) while some later investigations saw a broad, two-sided cerebrum damage (Soares et al., 1992, Smith et al., 1994, Pierce et al., 1996). One basic factor deciding the result

seriousness in this model is by all accounts the situating of the brain skull as effectively a little move in the craniotomy site is related with checked contrasts in neurological result, sore area and size (Vink et al., 2001, Floyd et al., 2002). Consequently, setting up a FPI model requests broad methodological calibrating to get an institutionalized result in regard to its seriousness and patho-physiology. When the FPI model is built up, the initiated cerebrum injury has all the earmarks of being profoundly regeneratable.

To allow the utilization of transgenically produced mouse, (Carbonell et al., 1998) adjusted the FPI models to the rodents. Like the rodent, the forced damage in mouse prompts intellectual issue, microglial initiation and neuronal and axonal damage (Carbonell et al., 1998, Carbonell and Grady, 1999, Spain et al., 2010).

2.5 **Controlled Corticals Impact Injury Models**

Controlled cortical effect (CCI) models apply a pneumatic gun to twist horizontally the uncovered Durras and give well-ordered influence and computable biomechanically constraints. This model was adjusted to rodent in 1991 (Dixon et al., 1991) and to mouse in 1995 (SMITH et al., 1995) and produces evaluated, reproducible mind harm.

Dependent on the seriousness of harm, CCI brings about ipsilateral damage with cortical wound, discharge and blood-cerebrum obstruction disturbance (Dhillon et al., 1994). Neural cell passage and deteriorating activities, astrogliotism, initiation of microglial cells, fiery occasions, diffused axonal damage, subjective shortfalls, excitotoxicity and spreading in cortical regions despondencies are accounted for to supervene (Adelson et al., 1998, KOSHINAGA et al., 2000, Chen et al., 2008). For the most part concerning cerebrum oedema, CCI is a significant exemplary as it likely reasons to cause cytotoxicity and a cacogenic mind oedema (Schuhmann et al., 2003, Elliott et al., 2008) and in this way it uncovers the clinical circumstance of post-horrendous improvement of mind oedema. For the most part central mind

damage brought about by CCI making this model to a valuable device for examining the patho-physiology of the auxiliary procedures actuated by damage. Astoundingly, CCI in mice and rats is related with post-traumatic seizure movement like the damage actuated epilepsy in people (Hunt et al., 2009, Statler et al., 2009). Along these lines this model is prevalently appropriate to think about patho systems of post-horrendous epilepsy.

2.6 **Cryogenic injury model**

The procedure of cryogenic damage in animals (Pappius, 1981, Tengvar and Olsson, 1982) prompts a central cerebrum injury. The cerebrum damage in this model is for the most part delivered by generating a chilly bar to the uncovered dura in rodents (for example on the parietal cortices utilizing a copper chamber loaded up with a blend of CH3)2CO and dry ice (- 78°C) (Rákos et al., 2007) or brain skull in mice (for example on the parietal cortex utilizing a copper chamber loaded up with fluid nitrogen (- 183°C) (Raslan et al., 2010). In certain examines, a dried ice capsule was straightforwardly smeared to the cranium of the rodent or mice (Giralt et al., 2002, Pifarré et al., 2010). Diverse harm brutalities can be accomplished by shifting the interaction interval to the uncovered cortices (Eriskat et al., 2003).

In rodents, cortical cryogenic damage brings about a central cerebrum injury and disappointment of the blood-mind boundary (Rákos et al., 2007, Raslan et al., 2010). The essential sore is circled by a penumbras territory where optional procedures can be the reason to an expansion of sore size joined by neuronal cells demise and cytotoxicity and cacogenic oedema (Eriskat et al., 2003, Stoffel et al., 2004). These auxiliary procedures likewise incorporate enactment of astrocytes and aggravation (Bordey et al., 2000, Penkowa et al., 2000, Albert-Weissenberger and Sirén, 2010). Moreover, it was accounted for as of late that a isolated cryogenicity causing sore to the parietals cortices of adolescent mice causes deferred worldwide neuro-degeneration (Sirén et al., 2005). Because of epileptic exercises

encompassing the central injury, this strategy is likewise utilized for impersonating certain highlights of epilepsy (Lewin, 1972, Coutinho-Netto et al., 1982, Redecker et al., 2000).

The cryogenic cerebrum sore model is predominantly fitting for exploring TBI-related blood-mind boundary spillage and vasogenic mind oedema. However, this central injury model does not have the countercoup and diffuse axonal wounds that traditionally confuse human head wounds (Iverson, 2005). In this way the cryogenic mind sore model just restrictively mirrors the clinical circumstance. Albeit different models reflect increasingly precise pathophysiological normal for Traumatic brain injury, the cryogenic cerebrum sore model has been in a significant improvement: The injuries brought about due to the cryogenic damage are unmistakably bound and can be profoundly reproduces in size and area, and for patho-physiological procedures of the auxiliary sore advancement at the cortically effect sites. The peak reproducibilities of the cortically sore is especially valuable to monitor the effect of pharmacologically used medicines or quality sensation on auxiliary sore advancement after central mind damage.

2.7 **Role of Secondary Complications in TBI**

Notwithstanding essential impacts of TBI, an assortment of further factors may convolute damage including horrible hematomas (for example subdural, subarachnoid, and intra-parenchymal hematomas), central or diffused cerebral oedema, raised intra-cranial weight, disruptive hydrocephalus, hypoxic-ischemic damage, and disease. Since TBI regularly happens with regards to different wounds (poly-injury) and restorative challenges, for example, volume consumption or blood misfortune, hypo perfusion, hypoxia, disease, and the issues related to it could be seen and might build post-awful transience and bleakness (Levasseur et al., 1989).

2.8 **Metals:**

Al is a known grown-up neurotoxic specialist (Golub and Germann, 2001) just as its neurotoxicant effects have been depicted in the developing stages likewise (Domingo, 1995, Golub and Domingo, 1996). Al has been related with the aetiology of a few neuro-degenerative conditions like Parkinson's infection (Yasui et al., 1992), dementia (Forbes et al., 1995), and Alzheimer's sickness (Kawahara, 2005), however, over-whelming restorative and logical estimations are due to the discoveries out-lined above don't compellingly set up a pivotal relationship among Alumnium and neuro-degenerative illnesses (Massey and Taylor, 1989, Dakanali et al., 2003). It is conceivable that not only expanded presentation to Aluminium alone but rather the raised degree of Al might be the dynamic element in starting and irritating the pathogenicity of neuro-degenerative sicknesses (Sethi et al., 2008), yet it isn't normally acknowledged that Aluminium is a influential aspect in the etiology of such neuro-degenerative ailments in light of the fact that the careful system of Alzheimer's ailment pathogenesis stays obscure, and is still the matter is disputable (Kawahara and Kato-Negishi, 2011). Aluminium admission have being accounted for to actuate oxidative worry by forcing damage to film lipid, proteins and against oxidative chemical protection framework (Jyoti et al., 2007). Long-standing impacts of formative presentation could not be concentrated in such manner in human populaces, albeit an ongoing report recognized a postponement in neuro-development in preterm babies presented to significant concentrations of Al through parenteral liquids (Bishop et al., 1997).

Inorganic arsenic is an omnipresent metal that is utilized in wood protection, as an insecticide, in electronically gadgets because of its semi-transmitter limits and furthermore as a chemo-remedial specialist (ATSDR, 2007). This metalloid effect which is recognized an epidemiologically significant common poison which could be found in arsenic containing minerals, metals and underground water. Around the world, greater than 200million of people

drink water with high concentration of iAs over the World Health Organization reference estimation of 10 mg/L. Expanded centralizations of iAs can be found in ground waters in Argentina, Bangladesh, India, Mexico, Taiwan and the USA where individuals are relentlessly presented to iAs by drinking water from tainted wells because of geothermal exercises, mineral disintegration or statement and enduring of environmental volcanic particles. Deficiencies in subjective capacities as appeared by diminished insight, verbal coefficients (Calderón et al., 2001) and disabilities in learning and cognition have been connected with interminable presentation to iAs. The neurological and psychological issue appear to be subject to the fixation, timing and length of introduction (Tyler and Allan, 2014). In human and in numerous mammalian species inorganic Arsenic is decreased, methylation in the trivalency and pentavalency methylated specie and combined with glutathione (GSH). These occasions are related with the age of oxidative pressure (Kumagai and Sumi, 2007).

2.9 **Lead**

The all-inclusive community is presented to lead from the air and sustenance in generally same extents. Lead in staple started from pots that are utilized for culinary and capacity, and lead acetic acid source had recently used to improve port wine. During the only remaining century, lead discharges to including air have additionally contaminated our condition, over half of lead emanations beginning from oil. In the course of the most recent couple of decades, in any case, lead emanations in created nations have reduced particularly because of the presentation of unleaded oil. Along these lines blood lead concentrations in the overall public have diminished.

Word related introduction to inorganic lead happens in coal face and smelters just as fusing of lead decorated metal, and in battery-operated plants. Elevated levels of air emanations may cause contamination in zones close to lead mines and smelters. Airborne lead can be kept on soil and water, in this way coming to humans via the natural way of life.

Half of the inorganic lead which is breathed might be invested in the lungs. Grown-ups take up 12–15% of lead in nourishment. Lead in blood is bind to erythrocytes, and end is moderate and principally transported through urine. Lead is amassed in the skeleton, and is just gradually discharged from this body compartment. Half-existence of lead in blood is around multi month and in the skeleton 20–30 years (Järup, 2003).

In grown-ups, inorganic lead doesn't enter the blood–mind obstruction, though this boundary is less created in youngsters. The high gastrointestinal take-up and the porous blood–mind hindrance make kids particularly defenseless to lead presentation and resulting cerebrum harm. Natural lead mixes infiltrate body and cell films. Tetramethyl lead and tetraethyl lead enter the skin effectively. These mixes may likewise cross the blood–cerebrum obstruction in grown-ups, and in this way grown-ups may experience the ill effects of lead encephalopathy identified with intense harming by natural lead mixes.

The side effects of intense lead harming are cerebral pain, crabbiness, stomach torment and several side effects associated with the sensory system. Lead encephalopathy is portrayed by restlessness and fretfulness. Kids might be influenced by conduct unsettling influences, learning and focus troubles. In extreme instances of lead encephalopathy, the influenced individual may be experiencing the ill effects of intense psychosis, perplexity and decreased cognizance (Lidsky and Schneider, 2003). Generally, Individuals who may have been exposed to lead for quite a while may be experiencing the ill effects of memory decay, delayed response time and decreased capacity to get it. People with normal blood lead levels under 4 μmol/l may give indications of fringe nerve manifestations with diminished nerve conduction speed and decreased dermal reasonableness. In the event that the neuropathy is serious the sore might be lasting (Luo et al., 2009). The traditional picture incorporates a dull bluish lead sulfide line at the gingival edge. In less genuine cases, the most evident indication of lead harming is

unsettling influence of hemoglobin blend, and long haul lead introduction may prompt iron deficiency (Järup, 2003).

Late research work has been demonstrated that long haul low-level lead presentation in kids may likewise prompt reduced scholarly limit (Mortada et al., 2001). Intense presentation to lead exposure is identified to be associated in causing proximal renal rounded damage (Rossman, 1998)(Mortada et al., 2001).

In spite of escalated endeavors to characterize the connection among body weight of lead and circulatory strain or different impacts cause to the cardiovascular framework, no formal relationship has been exhibited in humans (Elliott et al., 1999).

Blood lead concentrations in youngsters beneath 12 µmg/dl have been so far viewed as satisfactory, however late information demonstrate that there might be toxicologically impacts of lead at low levels of introduction than recently envisioned. There is likewise proof that specific hereditary and natural elements can expand the negative impacts of lead on neural advancement, in this manner rendering certain youngsters progressively defenseless against lead neurotoxicity (Lidsky and Schneider, 2003).

IARC arranged lead as a 'conceivable human cancer-causing agent' in light of adequate creature information and deficient human information in 1987. From that point forward a couple of studies have been distributed, the general proof for lead as a cancer-causing agent being just feeble, the probably competitors are lung malignancy, stomach disease and gliomas (Steenland and Boffetta, 2000).

2.10 **Arsenic**

Arsenic is a commonly discovered metal, found to be in shake, soil, water and air. Inorganic arsenic has been found in underground water consumed for cherishing a few nations all over in the world (e.g. Bangladesh, Chile and China), though natural arsenic mixes, (for example, arsenic betaine) are principally originate in fish, which along these lines may offer ascent to human exposure (Organization, 2003).

Delivering of non-ferrous metals and the generation of vitality from petroleum product are the two significant mechanical procedures that lead to arsenic sullying of air, water and soil, purifying exercises being the biggest single anthropogenic wellspring of climatic pollution (Page et al., 1987). Different wellsprings of pollution are the creation and utilization of arsenical insecticides and wood additives.

Focuses in air in rustic territories go from 2 to 6 ng/m^3, though fixations in urban communities might be as high as 210 ng/m^3. A lot higher focuses (more prominent than1000 ng/m3) have been estimated close to modern sources. Water focuses are typically under 10 µg/l, albeit higher fixations may happen close to anthropogenic sources. Levels in soils for the most part run from 5 to 45 mg/kg, yet insecticide application and waste transfer can bring about a lot higher concentrations (Annest, 1934)(Saha et al., 1999).

Retention of inorganic arsenic in breathed in airborne atoms is exceptionally subject to the dissolvability and the bulk mass of particles. Dissolvable As mixes are effectively assimilated from the gastro-intestinal tract. Be that as it may, inorganic arsenic is broadly methylated in people and the metabolites are discharged in the urine (Singh et al., 2007).

Arsenic fixations in different parts of body like blood, hair and pee etc. have been utilized as biomarkers of presentation. As in hair and nails may be used as helpful markers of past arsenic

introduction, if proper care is done to stay away from outer arsenic pollution of the examples. Associated metabolites in pee communicated as either iAs or the aggregate of metabolites (iAs + MMA + DMA) is commonly the best gauge of ongoing arsenic portion. Be that as it may, utilization of certain fish may perplex estimation of inorganic arsenic presentation, and should in this way be maintained a strategic distance from before pee sampling (Singh et al., 2007).

Arsenic is extremely poisonous and exposure of enormous quantities prompts gastro-intestinal signs, extreme aggravations of the cardiovascular and focal sensory systems, and in the end demise. In survivors, bone marrow sorrow, haemolysis, melanosis, polyneuropathy and encephalopathy might be watched. Ingestion of iAs may actuate fringe vascular malady, which can then cause outrageous structure prompts gangrenous changes (dark foot sickness, just revealed in Taiwan).

Populaces presented to inorganic arsenic via ingestion of water show overabundance threat of mortality from lung and kidney malignancy. There is additionally an extended danger of skin malignant growth and other skin injuries, for example, hyperkeratosis and pigmentation changes.

Concentrates on different populaces presented to arsenic by inward breath, for example, smelter laborers, insecticide producers and excavators in a wide range of nations reliably show an overabundance lung malignant growth. Albeit every one of these gatherings are presented to different synthetic substances notwithstanding arsenic, there is no other regular factor that could clarify the discoveries. The lung malignant growth hazard increments with expanding arsenic introduction in every single important investigation, and bewildering by smoking doesn't clarify the discoveries.

The most recent WHO evaluation has stated that arsenic exposure via drinking water has been casually identified with malignant growth in the lungs, kidney and skin, the remainder of which is gone before by straightforwardly perceptible precancerous injuries. Vulnerabilities in the approximation of past experiences are significant when evaluating the presentation reaction connections, however doubtlessly drinking of water containing arsenic convergences of roughly 102 μg/l have prompted malignancy at these locales, and that antecedents of skin disease have been related with levels of 52–105 μg/l.

The connections between the arsenic presentation and other wellbeing impacts are less identified. There is generally solid proof for the association of hypertension and cardio-vascular ailment, however the proof is intriguing for diabetes and conceptive impacts and frail for cerebrovascular malady, long haul neurological impacts, and disease at locales other than lung, kidney and skin.

MATERIALS AND METHODS

3.1 Animals:

The investigation was led on Balb/c mice, provided by the Laboratory Animal House at our institute and the mice were divided into six groups. All the animals fall in the scope of 3-4 months of age and normal weight of 30-45g during the start of the experiments.

3.2 Ethical Statement:

Endorsement of the conventions was gotten by the Internal Review board (IRB) of 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).

3.3 Feed

All animals were being kept in plastic cages, under standard housing conditions with feed and water and 12-hour light/dark cycle. The mice were kept at room temperature. Around 5 mice were kept in a single confine of 40 cm × 25 cm ×15 cm measurements with wood shavings as bedding.

3.4 Reagents

Chemicals in the experiment were purchased from Scharlau, Spain. Metal treatment was given to mice by mixing their compounds in water about 20mg/kg of each metals. These compounds were also purchased which are, Aluminium chloride hexahydrate $AlCl_3.6H_2O$, Lead acetate trihydrate Pb (CH3CO2)2 · 3H2O and sodium arsenate Na_3AsO_4.

3.5 Study Design

Mice of age 6-8 weeks were taken and were divided into 6 groups (table 3.1). Each group containing 12 animals. Metals 20 mg/kg treatment was given to the respective in oral doses mixed with water for the period of seven weeks after inducing trauma in them. Effects of metals were assessed then by behvariol analysis. The schematic diagram of study design is given in figure 3.1.

3.5.1 Groups Division

Mice were divided on the basis of treatment given. 12 mice were kept in each group. There were two control groups, one was negative control and other was positive control, and four were experimental groups as shown in table 3.1. Control was given only incision while trauma group was induced with trauma and both were treated with normal saline water. The experimental group was distributed by the treatment the get. Three groups were given individual metals treatment and fourth group was exposed to all of the three metals combine. All the metals were given orally mixed with water

Table 3-1: Group distribution and their treatment

Group	Name	Type	Treatment	No of Animals
G1	Control	Non-traumatic	Normal water	12
G2	Trauma	Traumatic	Normal water	12
G3	PoTAl	Traumatic	Aluminum with water	12
G4	PoTPb	Traumatic	Lead with water	12
G5	PoTAs	Traumatic	Arsenic with water	12
G6	PoTM	Traumatic	Aluminum + Arsenic +lead with water	12

G1 = Group 1, G2 = Group 2, G3 = Group 3, G4 = Group 4, G5 = Group 5, G6 = Group 6, PoTAl = Post Trauma aluminium treatment, PoTPb = Post trauma lead treatment, PoTAs = Post trauma arsenic treatment, PoTM = Post trauma metals mixture treatment

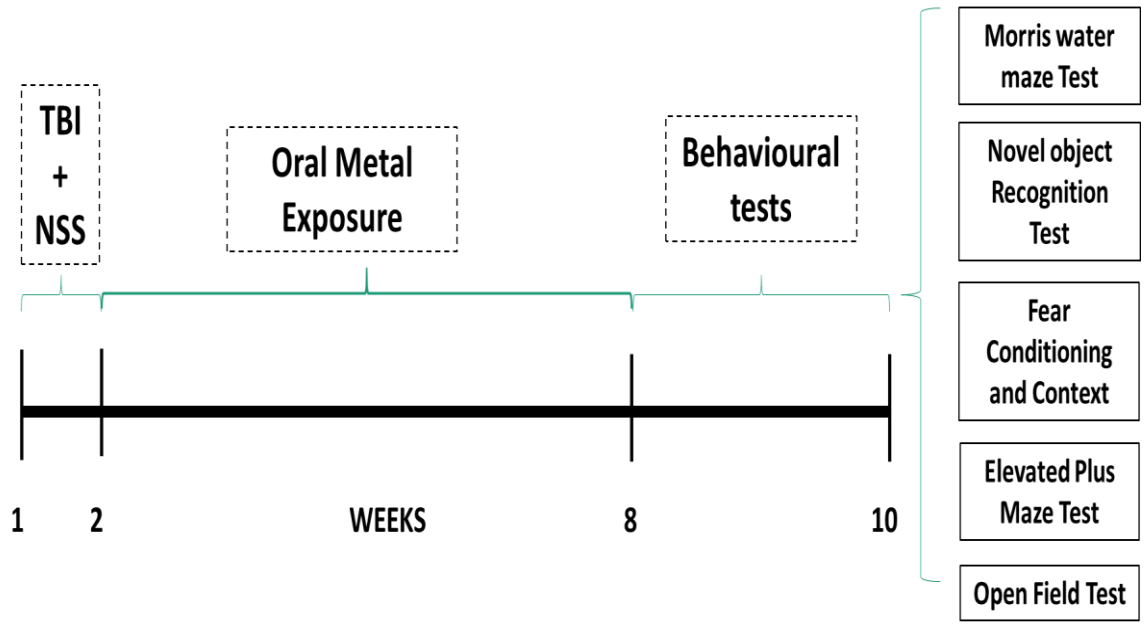

Figure 3-1: schematic representation of the study.

3.6 Traumatic Brain Injury

Trauma were induced by using weight drop model. Traumatic brain injury was induced in mice using the procedure described by (Chen et al., 1996). First of all the animals were given anaesthesia and a longitudinal incision was implemented by using the scalp in order to expose the skull. Mice have been positioned on a platform under the rod in weight drop instrument. Focal brain injury in the skull was induced by using a metal rod of 334 g (with a blunt tip) which was freely drop from a height of 3 cm on the exposed skull (producing final impact of 0.065 J). After inducing injury, the incision was locked using silk sutures and animals were then allowed for recovery.

3.7 Injections and Anaesthesia

Anesthesia was injected intraperitoneally via insulin syringes (30 gauge×0.3mm×8mm needle). Xylazine and ketamine were used as an anesthesia. Mice to be injected were 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.

3.8 Metals Treatment

Metals were mixed in normal doses of drinking water. Control and Trauma group of mice were given normal drinking water while 20 mg/kg doses of Aluminium, Lead and Arsenic were given to group 3-5 i.e. PoTAl, PoTPb, PoTAs, respectively. And 20mg/kg of each metal were given to metal mix group of mice i.e. PoTM. Metals treatment was started from the first day of experiment and was given for 6 weeks after inducing traumatic brain injury. Metals treatment was also continued during performing of behaviour tests in week 7-8.

3.9 **Neurological Severity Score (NSS)**

NSS were done in the first week after trauma and in the seventh week after exposure to metals. Neurological Severity Score (NSS) was being recorded at 4h, 24 h, 48 h, 72 h and lastly at 1000h post injury. Motor functions and reflexes of traumatically injured mice were examined at a particular time period by evaluating NSS. The parameters and scoring paradigm of NSS is shown in Table 3.2.

Table 3-2 NSS scoring

Function	Parameters
Vestibulo-Motor	Straight walk (0-2)
	Surface righting reflex (0-1)
	Twisting (0-3)
	Beam walk (0-3)
	Beam balance (0-3)
	Round stick balance (0-1)
	Wire suspension test (0-6)
	Hemi-Monoparesis (0-1)
Sensory Reflexes	Flexion reflex (0-1)
	Pinna reflex (0-1)
	Corneal reflex (0-1)
	Acoustic startle response (0-1)
Cognitive	Exit circle (0-1)
	Seeking behavior (0-5)

NSS = Neurological severity score

3.10 **Behavioral Analysis**

Behavior tests were performed on mice after 7 week of trauma. Behaviors were performed during the light cycle of mice i.e. between 9am 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 25 ± 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.

3.10.1 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 that depends upon distal cues to navigate from specific start locations of an open swimming arena to locate a submerged escape platform. 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.

3.10.1.1 Training

For five consecutive days the mice were put to training to locate the hidden platform. The mice had to undergo five trials session 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 being allowed to locate the platform for at least 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.

3.10.1.2 Probe trail

A single probe trial was performed after Morris water maze test on day 6 for all mice. All the procedure was same as MWM except for that the platform was removed and the mice were being released to the tank, trying to locate the platform. The trial was recorded with a camera, and was analysed for the number of entries to the target quadrant, time spent to search for the platform in the target quadrant and number of crossing of mice in the area where platform was supposed to be placed.

Table 3-3 the direction release of the mice for Morris Water Maze test

No. of Days	Release Direction				
	Trial 1	Trail 2	Trail 3	Trail 4	Trail 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
Day 6	Probe Trial without Platorm. Release direction, West.				

3.10.2 Open Field Test:

Open field test is being used to evaluate locomotory and exploratory activity as well as anxiety levels in mice (Gould et al., 2009). Spending more time in the centre of the arena and rearing on the side walls depicts exploratory activity, while grooming behaviour indicates stress and anxiety; the quicker the mice are to begin grooming in the arena, the more the anxious they are (Komorowska and Pisula, 2003, Negishi et al., 2005). Animals were placed in a square shaped arena (40x40x40cm) and their activity was recorded with a camera for 30 minutes. The apparatus was thoroughly cleaned after each mouse, so that the behaviour of next mouse would not be affected. The following parameters were assessed during the test.

- Total time spent by each mouse in the centre and peripheral area of the arena for entire 30 minutes. Mice preferring peripheral region are more likely to be depressed and more anxious. The mice crossing the line from peripheral region to central region shows more locomotor and exploratory activity and hence having low anxiety levels.
- The number of times the mice reared on their hind-paws and the time when the mice started to display grooming behaviour by licking parts of their body (grooming latency). These two parameters were measured for the first and last 5 minutes of the experiment, to compare how the mice adopt to new environment.

3.10.3 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 mice will expend extra time exploring and investigating the novel object with which it has no interaction in familiarization session. The NOR protocol earlier described by (Silvers et al., 2007) was adopted with few adjustments. The test has been carried out in a square

box which was made-up of iron with dimension of 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 protocol was identical as familiarization session except for that one of the two objects from familiarization was replaced by 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 has been determined through:

[(Time Spent on the Novel Object/Total Time) × 100]

3.10.4 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 22 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 in 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 mice had made in each arm. The pr otocol was adopted from (Walf and Frye, 2007) with few modifications.

3.10.5 Fear Conditioning

The test protocol was the same as (Dineley et al., 2010) with slight modification. The procedure was started by placing the mice in the chamber for five minutes for habituation process to avoid any depression and anxiety level due to new environment. The test protocol was comprised of five tones of 80 db, each tone lasting for 30 sec called as conditioned stimulus, which is followed by 1-s 0.5-mA foot shock. These shocks were given with an interval of 3 min. The reaction to the Conditioned Stimulus was analysed as freezing response. Freezing response was measured with the help of ANY-maze software. Each freezing response against the tone was being expressed in percentage by using the following formula.

$$\% \text{ freezing} = \frac{\text{time of freezing}}{30 \text{ s}} \times 100$$

3.10.6 Contextual Fear Conditioning

Contextual fear Test is done on the next day of fear conditioning to evaluate fear memory retrieval of the mice. The procedure as same as fear conditioning. The freezing response is assessed in the absence of shock stimulus for five minutes interval. Freezing response is expressed as

$$\% \text{ freezing } = \frac{\text{time of freezing}}{5 \text{ min}} \times 100$$

3.11 Statistical Analysis

Statistics of the data was made by means of GraphPad Prism Software (Version 5.03). Analysis of variance (ANOVA) was applied followed by Dunnett's Multiple Comparison Test and Bonferroni multiple comparison test to the data as the statistical tool for the analysis. P value less than 0.05 was considered significant. The data was shown as mean ± Standard Error of Mean (SEM).

RESULTS

To evaluate how much the metal treatment effect the recovery and outcome from traumatic brain injury different behavioural test were conducted. Morris Water Maze, Neurological severity Score, Fear conditioning and fear context were performed to check retrieval of memory formation. Elevated pus maze test and open field test was conducted to check the levels of anxiety and depression in mice after traumatic brain injury.

4.1 Effect of metals exposure after Trauma on cognitive behaviour

Neurological Severity Scoring (NSS) at particular time after traumatic brain injury was measured to evaluate the effect of metals after traumatic brain injury on cognition and motor abilities. All of the trauma induced mice showed distinct impairments in neurological performance at post 4h post-injury depicting the severity of injury. However, Steady progress in neurological performance was observed in all the groups during observation period of 24h, 48h, and 120h. However the metal treated group showed a little progress in NSS.

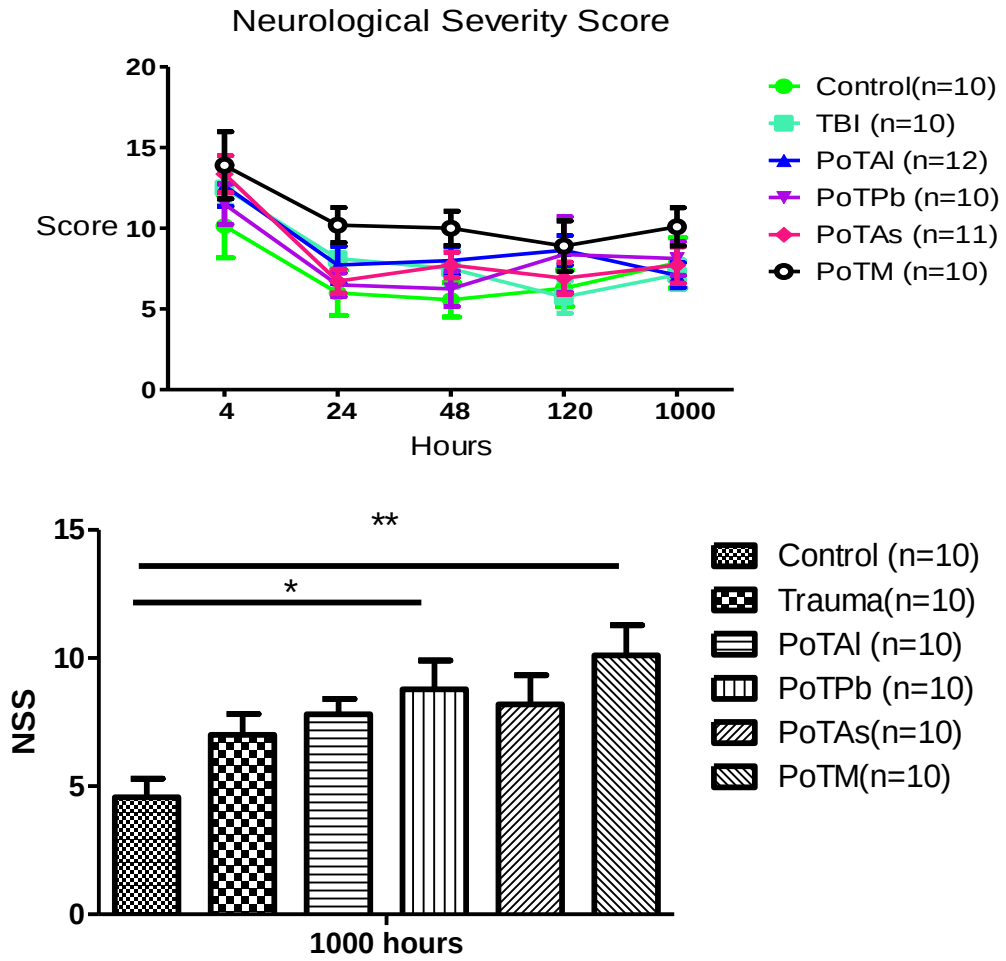

Figure 4-1 Effects of metals exposure after trauma on cognition (a) the line graph shows comparison of neurological severity score of different groups Contol, Trauma, PoTAl, PoTA, PoTPb and PoTM at 4h, 24h, 48h, 120h and 1000h. (b) The bar chart shows severity score of different groups at 1000h. Data is represented as mean ± SEM.

4.2 Effects of metals exposure after trauma on learning and memory

4.2.1 Effects of metals exposure after trauma on spatial memory

Morris water maze was performed for cognitive and behavioral assessment. Effect of metals after traumatic brain injury 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 significant improvement (P value < 0.001). Control showed the most improvement from day 5 (58.75±6 sec) to day 1 (5.75±1.2 sec); While the escape latency time at day 5 of Trauma, PoTAl, PoTPb, PoTAs and PoTM were 9.33±1.4, 28.71± 9.22, 11.57±3.6, 11.1±2.5, 6.8±1 .2 (P value < 0.001) respectively.

After two days of the training probe trial was implemented for assessment of reference memory. The platform has been detached for the probe trial to evaluate memory retrieval. And it was done by measuring total time spending in required quadrant, number of entrances to the required quadrant and quantity of platforms crossing at the location where platform was placed during training session.

Control spent the most time as compared to the meals treated groups ($P<0.05$). Both the control and trauma groups had the most number of entries into the target quadrant, showing sharp reference memory for the platform. The number of times the mice crossed the location where platform was previously present was also recorded to further confirming the reference memory for the platform, and the metals treated group especially metals mixed treated group has lowest number of platform crossing ($P<0.001$).

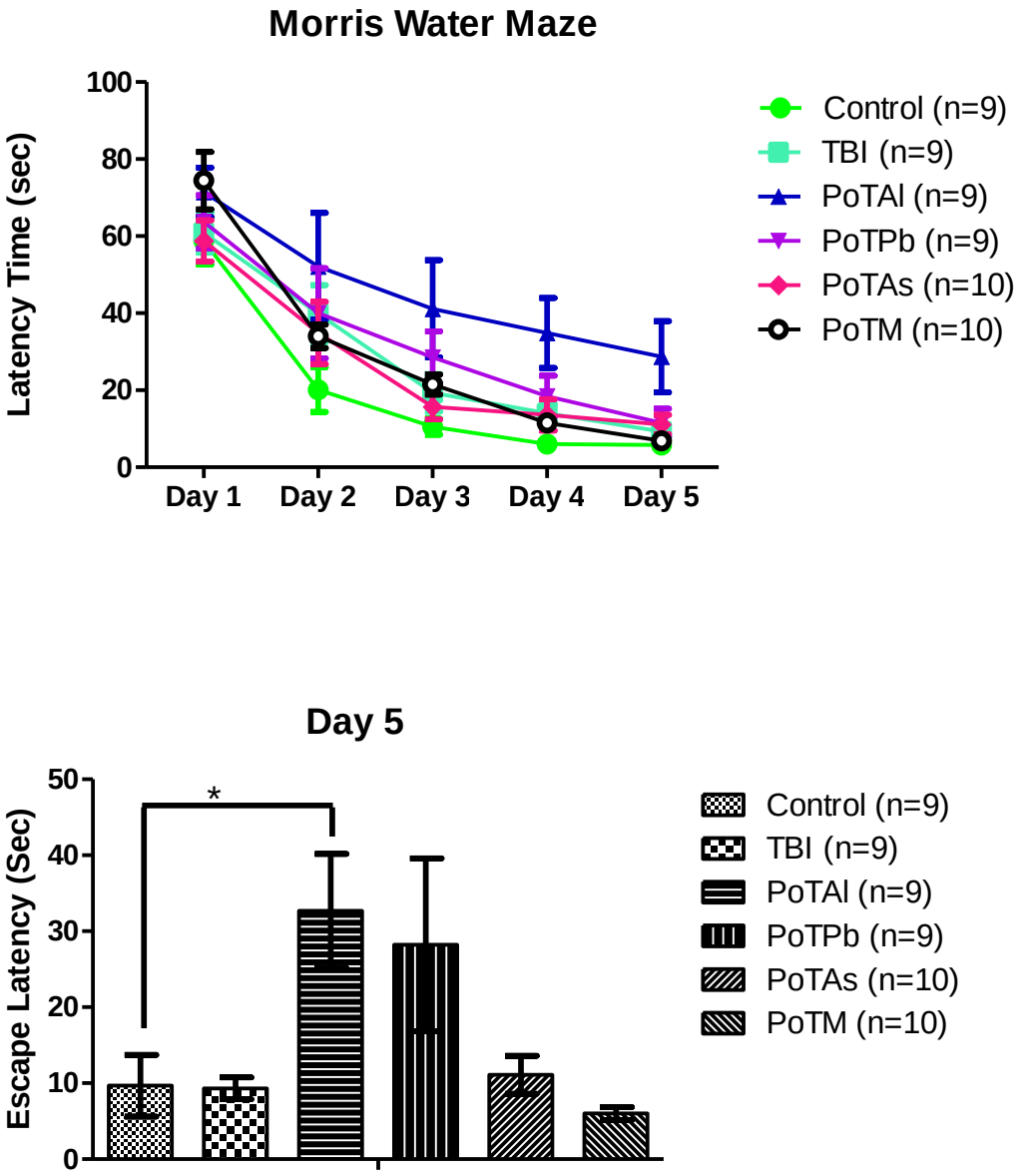

Figure 4-2 Effects of metal exposure after trauma on spatial memory (a) The graph showing escape latency (sec) of the Control (Sham), Trauma (Traumatic), PoTAl (Aluminium treated), PoTPb (Lead treated), PoTAs (Arsenic treated) and PoTM (metals mixed treated), training across 5 days acquiring spatial memory to locate hidden platform. Error bars shows mean ± SEM for two-way ANOVA. (b) The bar chart shows the escape latency of different groups at the last day of training.

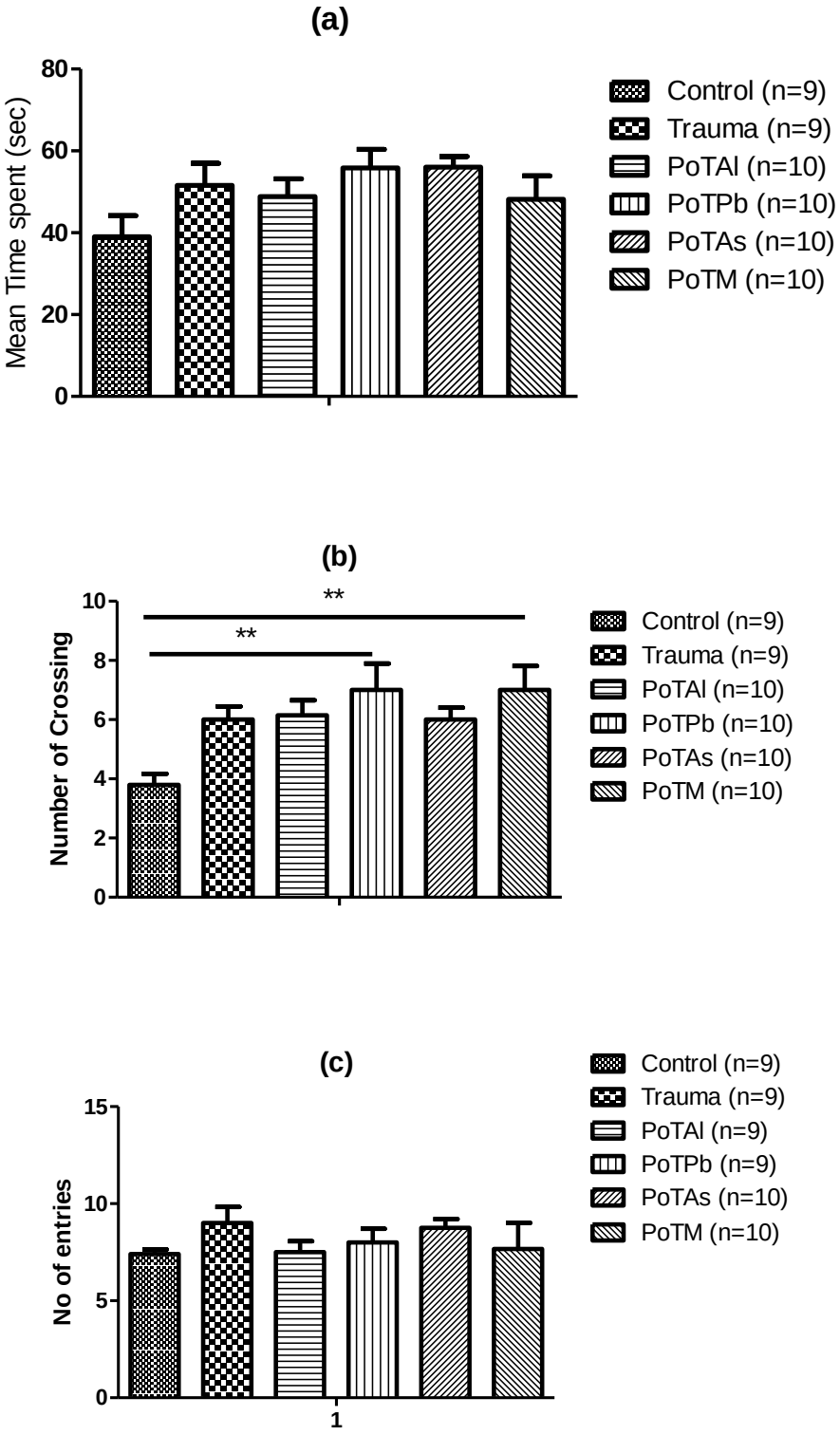

Figure 4-3: Morris Probe Trail (a) shows mean time spent (sec) by the mice in the target quadrant (b) shows number of times mice crossed the location of the platform (c) number of entries made by the mice to the target quadrant . The error bars represents mean ± SEM.

4.2.2 Effects of metals exposure after trauma on fear memory

Fear conditioning determines fear memory acquisition by evaluating percentage freezing across five cue foot shock trails. Freezing response is measured by analysing percentage freezing among the groups as well as across the five cues foot shock trails. Control group showed high freezing response (Mean value 43 ± 3.66) as compared to metals mixed treated group (mean value 23.26 ± 4.26)($P<0.01$). In fear context memory is acquired based on fear conditioning test. It tests if the animal remembers the environment linked to fear to which it is already exposed or not. Similar to fear conditioning freezing response is analysed in this test without the conditioned stimulus.

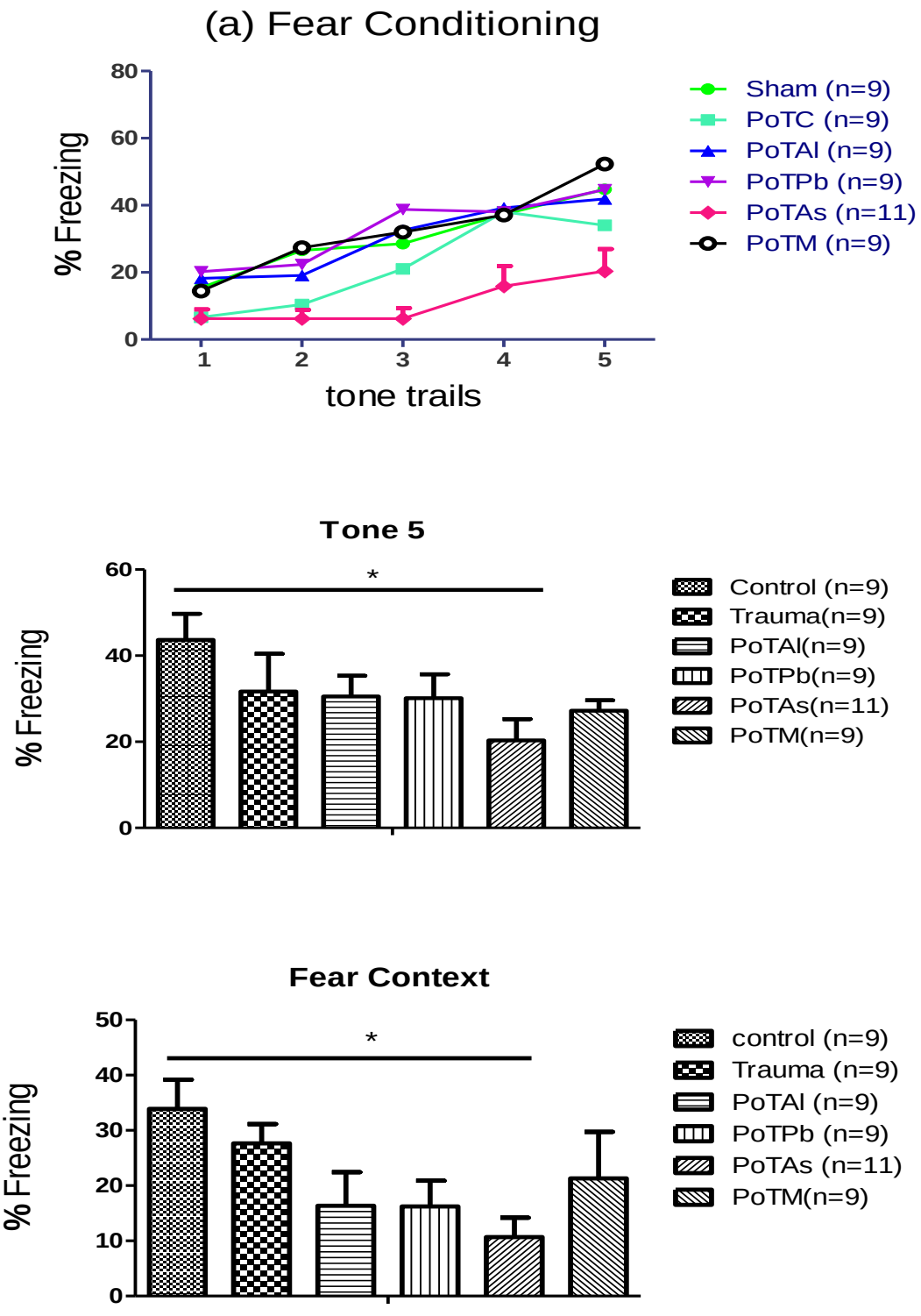

Figure 4-4: Effects of metals exposure on fear memory (a) Figure showing amygdala dependent fear memory acquisition across all five tone trails in all groups (b)Freezing response at tone 5 (c) This graph shows percentage freezing to determine fear memory acquisition

4.3 **Effects of metals exposure after trauma on recognition memory**

Spatial memory and exploratory behaviour was determined using novel object recognition test. Metals treated groups showed low interaction time with the novel object while control groups showed high interaction time with novel object. Discrimination index showed significantly lower ($P<0.01$) object recognition memory in metals treated groups.

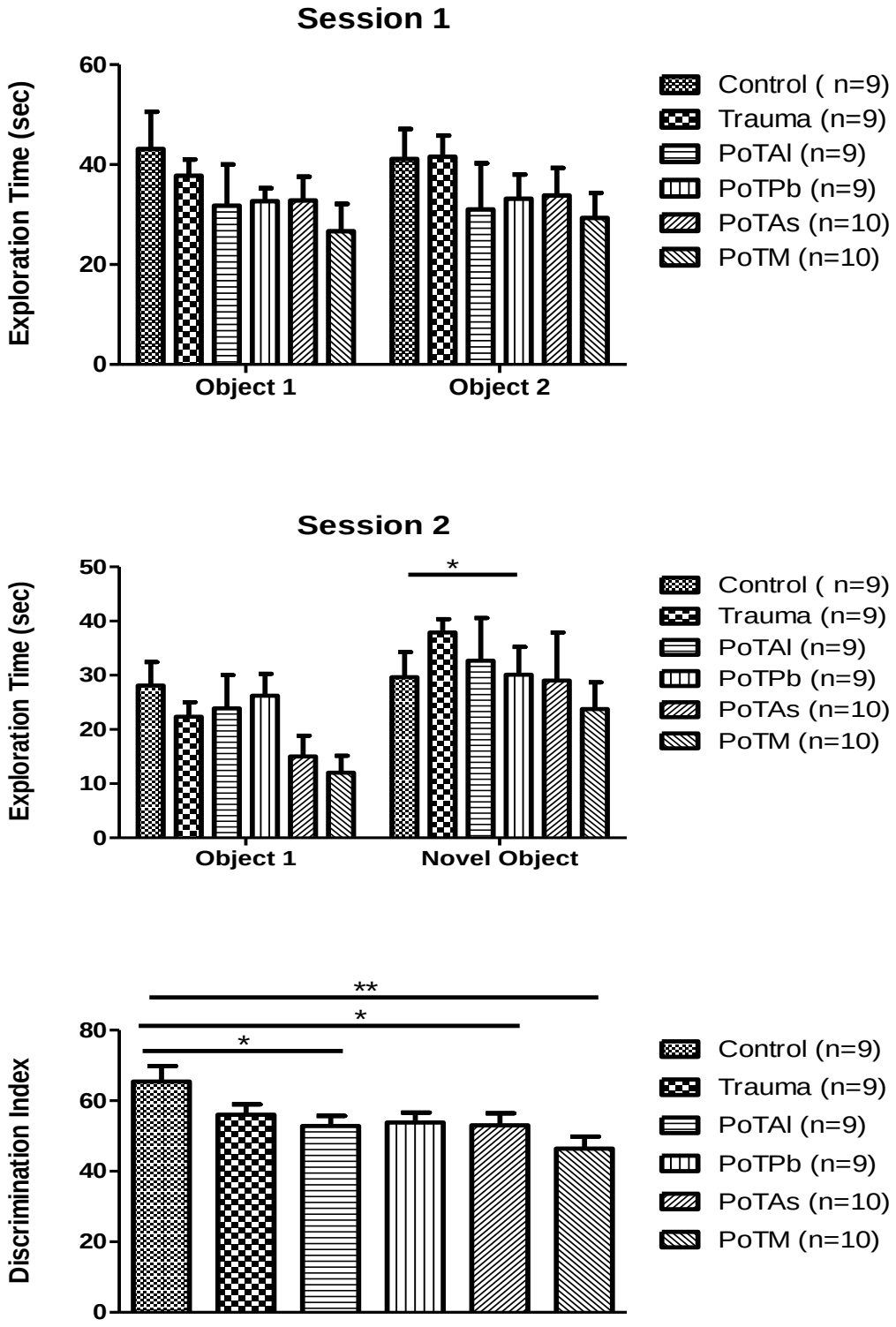

Figure 4-5 Effect of metals exposure on object recognition memory: (a) Comparison between different group interaction with similar objects in familiarization session (b) Interaction with novel object (c) Discrimination index of different groups. Data represented as mean ± SEM.

4.4 Effects of metals exposure after trauma on anxiety

4.4.1 Effects of metals exposure after trauma on anxiety and activity

Anxiety induced by open spaces and height was assessed in elevated plus maze. Number of entries to open arm and time spent in the open and closed arm had been used to evaluate anxiety and depression behaviour in mice. Control groups spent greater time in open arm suggesting low level of anxiety and depression while metals treated groups spent lower time in open arm showing high level of anxiety and depression. The data was non-significant.

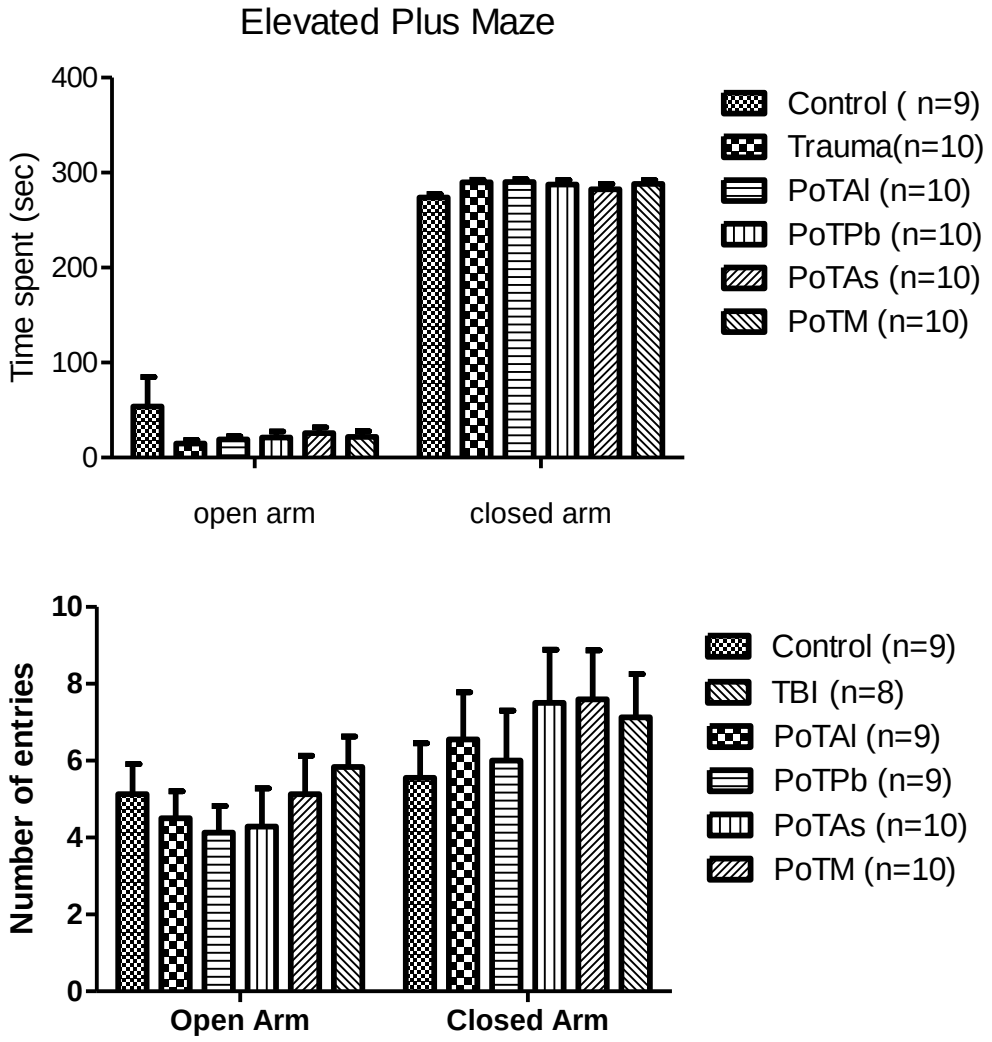

Figure 4-6 Effects of metals exposure on anxiety and activity: (a) shows total time spent by the mice in open arm and closed arm (b) Number entries to the closed arm. Data was analysed by two way ANOVA. Data is shown as mean ± SEM.

4.4.2 Effect of metals exposure on anxiety and exploratory activity

Open field test had been used to evaluate the effects of metals on anxiety and exploratory behavior in a traumatic brain injury mouse model of neurotoxicity. Time spent in the center, number of rearings and grooming latency were the parameters recorded for this test. The control and traumatic groups spent significantly less time in the centre of the arena (430 ± 22), as compared with the metals treated groups (230 ± 33)(figure 4.7) suggesting low levels of anxiety and depressive behavior($P<0.01$). Number of rearing was also greater in control group from metals treated group showing that control have less depression and anxiety than metals treat group ($P<0.001$).

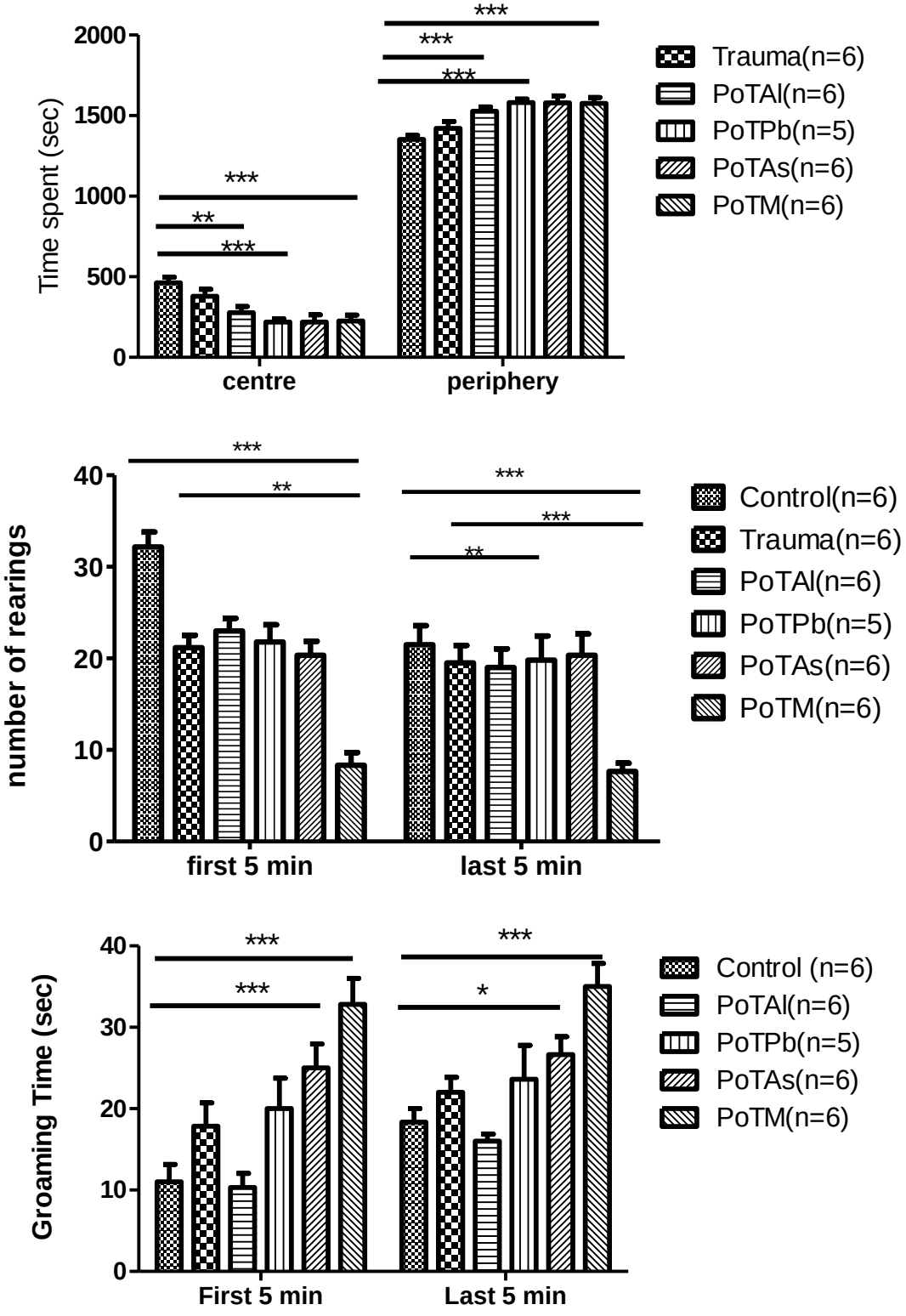

Figure 4-7 Effects of metals exposure on anxiety and exploration: (a) time spent at the centre and periphery of the arena (b) number of rearings of different groups at first and last 5 minutes of the test (c) total groaming time of all the groups during first and last 5 minutes of the test.

DISCUSSION

Animals model provide significant insights on treatment approaches fitting to a disease. In this study of traumatic brain injury are extensively examined by using weight drop model of traumatic brain injury. Numbers of animal models for TBI have been established over many decades to study clinical conditions associated with traumatic brain injury (Johnson et al., 2015). Traumatic brain injury pathology is quite complex and diverse (Finnie and Blumbergs, 2002); structural damage strongly correlate with the type of the injury. In the present study, diffuse brain injury was produced in mice and metal treatment was given to study the effect it causes after brain injury.

Heavy metals especially aluminium, arsenic and lead have long been known to produce cognitive deficits in humans and these effects have been verified and studied in animal models. Main sources of these metals include of drinking of water from industrial areas. The present study focuses on how these metals effect the traumatic brain injury outcomes and how their individual effects is differ from their combination effect.

NSS is the product of a test battery focused on interest, motor ability and reflexes. It was widely used to gage cognitive disorders following traumatic brain injury in rats in non-clinical studies. The NSS analysis indicated a decrease in intracranial and cerebral oedema due to osmotic therapy, improvement in motor coordination, responding to auditory stimulus and vigilance. (Rao and Lyketsos, 2000, Khandelwal et al., 2017) works shows impairment in subsequent to weight drop injury mice displace various neuropsychiatric deficits that are common in traumatic brain injury such as motor and sensory functions impairment loss of reflexes, cognitive deficits and impaired emotionality (Rao and Lyketsos, 2000). Neurological severity score (NSS) was utilized to determined immediate neurological deficits. NSS is based on multiple of tests that examine motor skills, reflexes and inquisitiveness; it is widely used in non-clinical studies to measure the neurological deficits after traumatic brain injury in rodents

(Khandelwal et al., 2017). All groups showed marked impairments at 4h post injury which depicted the severity of injury.

Control group severity score at 120h showed a decrease compared to the Trauma group and metals treated traumatic groups showing no decrease. Exposure of metals was found to potentiate the motor impairment caused by trauma. Their combined effect yielded deficits than their individual effect, suggesting that they potentiate the effect of each other in causing malfunctioning of pathways mediating motor abilities, while motor deficits remain the most common sequalae of TBI, a few studies have reported the exposure of metals like Al and As also impaired locomotor and vestibular activity to some extent (Rodriguez et al. 2012, Yadav et al. 2009). Testing of NSS suggests that chronic exposure to non-toxic metals dose of metals worsen the trauma impaired motor and sensory skills and delays recovery.

Learning and memory retrieval of Morris Water Maze test were used to assess spatial memory retrieval in rodents. 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 resulting in longer escape latency time and also significantly deficited in search time. Similarly, for metals treated groups the escape latency decrease rate was slow on the third and final day (Chen et al., 2015), findings show that mice that were treated with metals 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 metals. Similar results were observed in our experiment; as the mice treated revealed no learning on first day but a gradual decrease in escape latency after Day 2 (Morales et al., 2005).

In the probe trials, spatial memory retrieval has been evaluated on the basis of their training in Morris water maze test by assessing their performances in the target quadrant by removing

hidden platform. Metals treated groups performed the worst among all groups; displaying an impaired spatial memory recall and impaired hippocampal activity compared to control groups in probe trial. Metals treated groups had elevated escape latency depicting an impaired hippocampal activity.

Open field test has been used to evaluate exploratory behaviour, hyperactivity and anxiety. When placed in a symmetrical arena, mice may desire to stay near the periphery, and travel to the centre in explore the arena. An anxious mouse will not spend time exploring the surrounding, instead it will display grooming behaviour, licking parts of its body in anxiousness; an active mouse will occasionally move to the centre and rear on its hind-paws to explore, and showing late grooming behaviour. A hyper active mouse will spend more of its time in the centre and display more rearing and will spend less time in the periphery (Negishi et al., 2005). The results from this test are consistent with MWM, in the respect that metals treated group exhibited that most irregular behaviour for time spent in the centre of arena, in this test as well, compared to other: trauma group as well as Control. Although a pattern was observed, there was, however no significant difference recorded among the activity levels of any group.

Metals treated groups spent lesser time in the centre than Control. Similar findings were extracted from the number of rearing as well for aluminium, arsenic and lead groups. Control group showed the most exploration in comparison to all other groups. Combination metals treated group exhibited the least exploration via rearing; this may suggest that probably potentiate their effect to decreases the urge to explore the environment and show vertical activity.

Latency to exhibit grooming behaviour measures anxiety levels; late grooming shows low levels of anxiety. The results overall indicate that control group was the most active group

eager to and explore show vertical activity, while the metals treated groups showed performance differ than Control, spending moderate amount of time in the center as well as rearing to the walls of the arena. What this may suggest is that metals exposure decreases the urge to explore the environment compared to control, induces anxiety in mice as shown by the results of groom in latency.

The Novel Object Recognition based memory test has been widely used model for the assessment of memory retrieval. Although, it can be constituted to analyze working memory, attention, anxiety, and inclination for novelty in rodents (Andersson et al., 1999). 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 (Ben-Yishay and Diller, 1993). Control group showed an 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 such statistical variations are found in the object exploration index in the test sessions compared to the training session, suggesting a memory deficit (Moojen et al., 2012). TBI markedly decreased the object based memory formation. This impairment was potentiated by metal exposure evident by the minimal difference between the time spent with the familiar and novel object. Metals in combination seem to enhance the blocking of memory formation in lateral inferior parietal cortex and affect object recognition as chronic administration of metals mixture before trauma resulted on highly diminished ability to recognize between the two objects (Buchsbaum et al., 2015).

Trauma and metal exposure causes significant decrease in formation of fear memory. While memory impaired due to trauma tends to improve during successive tones and fear stimuli, exposure of target metals slow down the rate of fear memory improvement where the most significant impairment was shown by the lead. Pb partly suppresses neurogenesis and changes

of the differentiation pattern of other newly formed cells, this action is implicated in inhibition of fear memory formation (Jaako-movits et al., 2005).

Metals exposure in trauma produced a marked failure in development of context based memory compared to sham operated mice. While aluminium and lead augmented the trauma induced deficit, the freezing response in arsenic treated mice suggests its opposite behaviour where As repaired the memory decline in injured mice. Though (Cicerone et al., 2000)reported. As to impair fear based and context memory formation equally. Combination of metals yielded similar results as As treated group where it could be responsible for suppressing the contextual memory impairing effect of aluminium and lead.

Elevated plus maze allowed evaluation of the locomotor, depressive and anxiogenic effects of trauma and metal exposure in mice which are controlled by the cortex, hippocampus and amygdala. Control mice spent highest amount of time in open arm showing least anxiety and had fair locomotion into the alternate open and closed arms. Anxiety like behaviors are commonly seen in mice with TBI (Petraglia et al., 2014), however, Luo and colleagues reported contradictory results where trauma caused no change in activity of mice in elevated plus maze test compared to control (Luo et al., 2014).

Glutamatergic and cholinergic signalling play important role in mediating the process of learning, memory formation, retrieval and other cognitive function (Blokland, 1995, Myhrer, 2003). Cognitive system is majorly controlled by the cholinergic signalling comprising of muscarinic and nicotinic receptors reacting to neurotransmitter acetylcholine and glutamatergic signalling reacting through glutamate and controlled by metabotropic and ionotropic receptors. A balanced glutamatergic signalling is required for normal cognitive functioning, learning and memory are likely due to the neurodegenerative and auto protective pathways activated as a result of chronic metal exposure and TBI.

CONCLUSION

Metal Exposure alters the pathways involved in sensorimotor function, cognition and memory potentiating the cognitive inhibitory effects of brain injury. Combined exposure of metals Al, Pb and As yield results different from their exclusive exposure, they either potentiate or suppress their individual effect on traumatic brain injury. Non-toxic doses of metals on chronic exposure produce long lasting neurological deficits, augmenting the effects of brain injury after trauma.

FUTURE ASPECTS

In vitro verification of these findings to confirm the role of environmental metal exposure on Traumatic brain injury outcomes. Development of treatment regime is needed for Traumatic brain injury considering the interfering role played by metals. Further investigation should be conceded on for the assessment of the neuronal effects of metals in combination instead of their individual effects.

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