Effect of Temporal Exposure of Aluminum on Hippocampus Dependent Cognitive Functions and Post Exposure Recovery

Master of Science (MS)

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Effect of Temporal Exposure of Aluminum on Hippocampus Dependent Cognitive Functions and Post Exposure Recovery

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science (MS)

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FORM TH-4

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Dedicated to my

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ACKNOWLEDGEMENTS

All praises for Allah Almighty, the most Merciful and the most Gracious. Foremost, I like to send my gratitude to my supervisor and HOD, HCB- ASAB, Dr. Touqeer Ahmed for his inordinate guidance and consistent support in my research work. I am pledged to say my thanks to Dr. Husnain Janjua (Principal ASAB), my GEC members Dr.Saadia Zahid, Dr. Maria Shabbir and Dr. Salman Khan for their guidance.

I want to dedicate this work to my beloved son Shameer and my husband Abdul Hanan whose unswerving support made my way through all the obstacles. I want to thanks my mother, my father, siblings and In Laws who supported me throughout this period. I cannot forget the support and help of my sister Maria Siddiqui, my colleague's Dawood Amjad and Maham Nawaz. I will also like to mention my thankful regards to Mahnoor Qureshi and Yumna Rehman for their kind help.

In the end I want to dedicate this thesis to all those incredible women who study after marriage bearing all the household responsibilities. But they prove that nothing can come between quest for knowledge and strong women.

Alveena Siddiqui

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ABSTRACT

Due to higher load of development and industrialization animals and humans both are at higher exposure to metals especially Aluminum because of its high usage in everyday life. Higher level of aluminum in drinking water has long been linked with Alzheimer's and other cognitive dysfunctions. However, effect of time dependent exposure of aluminum on hippocampal dependent learning and memory has not been completely established. Mice model of temporal exposure of Aluminum ($AlCl_3.6H_2O$) with same total exposure of 5850 mg/kg was used to elucidate whether Control group, 50 day exposure group (117 mg/kg/day), 20 day exposure group (292.5 mg/kg/day) or 35 day exposure group (175 mg/kg/day) produce reversible or irreversible damage to learning and memory. Behavioral tests used to determine the effect were Morris water maze, Y-maze test, Social interaction and novelty preference and Hole board test. The result of Morris water maze test showed that 50 day exposure group presented highest impairment than Control group in learning and memory with no significant recovery after recovery period. Y-maze result depicted highest learning and memory deficit in both, 50 day exposure group and 20 day group with almost irreversible damage. 35 day group exhibited least deficit in learning and memory and also presented significant recovery on their own after 20 days of with drawl of Aluminum exposure. Social interaction results were also consistent with previous results and presented 50 day exposure group with highest deficit in social novelty performance. 20 day exposure group showed slightly better performance than 50 day exposure group as 20 day exposure group showed slight recovery in 20 day recovery group. Hole board test for the analysis of reference and working memory presented highest impairment in 20 day exposure group and 50 day exposure group as compared to Control group with almost no recovery in their respective recovery groups. Whereas 35 day exposure group showed very little

impairment in learning and memory and also better recovery in 35 day recovery group. It can be inferred from the results that both long term exposure with low dose and short term exposure with high dose both cause almost irreversible on its own hippocampal dependent learning and memory impairment. Future studies can reveal mechanism of temporal exposure of Aluminum suitable treatment depending on Aluminum daily exposure and duration.

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 Higher Cognitive Functions:

Cognition is the process of acquiring knowledge and comprehending it to perform various everyday life tasks. It forms the basis of our behaviors to achieve various goals through perception, learning, memory and thinking. Higher cognitive functions are executive function performed by brain comprising of thinking, problem solving, attention and decision making (Nelson et al., 2015). Cognitive functions are modified throughout our life time depending on neuronal plasticity.

Learning is a process of acquiring new knowledge, information or skill by experience. It can be defined as a process of assembling new information to make sense or alter the behavior accordingly. Learning can happen by paying attention or simply through interaction with new information. Memory is the usage of pre stored information to stride through a situation or achieve certain goal (Chan et al., 2008). Memory can outlive the stimulus which triggered it (Waddell et al., 2006). When memories are formed they can be long term, short term or working memory. Long term memory is then further divided into two types, one is declarative memory (memory of facts and figures) and other is procedural memory (unconscious memory of a skill like cycling). Declarative memory is referred to those fact and events that can be recalled consciously (recalling answer to the exam question) while procedural memories are related to unconscious working and are improved or enhance by practicing (riding a bike) (Knudsen et al., 2015). Working memory is a limited capacity information storage while performing a task. Information is retained and manipulated to achieve the goal (Baddeley et al., 1986).

1.2 Brain regions involved in learning and memory

Formation and storage of learned behaviors and memories are associated with the change in neuronal connections and neuronal plasticity in different regions of brain. Synaptic transmission (functional plasticity) and changes in synaptic connections (structural plasticity) forms the basis of any memory storage that occur (Korte & Schmitz, 2016). Various region of brain are involved directly or indirectly with the function of memory. Some of the major are hippocampus, prefrontal cortex, amygdala and cerebellum.

1.2.1 The Hippocampus

Hippocampus is responsible for converting short-term memory into long term memory. It forms the neural foundation for attainment and packaging of configure association between events. There are two kind of memory process: a simple associative process that does not require hippocampus network and configure associative system that requires hippocampal formation. The configuring associative system creates a unique representation of an elementary stimulus event and builds association between different elementary representations (Sutherland & Rudy, 1989).

Many neuroscientists believe that hippocampus is important in forming new memories as it helps in identification of new stimuli event, experience and places (Cohen & Eichenbaum, 1993). It is also regarded as a medial temporal lobe, memory system, for declarative memory, memory that can be verbalized such as facts. It also encrypts emotional data from amygdala. Episodic memories and places are connected (Tomer et al., 2014). It also [plays role in working memory, spatiotemporal situation tagging, temporal and spatial mapping, anxiety, storage of neocortical

cell-assembly addresses, change in irrelevant events, response inhibition, memory-retrieval processes, and associations (Sutherland & Rudy, 1989).

Hippocampus also plays role in spatial memory and navigation by help of place cells. Pyramidal cells are known to show response for place cells. Another important function is approach avoidance conflict. The anterior portion of hippocampus can detect conflicts whereas larger cortical and subcortical makes the decision. It occurs in a decision-making situation that requires a certain decision, either rewarding or punishing, the decision making is influenced by anxiety (O'Neil et al., 2015).

The loss of hippocampal formation results in impaired learning and memory. The bilateral symmetry is important, if one hemisphere gets damaged the other structure and functioning remain unaffected. Severe damage of both hemisphere results in anterograde amnesia, which is described as inability to form new memories and retrograde amnesia, in which the memories before damage are difficult to retrieve (Di Gennaro et al., 2006).

1.2.2 The prefrontal cortex

Prefrontal cortex located in cerebral cortex is important for human memory. Many neurologists and psychologists believe that the functioning of PFC and a person's personality are linked (DeYoung et al., 2010). PFC controls decision making, speech, language, social behavior and complex cognitive behavior (Yang & Raine, 2009). Basic function is the arrangement of thoughts according to person's will (Gabrieli et al., 1998).

Working memory, including all executive functions are controlled by PFC (Miller et al., 2002). Goldman-rakic determined this creates the representational knowledge which then helps in guiding actions, thoughts and emotions (Baddeley et al., 1986). Fuster proposed that PFC allows

connection of future and past which is necessary in determining goals. According to dynamic filtering theory PFC directs processing levels such as maintain information, selecting and retrieving information. It provides guidance to other parts of brain for proper processing of a given task (Miller & Cohen, 2001).

Some region of PFC is involved in generating language, speech and response before speaking. Words and sentences are processed majorly by left ventrolateral PFC. The retrieval of explicit memory is controlled by right prefrontal cortex for use of that memory in speech. The deactivated left is retrieves implicit memory for producing verbs. In amnesic patients there is impairment in nouns recollection (Hoffman, 2019).

Any injury in PFC affects cognitive memory. Such as loss in motor control, difficult to concentrate, loss of creativity and reasoning, short term memory deficits, temporal and source memory problems and difficulty in associative learning (Hoffman, 2019). Amygdala is located as two almond structures in the brain. Its major role is in generating emotions, processing memory and making decision (Amunts et al., 2005). Amygdala projections are extended to many parts of brain such as hypothalamus, thalamic reticular nuclei, facial nerves, to the ventral tegmental area, the lateral dorsally tegmental nucleus and trigeminal nerve nuclei and nucleus accumbens (Taskar et al., 2004). Thus it is involved in receiving information from olfactory bulbs and pheromone processing. It basically forms the connection with different parts of brain and then aids in processing information (Nieh et al., 2013).

1.2.3 The amygdala

Emotional learning is the major role of amygdala. It processes the emotional information and then store the related memories. Long term potentiation refers to relation between stimuli and

unpleasant event, which usually occur during fear conditioning. It is responsible form retrieval of long term fear associated memories (de Calignon et al., 2012). Memories related to emotions are usually stored in synapse all over the brain. Such as memories related to fear are stored in neural connections that extend from lateral nuclei of amygdala to its central nuclei. Nuclei of amygdala also process information from other brain parts that are important in making memory (Lalumiere, 2014).

Amygdala not only creates fear conditioning but it also creates positive (Appetitive) conditioning through distinct nuclei. Different nuclei within amygdala have different role in interpreting appetitive memory. Amygdala also has role in generating reward system. It is influenced by dopamine, primary pheromones and secondarily attractive odorants (Lalumiere, 2014). Another important function of amygdala is in memory modulation. For any event the long-term memory is formed immediately instead it is solely stored through long term potentiation. And during this process the memory might get modulated. Greater emotional arousal and stress related to event, greater are the chances that the event gets retained in memory as it is.

Any damage to amygdala results in loss of long-term potentiation function. It impairs generation of emotional response. The emotional memories are not formed if the neuromodulators in amygdala gets affected in damage (Uematsu et al., 2017).

1.3 Metals and Health Hazard:

Metals in spite of being unsafe to wellbeing have been utilized by people for a long time in numerous ways. Some of the metals are essentials for human body but some metal like arsenic and lead are lethal. Some essential metals required by body can also become dangerous when their amount exceeds permissible limits like Aluminum (Shekhar et al., 2008). Exhibition to these metals and their health-related issues has been expanding day by day around the world, particularly in developing countries. Various studies and investigations conducted in Pakistan have clearly depicted hazardous effects of metals in general population. Exposure to these harmful effects are most visible in children and older age people in the form of growth disorder and neurodegenerative diseases respectively.

1.3.1 Metal toxicity in Pakistan

Due to unpredictable and low economic and social conditions, Pakistan is facing environmental challenges. Due to high expansion of population various region are expanded in an unplanned way, which has resulted in haphazard environmental load. Due to high urbanization resources are limited that resulted in poor quality of natural resources like soil water and air (Merolla et al., 2014). Population of Pakistan is at disposal of risky metals through routes like unclean drinking water consumption, air pollution and industrial waste. Hazard index of toxic metal lies high in contaminated water and air. Areas of Pakistan that are at high threat of metal toxicity are Central area of Khyber Pakhtunkhwa, Central and Northern areas Punjab and Southern area of Sindh. Consumption of toxic metals like lead arsenic and aluminum is highest through drinking water in areas of Khyber Pakhtunkhwa. Through the route of air inhalation metal toxicity is highly concentrated in Punjab region (Shakir et al., 2017).

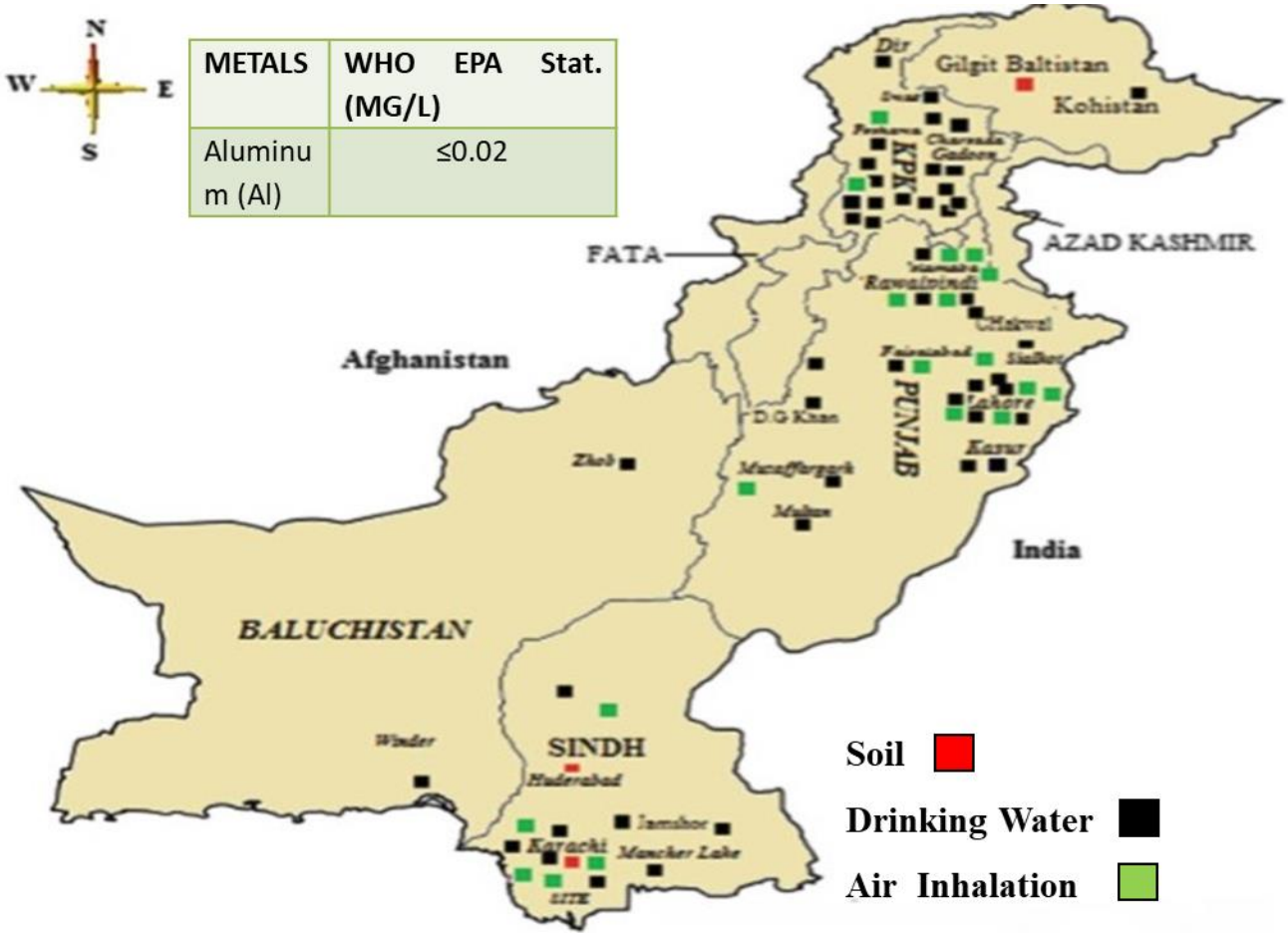


Figure 1.1: Modified image of Pakistani map indicating the areas with HI > 1 with heavy metals pollution in water, air and soil. Only those areas are shown here which pose the higher possible risks of metals contamination (Shakir et al., 2017).

1.4 Aluminum toxicity

Aluminum is the 3rd most abundant metal in earth crust. It is ubiquitously present in our environment in different forms. Aluminum is highly reactive metal and is found always in form of complexes with other chemical compound (L Blaylock, 2012). It has high affinity for oxygen, fluorine and silicon. Its property of being light weight has made its usage very prominent in modern everyday life. Humans are exposed to Aluminum through various routes like inhalation, oral route and skin (Exley & House, 2011).

Through inhalation excessive amount of aluminum can lead to various lung problems and nervous system diseases. Some studies have depicted oral intake of high doses of Al associated with Alzhiemer's (Kawahara, 2005). Some studies have negated it too. Aluminum accumulation in body can occur due to compromised kidney function and result in brain and bones disorders. In children bones diseases are observed due to high aluminum consumption as it prevent phosphates to be absorbed from stomach resulting in weak bones. Slow growth rate in children has also been observed. Highest toxic effects of aluminum are observed in nervous system and result in poor performance of various neurological tests (Cassidy-Stone et al., 2008).

1.4.1 Sources of Aluminum intake

Aluminum, an extensively distributed metal; its ingestion resources are both natural and anthropogenic. In soil, aluminum is the 3rd most frequent element. It is present in water, soil and atmosphere (Cuciureanu et al., 2000). Whereas anthropogenic ally it is produced in industries and mining process. Food packaging and cooking utensils, drugs, cosmetics and food components residues are also a source of Aluminum. In industrial waste, high degree of aluminum contaminates the surroundings and influences the residing population (Soni et al.,

2002). The average oral intake of Al in ingesting water is about 0.3%, whilst 0.1% in food and drinks. The level of aluminum exposure on daily basis as stated by European Food Safety Authority (EFSA) is 28.6-214 µg/kg (Authority, 2011).

1.4.1.1 Food

Typical Al intake for humans is 10mg/day. In food it is naturally present, by cooking or processing the aluminum amount might get increased. In diet Al is mostly consumed from processed food like cheese, cereals, salts, baking powder, coloring agent, anticaking agent, herbs, spices and food additives. In fresh fruit and vegetables Al is present as it is a major element in soil. Tea leaves have a naturally high level of Al as they are grown in acidic soils (Tze et al., 2012). Everyday intake of Al in kids is determined to be 2-6 mg/kg and in adults 6-14 mg/kg according to FAO/WHO Experts Commission for food in 1989. In the United States an adult intake about 7–9 mg/day from food.

Al cutlery and kitchen utensils are also a primary source of Al. Amount of leaching of al from metal utensil is observed in many researches. The amount of leaching is significantly high in food after cooking (Aini et al., 2007). The highest aluminum amount was recorded in 2010. It was recorded in fruit and vegetables from the Canary Islands (Spain), due to volcanic nature of island the soil is highly acidic (Gonzalez-Weller et al., 2010). The differences in aluminum concentration might be due to the wide difference in area from where samples were collected or it may be due analytical techniques but it was that packaging of preserved food and acidification of soil can increase the level of Al significantly.

Table 1: Estimated intake of Aluminum through diet.

Food group	Al mean content (mg/kg)[a]	Adults(≥17 years old, 68.48 kg[b])	
		EDI (mg/day)	EDI (mg/day)
Beverages	1.11	0.05	0.14
Fish and seafood	11.9	0.75	1.12
Meat and its derivatives	5.98	0.92	0.99
Fruits	6.84	1.45	1.78
Milk and its derivatives	3.05	1.31	1.07
Vegetables	16.8	1.60	3.18

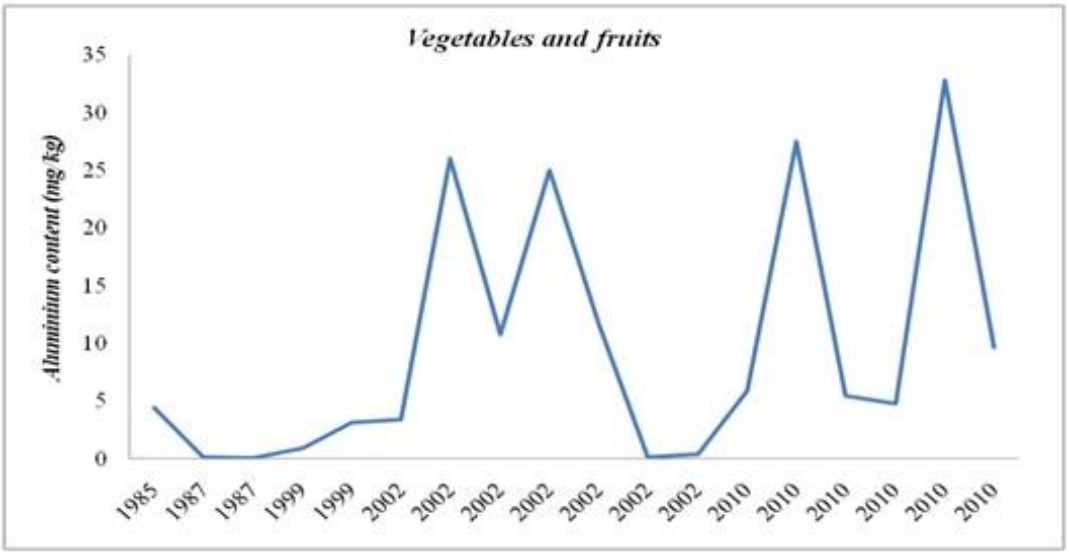


Figure 1.2: Aluminum evolution in fruit and vegetables in the period 1985 – 2010.

1.4.1.2 Water

Water is forms up to 60% of the human body. Aluminum can be beneficial and harmful it depends upon its concentration in the drinking water. Dissolved Al concentration in water at normal pH is from 0.001 to 0.05 mg/L. In acidic water or water rich in organic matter the value can increase (Liu et al., 2011). Al level also vary according to aluminum coagulants used for water treatment. Most commonly Aluminum Sulfate ($Al_2 (SO_4)_3$) is used (WHO, 2001). Table 2 refers to the different levels of Al identified in water and selected drinks by different authors.

Table 2: **Mean aluminum content in drinking water and other drinks.**

Product	Al mean content (mg/L) ± SD
Water	0.12 ± 0.06
Mineral water, spring water and table water	2
Drinking water	0.016 ± 0.0004
Soft drinks (Cola, cans)	0.66
Fruit juice and fruit juice drinks	3
Sweetened tea	2.2 ± 0.1
Herbal teas	0.14-1.065
Instant coffee	0.02-0.581
Whole coffee	0.235-1.163

1.4.1.3 Pharmaceuticals and Agrochemicals

The average intake of Al in drugs is 50-1000 mg/day. 104-208mg of aluminum is present antacids, current as aluminum hydroxide 300-600mg per tablet or 5ml as liquid dose. A buffered aspirin incorporates 10 to 20mg of Al. Some intravenously administered vaccine would possibly incorporate 684–5977 μg/g of Al. In vaccines, Al formulations are extensively used as adjuvant, these consist of aluminum hydroxide and aluminum phosphate. World Health Organization and Food and Drug Administration has identified a particular level of Al that is up to 0.85 mg/dose in vaccine as it is going in the blood. (Faroon et al., 2008) Antiperspirant, dentifrices, disinfectants, fumigants, pesticides, also are most important source of exposure to Al. Livestock food and water gets contaminated with Al. Litter and waste is additionally treated with aluminum sulfate and zeolite to stop phosphorus loss from lands (Moore Jr. et al., 2000). Al is also inhaled due to agrochemical fumigants and sprays. This indicates that people are directly exposed to Al through food and water. Pharmaceutically produce are predominant supply of Al as they are administered orally or parenteral administration to individuals. Also, agrochemicals contaminate water and food taken by individual.

1.5 Aluminum neurotoxicity:

The interference of any biological, physical, or chemical agent on the functionality of peripheral nervous system is termed as neurotoxicity. Elements like Aluminum, Arsenic, Mercury, Copper, Lead, and Manganese etc. are known to be neurotoxic in trace or high amounts depending on their periods of exposure and can lead to neuronal cell degeneration. (Hashmi & Hong, 2015) Strong evidences suggest that there is an undeniable link between the accumulation of Al in the brain and the occurrence of Alzheimer's disease. To test this, rats were orally administered with Al for 15 months and the morphological changes observed in the rats' brains were similar to

those present in Alzheimer affected patients' brains. Furthermore, Al accumulation in the brain was detected in patients suffering from Alzheimer's disease. These suggest that there must be some connection between Al and Alzheimer's. (YUMOTO et al., 1992) The cholinergic system refers to nerve cells in which the neurotransmission is done by acetylcholine. Since neurotransmission in many cells is mediated by acetylcholine, thus cholinergic system has a pivotal role in monitoring different functions of central and peripheral nervous system. iii (Hashmi & Hong, 2015) The principal neurotoxic effect of Al is the damage to learning and memory functions of CNS and this is caused by the damage to cholinergic system; thus Al must have a role in the dysfunctional performance of cholinergic system (De Jager et al., 2014). Muscarinic acetylcholine receptors (mAChR) are receptors of cholinergic system which mediate Acetylcholine's transmission. In higher brain functions i.e. learning and memory, nerve transmission takes place via muscarinic receptors. Muscarinic receptors have five genes and all are expressed in the hippocampus whose prime role is in learning and memory. In the hippocampus, they have diverse pre- and post-synaptic actions. (Levey, 1996) The overexpression of Amyloid Precursor Protein (APP) is one of the events that take place in the initial stages of Alzheimer's pathogenesis. The AP protein has many variants of different amino acid lengths emerged as a result of alternate splicing of mRNA from different sites. The full-length APP consists of 18 exons and has a length of 770 amino acids (Roßner et al., 1998). There are two types of APP cleavage; amyloidogenic and non amyloidogenic cleavage is done by β-secretase enzyme and results in β-amyloid protein (Aβ). Non amyloidogenic cleavage is done by α-secretase. The β-Amyloid (Aβ) protein forms clumps called amyloid plaques which are evidently found in all Alzheimer's patients (Ehehalt et al., 2003). An interrelationship exists between the functioning of cholinergic system and the production and deposition of beta amyloid

protein in the Alzheimer's disease which is the main dementia causing disease. Along with amyloid plaques, neurofibrillary tangles are also primary markers of

Alzheimer's and are caused by hyper phosphorylation of tau protein which is a CNS protein. These neurofibrillary tangles are also caused by beta amyloid(Pavia et al., 1998). An inverse relation exists between cholinergic functioning and Aβ; the decline in the performance of cholinergic system leads to increased production of Aβ through amyloidogenic cleavage of Amyloid Precursor Protein (APP). The M1 and M3 subtypes of muscarinic receptors of cholinergic systems promote the signal transduction and non amyloidogenic cleavage of APP. (Hashmi & Hong, 2015). Several experiments to test this hypothesis have been designed where M1 expression was suppressed through genetic deletion. The results showed that M1 receptor deletion leads to amyloid pathology due to enhanced amyloidogenic cleavage. This increases concentration of Aβ and thus deposition of amyloid plaques which are morphological markers of Alzheimer's (Jeon et al., 2010).

Aluminum accumulation in brain affects nerve transmission in the cholinergic system as it affects the synthesis, binding as well as degradation of acetylcholine which is the main component of cholinergic system. Due to weak binding of acetylcholine with its muscarinic receptors, signal transduction through these receptors is diminished and as a result, there is an increase in Aβ levels in brain due to amyloidogenic cleavage of APP. This leads to formation of amyloid plaques in the brain which is an event that takes place in the start of Alzheimer's pathology. (Mehpara Farhat et al., 2019) Another way through which Al shows its neurotoxic effects is by interfering with the balanced metal ion concentrations of neurons and disturbing their homeostasis. Metal ions regulate the functioning of many proteins and are thus important for normal cognitive functions like thinking, reasoning, remembering, and problem solving etc.

Imbalance in the normal concentrations may lead to protein aggregation or generation of reactive oxidative species (ROS) as a result of metal-protein association and metal-catalyzed protein oxidation respectively. Moreover, Al causes increased expression of cyclooxygenase2 enzyme which has role in inflammation genesis of cancer. It can also cause apoptotic cell death in hippocampal cells by up-regulation of bax mRNA and down-regulation of bcl-2 mRNA. This degeneration of neurons in hippocampus directly induces learning and memory impairment in laboratory mice (De Jager et al., 2014).

1.6 Excretion of Aluminum:

Almost 95 % of Aluminum is excreted through renal system and only 1% through fecal route (Sjögren et al., 1988). Some of the other routes are illustrated in Figure 1.3.

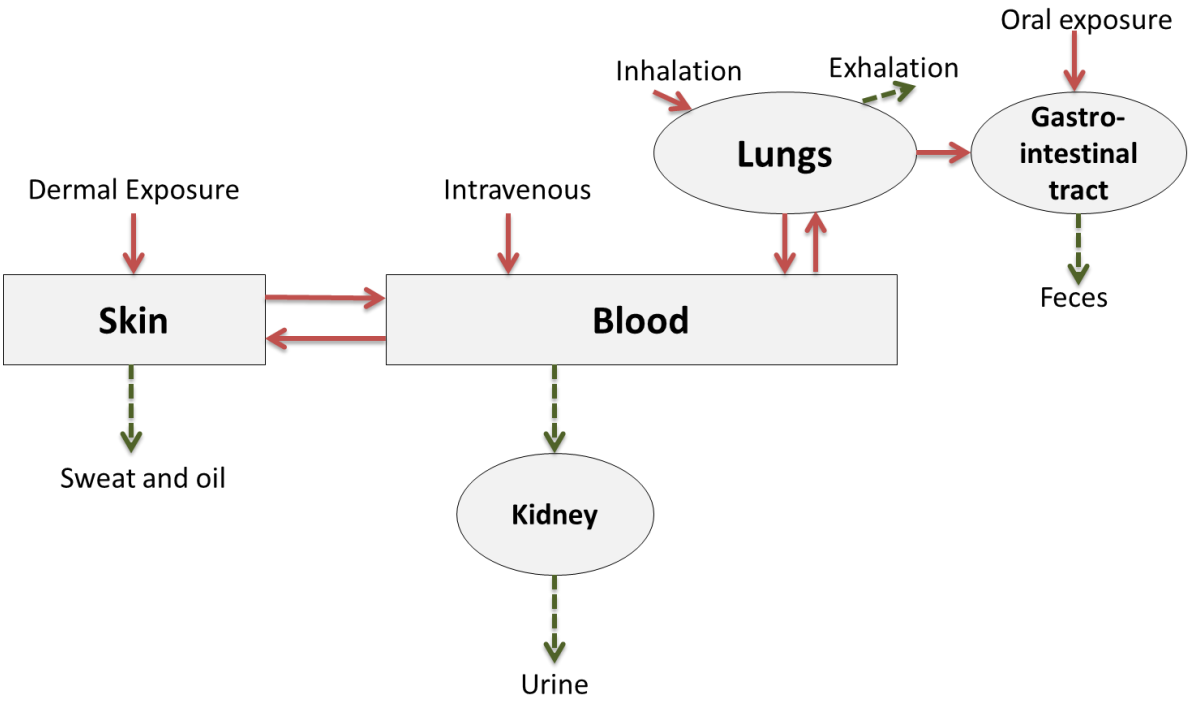


Figure 1.3 Illustration of excretion of Aluminum through body.

1.7 Aluminum Neurotoxicity Models:

Table 3: Aluminum neurotoxicity in animal models.

Animal model	Age	Route of administration	Aluminum dose	Dose duration	References
Male Swiss albino mice	8 weeks	Orally	$AlCl_3$ 50 mg/kg body weight per day	6 weeks	(Al-Amin et al., 2019)
Male wild type kunming mice	8 week	Orally	$AlCl_3$ 100 mg/kg/day	90 days	(Feng et al., 2018)
Male/female ICR mice	Adult	Nasal drip	AlNp 50 mg/kg of body weight	2 weeks	(Zhang et al., 2018)
Kunming mice	Not specified	Orally	$AlCl_3$ 40 mg/kg/day	4 week	(Li et al., 2017)
Male balb/c mice	3 months	Orally	$AlCl_3$ 250 mg/kg	42 days	(Farhat, Mahboob, & Ahmed, 2017)
Male balb/c mice	3–4 months	Orally	$AlCl_3$ 250 mg/kg/day	42 days	(Farhat, Mahboob, Iqbal, et al., 2017)
Male balb/c mice	3–4 months	Orally	$AlCl_3$ 250 mg/kg	42 days	(Mehpara Farhat et al., 2019)
Female cd1 mice	8 weeks	Im injections	Al hydrogel at the doses of 200, 400 or 800 µg al/kg	180 days	(Crepeaux et al., 2017)
Male swiss albino mice	Not specified	Orally	$AlCl_3$ 100 mg/kg/day	42 days.	(Jangra et al., 2015)
Balb/c and c57bl/6 mice	Not specified	Intra Peritonial	$AlCl_3$ 40 mg/kg/day	45 days	(Shati et al., 2011)

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Ethical Statement and Letter of Permission:

Atta Ur Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST) laboratory animal house maintained the animal under standard environment. All the testing protocols and procedures of the study were approved by Institution Review Board at Atta Ur Rahman School of Applied Biosciences, NUST (IRB # 135). All the test and procedures were performed under the regulations by the Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA .

2.2 Animal Model:

Study is carried out on male Balb/c mice, 40 provided by Laboratory Animal House, ASAB, NUST and 50 purchased from National Institute of Health (NIH), Islamabad. Animal were kept under standard condition in 14 plastic cages of size (40cm×25cm×15cm). 5 Animal were kept in each cage with soft wood shavings as bedding. Housing conditions were maintained at temperature of 22±2°C and 12-hour light/dark cycle with standard feed and water provision.

2.3 Chemical and Reagents:

Aluminum salt in this study used is aluminum chloride hexahydrate $AlCl_3.6H2O$ by Sigma Aldrich. Solutions were made using standard distilled water.

2.4 Study Design:

2.4.1 Animal Groups:

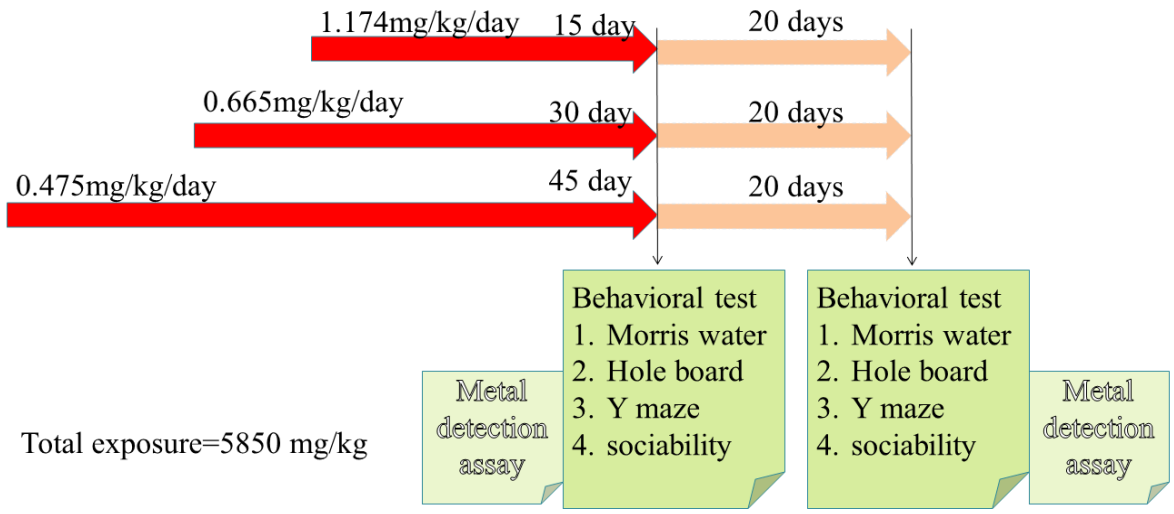
The animals were separated arbitrarily into the following groups:

Table 4: Animal groups employed in study.

Group No.	**Description**	**No. of Mice**
1	Control	10
2	20 days group	10
3	35 days group	10
4	50 days group	10
Total No. of mice		**40**

Control group was sustained at normal water. 20 day group was provided with Aluminum salt solution in distilled water at a dose of 1170 (mg/day/liter). 35 day group was given aluminum salt in distilled water at a dose of 668.56 (mg/day/liter). And 50 day group was sustained at aluminum salt in distilled water at a dose of 468 (mg/day/liter). Thus total dose for all the exposure groups were 5850 (mg/kg). All the groups were given standard feed.

2.4.2 Methodology layout:



All the exposure groups were given aluminum dosage according to the days specified i.e. 20 days, 35 days and 50 days. After the completion of exposure time, a recovery period of 20 days was provided to each group. In recovery group animals were given normal water and feed.

2.5 Behavioral Tests:

Behavioral tests were performed on all the animals under standard environmental condition. All the animals were habituated in a different room. And tests were carried out in mice day cycle to

prevent variations in the performance due to circadian rhythm disturbance. Inter test interval was kept 45 minutes minimum. Temperature of both the habituation room and testing room was maintained at 22±2°C. Behavioral test performed are mentioned in Table 5.

Table 5 : Behavioral test used and their association with learning and memory.

Test	**Brain region involved**	**Behavior**
Y maze test	Hippocampus prefrontal cortex	spatial learning and memory
Morris water maze	Hippocampus	spatial learning and memory
Hole board test	Hippocampus Amygdala	exploratory behavior/anxiety
Three chamber assay	prefrontal cortex	Sociability

2.5.1. Morris water maze test (MWM Test)

Purpose:

Morris water maze test is one of the most reliable test to assess hippocampal synaptic plasticity. Many type of Morris water maze have been developed but the best one is originated by Richard G. M. This test is used to determine spatial learning and memory through spatial cues provided to animal in its surrounding environment. Spatial learning is based on time taken by animal to reach submerged hidden platform in repeated trials. Reference memory is analyzed by animal tendency toward platform area when the platform is removed (Vorhees & Williams, 2006).

Apparatus:

Morris water maze test consist of a circular tank filled with water that is made opaque. A platform is placed in one of the quadrant of tank and is camouflaged due to the opacity of water. The level of water in the tank is such that the platform is just submerged in it. Temperature is maintained at 23±2°C throughout the experiment.

Procedure:

Morris water maze test has a protocol of 6 days. Training trials are conducted for 5 days then probe trial is performed.

Training period

Training period comprised of 5 days in which 5 trials are conducted each day with platform submerged in the water. Animal were dropped in the tank from different directions in each trial. Directions for each day were determined according to the table 4. Each trial is of 90 seconds in

which animal is allowed to find the platform by observing cues. If the animal finds platform before 90 seconds and sits on it for a minimum of 5 seconds then the trial was stopped and time was noted. If animal is unable to find platform in 90 seconds it will be placed on the platform manually for at least 20 seconds. It is then removed from tank and placed in its cage. Inter trial gap of 10 minute was maintained in training session. Time recorded in training session is then used to analyze escape latency of mice.

Table 6: Training sessions for Morris water maze test.

No. Of Days	Direction of Release				
	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**
1	West	South	North	East	South
2	North	West	East	West	South
3	North	East	West	South	North
4	East	South	West	East	North
5	West	South	North	East	South
6 (Probe Trial)	Single probe trial without platform. Direction of release: West				

Probe trial

After the successful completion of training period probe trial was conducted on next day. Platform was removed from the tank. Animal was dropped from the south direction in the tank and allowed to explore and search for platform for 90 seconds. Video was recorded through camera above the tank. Video will then be analyzed for following parameters.

1. Number of entries in target quadrant
2. Time spent in target quadrant
3. Number of platform crossings

2.5.2 Y-Maze Test

Purpose of test

Y maze test is a behavioral test based on hippocampus dependent spatial learning and memory. It is used to analyze the performance of working memory as well as recognition memory. This test is based on rodent natural curiosity to explore their environment. Spontaneous alternations and exploration of novel arm instead of visiting the already explored arm is the basis of this test. Various regions of brain are involved which are directly related to spatial learning and memory. Main regions involved are hippocampus, prefrontal cortex, basal forebrain and septum.

Apparatus:

The Y-maze is made up of 3 rectangular arms with a dimension of 50 x 16 x 32 cm . These three arms are unified at angle of 120º. Arms are named as “Start arm”, in which animal is placed when trial is started. Second is “Other arm” that is kept unblocked during habituation. And the

third is "Novel arm". The ends of these three arms are labeled with different white and black pattern to provide spatial cues.

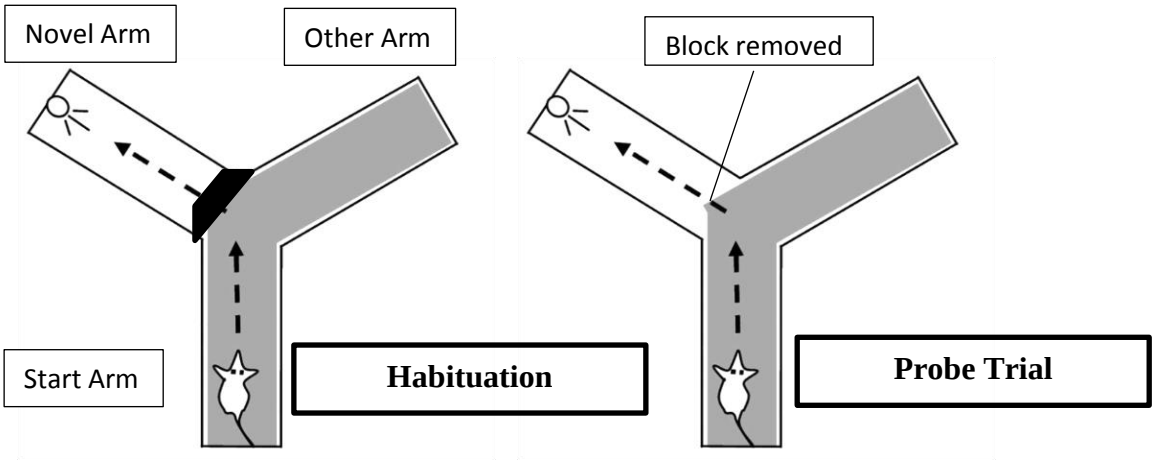


Figure 2.1: Diagrammatic representation of Y maze test.

The rodent enters the maze in the arm labeled "Start arm", facing away from the center. The rodent is then allowed to explore the two arms, in the training session, while in the probe trial, the "novel arm" is opened and the rodent is allowed explore all three arms freely. The rodent is expected to show a tendency towards exploring a less recently visited arm. Total number of arm entries, total time spent in each arm, and the numbers of triads are recorded to determine the spontaneous alternations. An entry is recorded when all four limbs are inside the arm.

Procedure

Y maze test was performed in two sessions. First is the habituation or training period and second is probe trial that is recorded by camera to analyze animal performance in the test. The experiment was executed with a little alteration to the y-maze protocol by Conrad et al., 1996.

Habituation:

In habituation period animal was given free access to two of the arms that are start arm and other arm. The novel arm was kept blocked by a removable wooden block. Session started when the animal was dropped in the start arm of the y maze with its face toward the wall of start arm. Habituation is carried out for 15 minutes in which animal explored its environment. After the end of habituation time animal was removed from the maze and placed back in its cage to provide inter trial time of 30 minutes between habituation and probe trial.

Probe trial

In probe trial the wooden blocked used to block novel arm was removed. Trial was started by dropping animal in the start arm with its head facing toward the center of the maze. Animal is then allowed to explore the maze for 5 minutes. The trial is recorded by a camera above the maze. Maze was thoroughly cleaned and wiped with 70% ethanol in between habituation, probe trial and next animal session to prevent any olfactory cues to the animal. Recorded videos will be used to assess following parameters:

1. Number of entries in each arm
2. Time spent in each arm
3. Spontaneous alternations
4. Alternate arm repeats and
5. Same arm repeats.

2.5.3 Social Preference and Novelty Test:

Purpose

This test is employed to assess animal general social interaction and preference for novelty object. Performance of an animal can be used to determine sociability and novelty deficit. It can also be used to check cognition by analyzing animal remembrance of familiar and unfamiliar novel mouse.

Apparatus

Three Chamber Assay is made up of glass rectangular box with three compartments in it. Compartments are separated by a glass wall with a door like hole in them so that animal can freely move between three chambers. 2 metal wire cages are placed in left and right chamber named S1 and S2 in which unfamiliar mice will be placed.

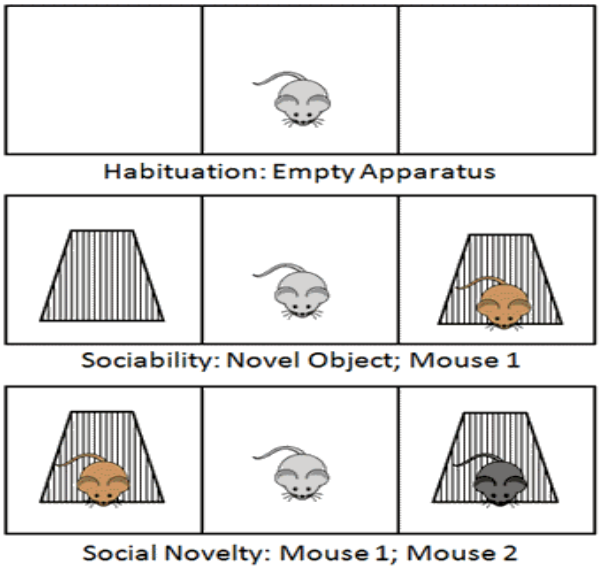


Figure 2.2: Representation of Social Preference and Novelty Test

Procedure:

Habituation

In habituation period empty cages were placed in chamber. Animal was dropped in the centeral chamber and was allowed to explore freely for 5 minutes. After habituation time animal was removed and placed back in its cage for 30 minutes before the start of session 1.

Session 1

In this session 1 wire cage was provided with a stranger mouse(S1) while other wire cage was left empty. Animal was dropped in the center compartment and was allowed to move and interact freely for ten minutes. Video was recorded to assess following parameters

1. Time spent in each chamber
2. Interaction time with empty cage and S1 cage.

Session 2

Session 2 will be carried out after 20 minutes of session 1. In this session S1 cage was provided with already familiar mouse of session 1 and a new non familiar stranger mouse in S2 cage. Animal was dropped in center compartment and allowed to move and interact freely for 10 minutes. In both sessions apparatus was thoroughly cleaned with 70% Ethanol. Videos of session was recorded to analyze following parameters

1. Time spent in each chamber.
2. Interaction time with S1 and S2 (novelty).
3. Percentage discrimination index.

2.5.4 Hole Board Test:

Purpose:

Hole board test was developed in 1970s to overcome the flaws of open field test. Modified form of hole board test used by Li et al. (2009). Modified hole board test can be employed to assess working memory and reference memory (spatial reference memory). Working memory is analyzed by observing the recurrent visit to wrong choices (empty hole). If in a trial animal visits a wrong hole (un baited hole) and in the same trial visits that hole again then it has committed a working memory error. Reference memory is based on long term and associated with spatial cues in surrounding. All the visits to wrong holes (unbaited holes) will be considered as reference memory errors (Li et al., 2009). Anxiety of animal can also assessed by hole board test through latency to reach the first hole.

Apparatus:

Hole board apparatus is a square box made up of sheen wood or board. The box is open from top to record the videos. The bottom of box contains 16 holes in it. These holes will be baited with feed or left un baited during trials. Different spatial cues were pasted on the walls of the hole board to assess reference memory.

Procedure

Pre habituation:

Animals were deprived of feed 24 hours before the start of habituation period to increase their quest for food. Water was provided normally.

Habituation

Animal were taken to the testing room prior day to acclimatize them to environment. All hole were baited with 100 mg of feed. 2 sessions of habituation were carried out. Animal was dropped in the center of apparatus for 15 minutes and was allowed to freely explore the box. After 15 minutes animal was removed and placed back into its cage. After an inter session interval of 3 hours habituation session was repeated again with the same protocol

Trials session

It was performed on the next day of habituation and continued for 4 days. Each session per day composed of 4 to 5 trials in which animal was dropped in the apparatus from different directions. Trial is of 3 minutes and in all the trial same 4 holes were kept baited with 300gm of feed. After each session and in between trials apparatus was thoroughly cleaned with 70% ethanol. Trials were repeated with same protocols. Videos were recorded for each trial and will be analyzed to assess parameter given in table 7.

Table 7: Parameters assessed in Hole board test.

MEMORY	PARAMETER	NARRATIVE	PURPOSE
Working memory	Working memory error	Any revisit to un baited hole in the same trial. (Animal nose should be below the rim of board floor)	To assess grade of hippocampal damage and effect on learning and memory.
Reference memory	Reference memory error	Any visit to wrong hole (un baited) in a trial. (Animal nose should be below the rim of board floor)	
Latency	Time taken to visit first hole	It is measured as the time required by an animal to visit the first baited or un baited hole when the trial starts.	To assess anxiety of an animal.
Activity	Nose pokes/ Head dips	No. of head dips or nose pokes in a minute	To assess locomotor activity and exploration rate.

CHAPTER 3

RESULTS

RESULTS

3.1 Morris Water Maze Test

Morris water maze test was employed to assess the effect of temporal exposure of Aluminum (5850mg/kg) on long term memory and learning. Memory deficit caused by Al in various temporal groups was analyzed through escape latency parameter. On first day all the three exposure groups have shown significant deficit in spatial learning as compared to control (58.49 ± 3.43). Highest deficit was seen in 50 day exposure group (77.66 ± 4.58) and 20 day exposure group (68.35 ± 6.69). Control group (58.49 ± 3.43), 20 day exposure (68.35 ± 6.69) and 35 day exposure group (58.65 ± 4.53) have shown almost similar learning behavior through all the next 4 days of training period. However, 50 day exposure group has shown decreased learning as compared to control group and other two exposure groups. Recovery groups have shown significant learning as compared to exposure groups. 50 day recovery group (25.10 ± 5.2) has shown least learning as compared to control (10.84 ±1.03), 15 day recovery (11.5 ±2.14) and 35 day recovery group (19.92 ±2.69). Comparison between exposure groups and recovery groups depict enhanced learning and memory after recovery period in all the recovery groups. Over all poor spatial learning behavior was observed in 50 day exposure group which did not improved after recovery period (Figure 3.1: A).

(A)

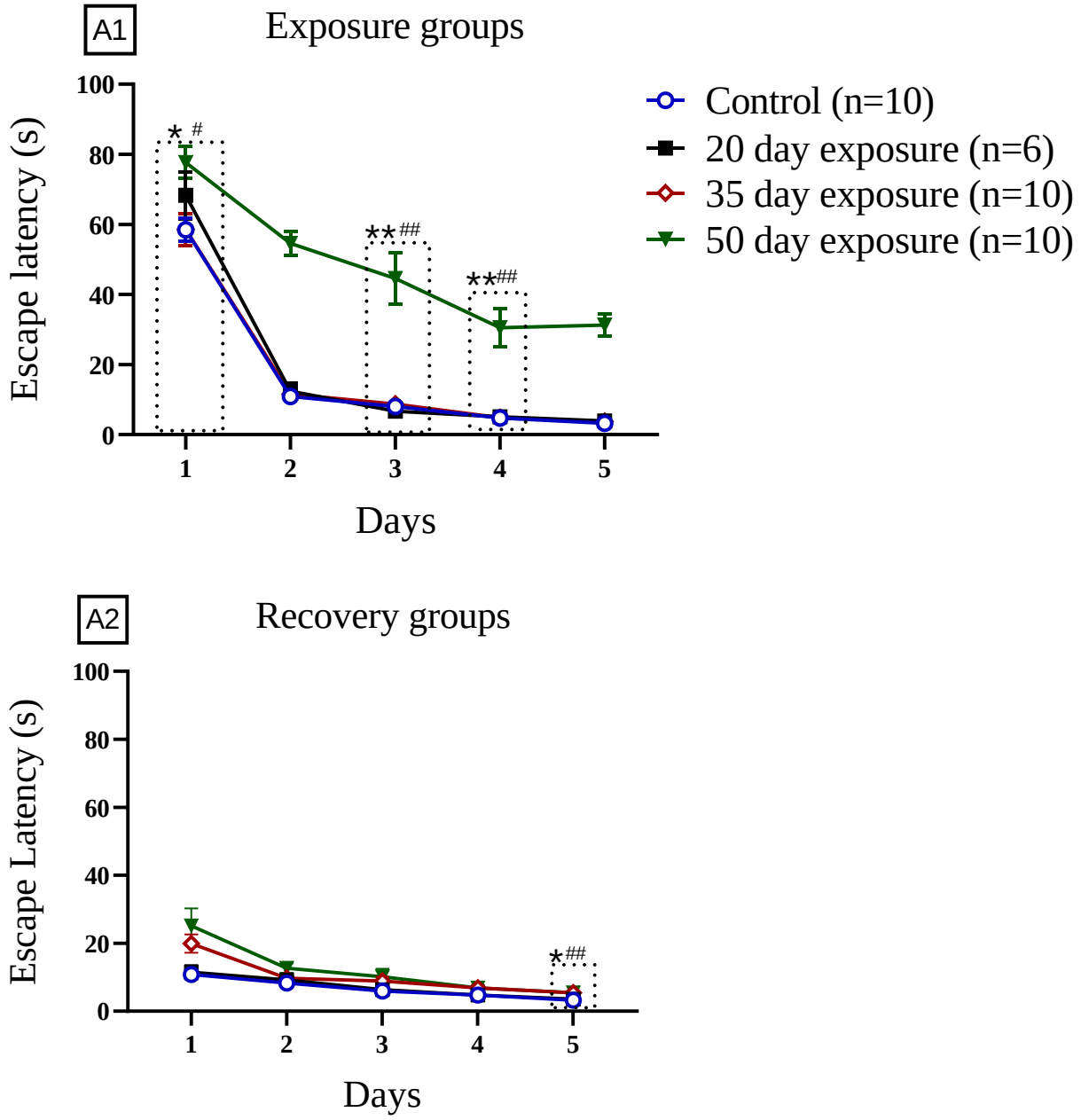


Figure 3.1 A: Effect of Aluminum on learning and memory in Morris water maze test; Escape latency. Graph shows escape latency (s) to assess the reference memory and learning among the control, 20 day exposure, 35 day exposure, 50 day exposure and their respective recovery groups. *=p<0.05 is significance between control and Al treated groups, ## = p< 0.01, ### = p< 0.001 are significance among Al treated groups. Error bars are represented as mean± SEM.

To assess reference memory platform was removed and probe trial was conducted after 5 day training period. Data was analyzed to observe difference in number of entries in target quadrant between all the groups. 20 day exposure group (8.16 ± 0.75) and 50 day exposure group (6.5 ±0.68) showed least number of entries as compared to control (9.2 ± 0.33) and 35 day exposure group (9.8 ±0.51). After recovery period no improvement in memory was observed in any recovery group in comparison with respective exposure groups (Figure 3.1 B)**.** The number of platform crossings was recorded and analyzed in probe trial. Within the exposure groups, 15 day exposure group (3.16 ± 0.30) and 50 day exposure group (2.5 ±0.52) showed highest deficit in memory as compared to control group (9.2 ±0.59). After recovery time period improvement in performance was observed in all the groups. 50 day recovery group (6.4 ±0.47) presented highest improvement in memory as compared to its respective 50 day exposure group (2.5 ±0.52) (Figure 3.1 C).Time spent in target quadrant (TQ) was analyzed to assess differential deficit between all the groups. 15 day exposure group (38 ± 1.26) and 50 day exposure group (38.9 ±3.49) spent least time in target quadrant as compared to control (64.20 ± 2.64). After recovery period all the three recovery groups spent more time in target quadrant. 50 day recovery group (52.50 ± 4.82) spent highest time in TQ as compared to 15 day recovery group (51.83 ±2.39) and 35 day recovery group (47.7 ± 3.33). 50 day recovery group (52.50 ± 4.82) presented improved referential memory in comparison with its respective 50 day exposure group (38.9 ±3.49) (Figure 3.1 D).

(B)

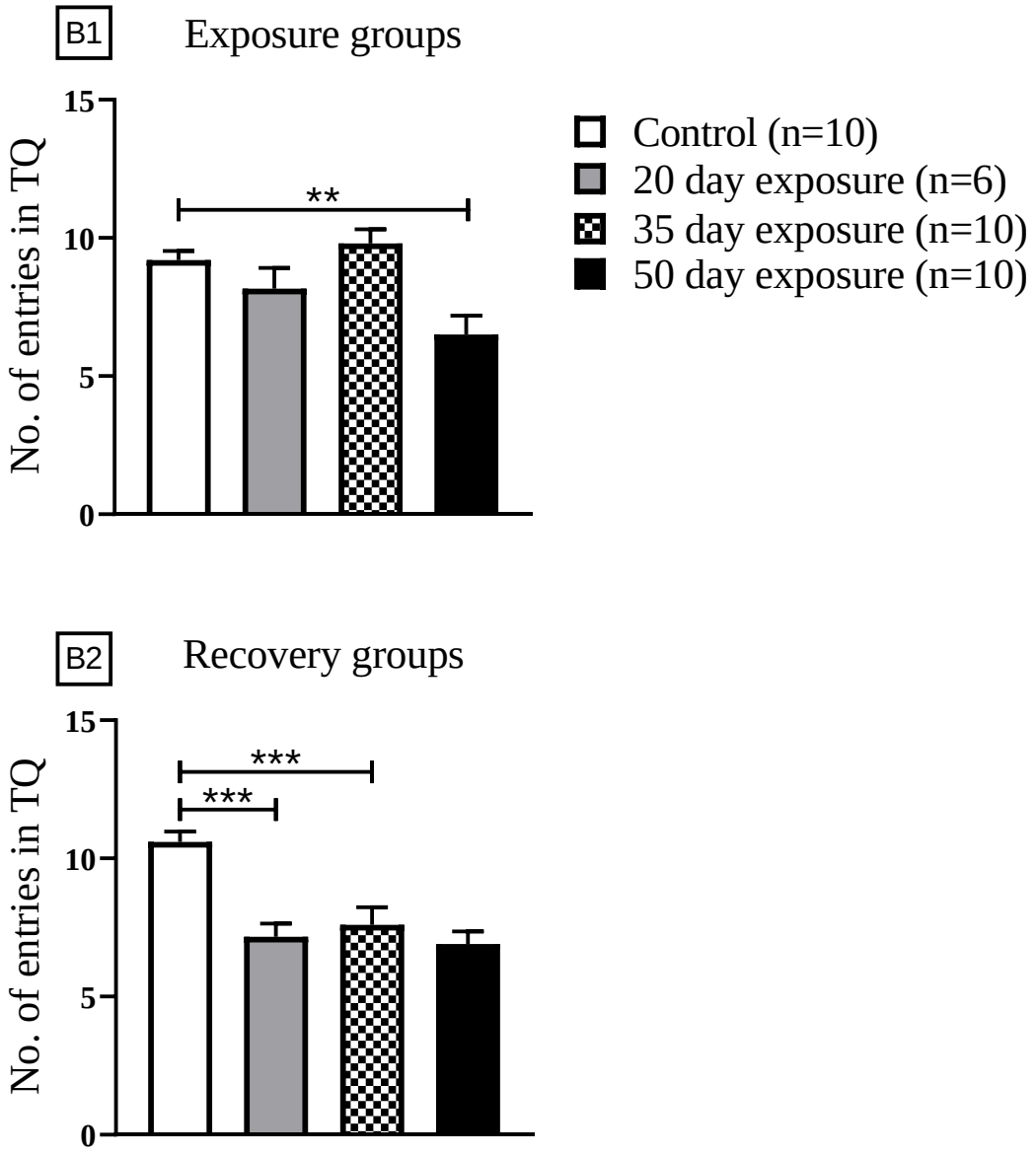


Figure 3.1 B: Morris Water Maze; Number of entries in target quadrant. Graph shows the number of platform crossings by all groups. * is used for significant difference between control and Al treated groups. # is used for significance among Al treated groups. Error bars are represented as mean± SEM for One-way ANOVA, followed by Bonferroni's multiple comparison test with *** = p< 0.001 as significance value. s = seconds.

(C)

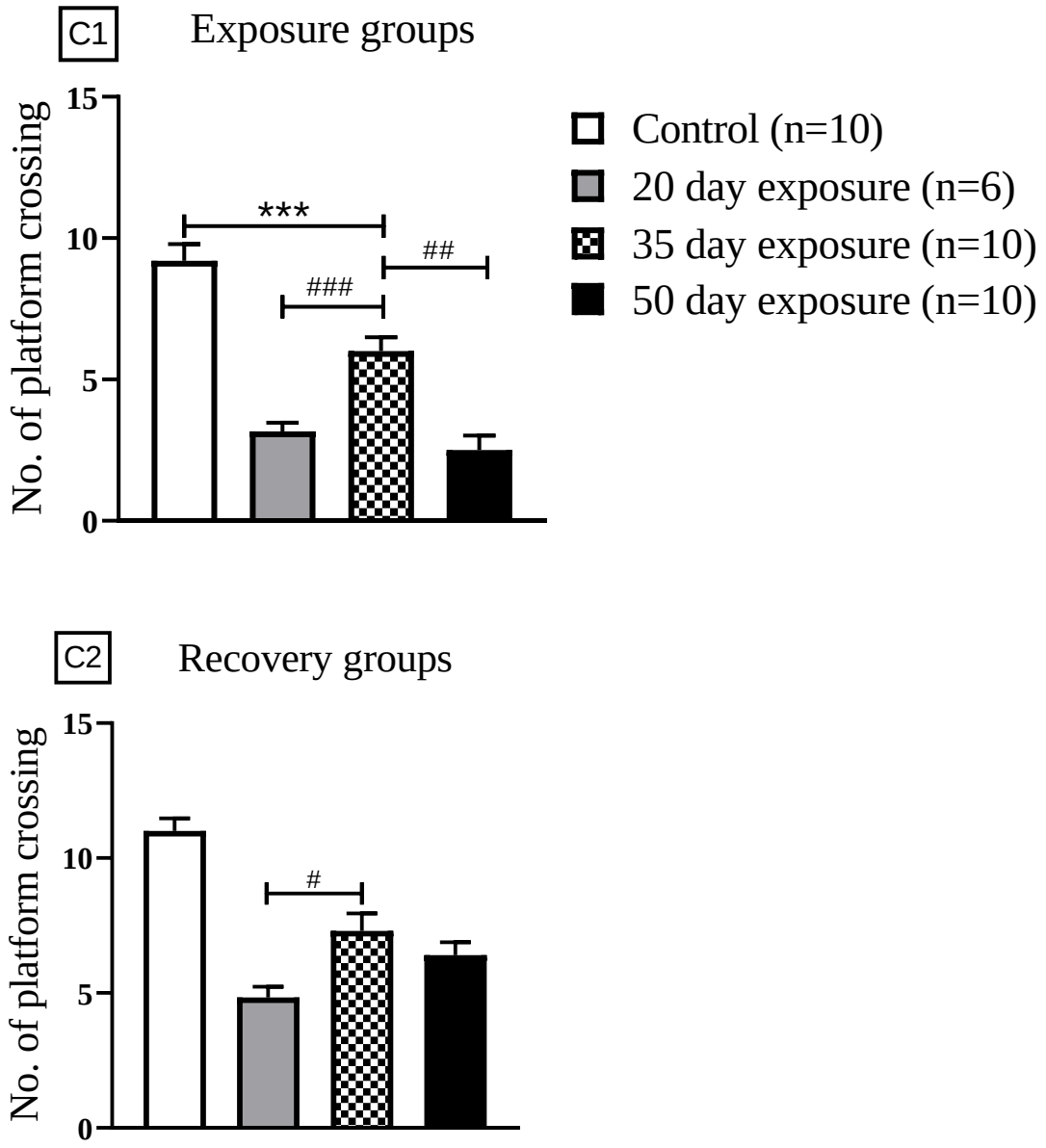


Figure 3.1 C: Probe Trial of Morris water maze; platform crossings. It shows the number of platform crossings by all groups. * is used for significant difference between control and Al treated groups. # is used for significance among Al treated groups. Error bars are represented as mean± SEM for One-way ANOVA, followed by Bonferroni's multiple comparison test with #=$p<0.05$ is the significance values. s = seconds.

(D)

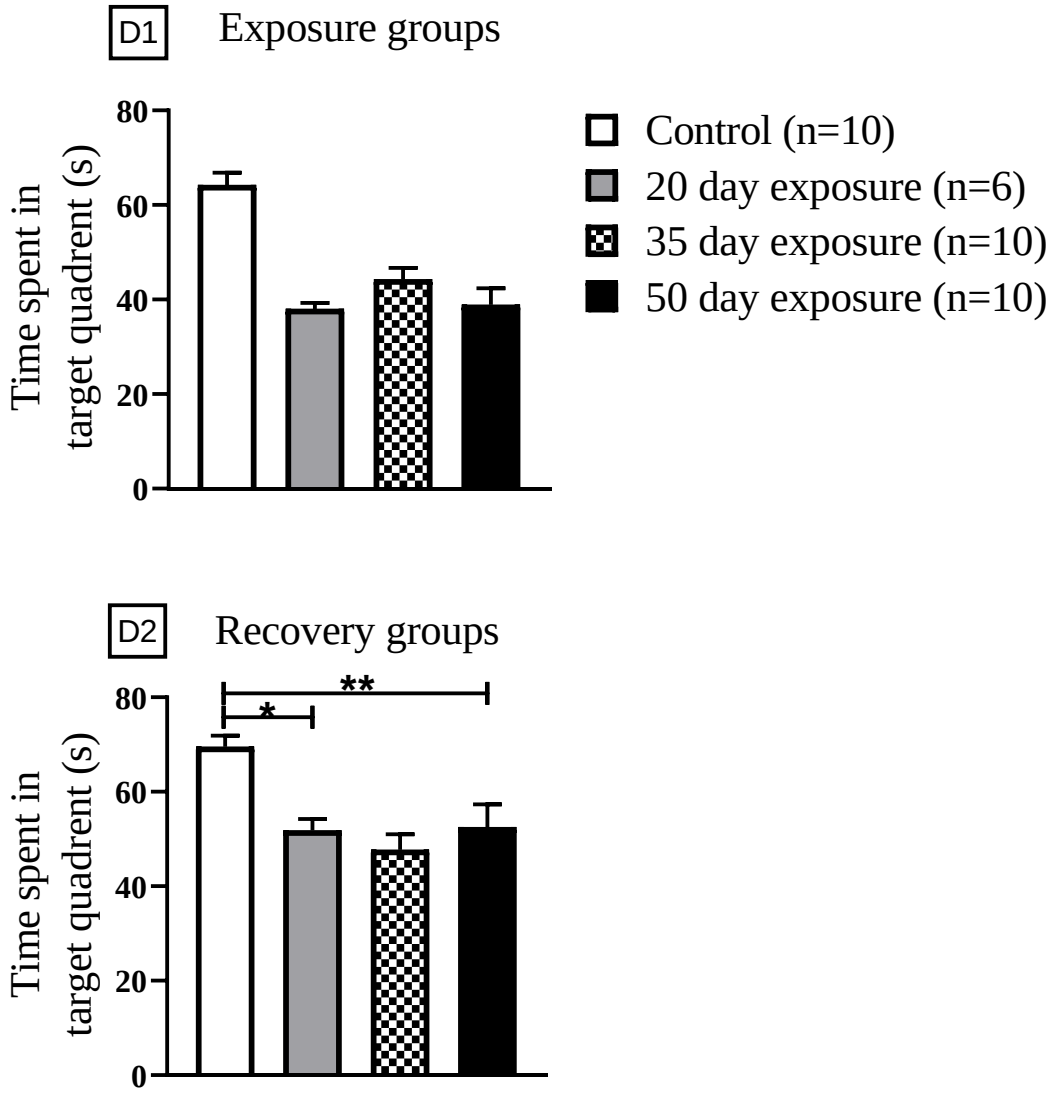
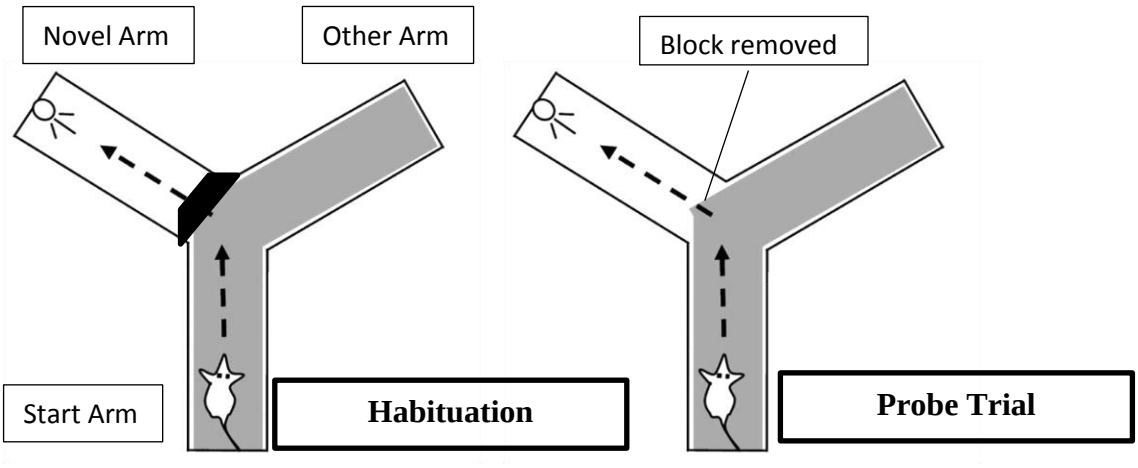


Figure 3.1 D: Morris Water Maze; Time spent in target quadrant. Graph shows time spent in target quadrant by all groups. * is used for significant difference between control and Al treated groups. # is used for significance among Al treated groups. Error bars are represented as mean± SEM for One-way ANOVA, followed by Bonferroni's multiple comparison test with *=p<0.05, ** = p< 0.01, are the significance values.. s = seconds.

3.2 Y Maze Test (Spontaneous Alternation Test)



Y maze test was employed to assess natural exploratory behavior of mice and to evaluate short term spatial learning memory. Animal hippocampus dependent reference memory was also investigated. All the four groups showed higher preference toward Novel arm through higher number of entries in Novel arm. Least preference was shown by 15 day exposure group (8.66 ± 0.42) as compared to control group (12.00 ± 0.36). After recovery period all groups showed increased number of entries in Novel arm. 35 day recovery group (18.20 ± 1.16) presented highest improvement in spatial memory as compared to its respective 35 day exposure group (7.6 ± 0.60) (Figure 3.2 A). Similar trend was observed while assessing time spent in Novel arm. 50 day exposure group (150.80 ±5.15) presented least preference to novel arm as compared to control group (177.30 ± 4.26), 20 day exposure group (188.17 ± 14.9) and 35 day exposure group (155.00 ±9.60). After recovery period no improvement in spatial memory was observed in recovery groups as compared to exposure groups. Only control showed enhanced spatial learning due to experiment repetition (Figure 3.2 B)

(A)

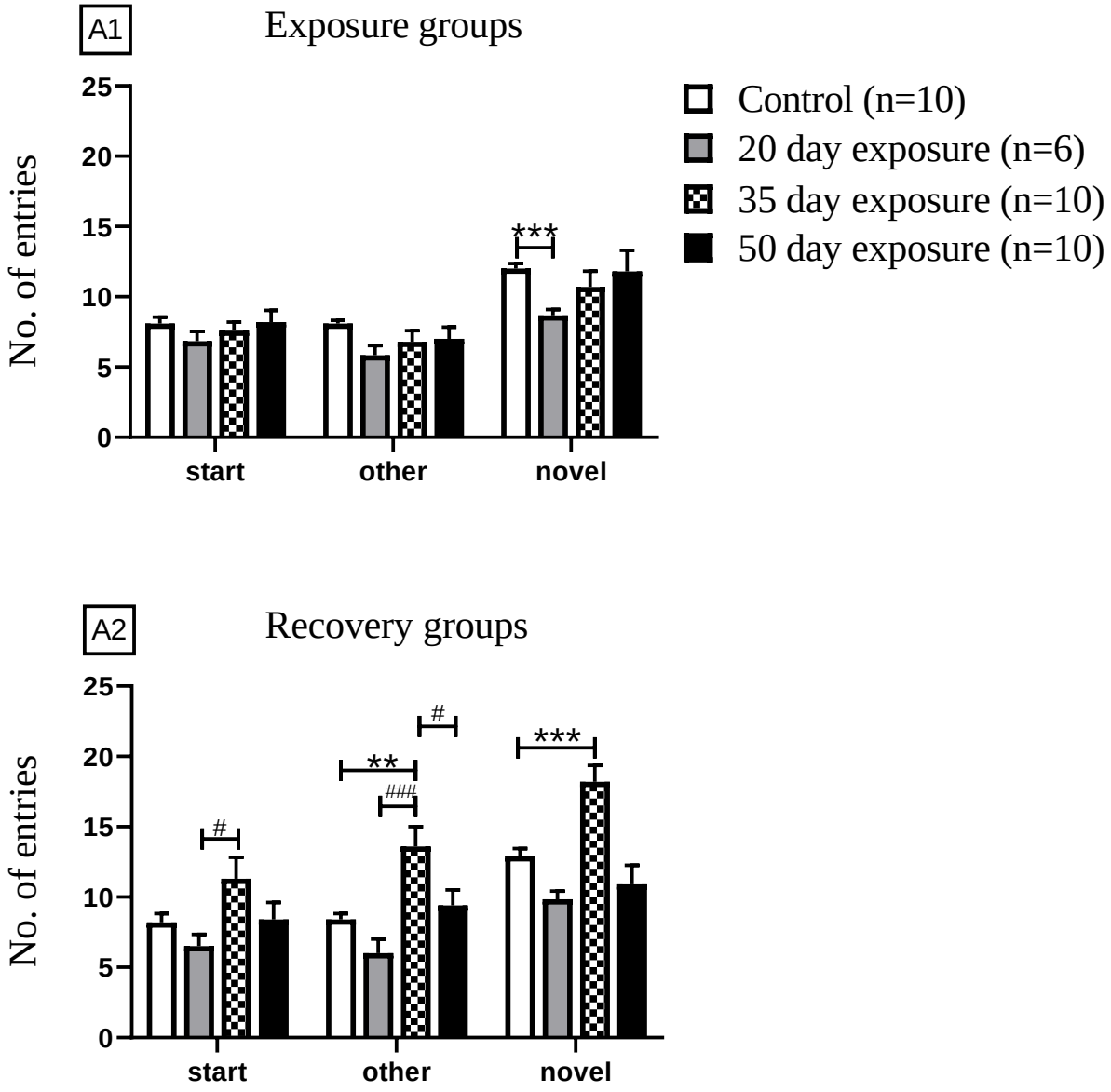


Figure 3.2 A: Performance of animals in Y-Maze test. The bar charts depicts the number of entries in each arm, by control, 20 day exposure, 35 day exposure, 50 day exposure, 20 day recovery, 35 day recovery and 50 day recovery groups. * is used for significant difference between control and Al treated groups. # is used for significance among Al treated groups. Error bars are represented as mean± SEM for two-way ANOVA, followed by Bonferroni's multiple comparison test with ** = p< 0.01, *** = p< 0.001, #=p<0.05 ## = p< 0.01, ### = p< 0.001 are the significance values.. s = seconds.

(B)

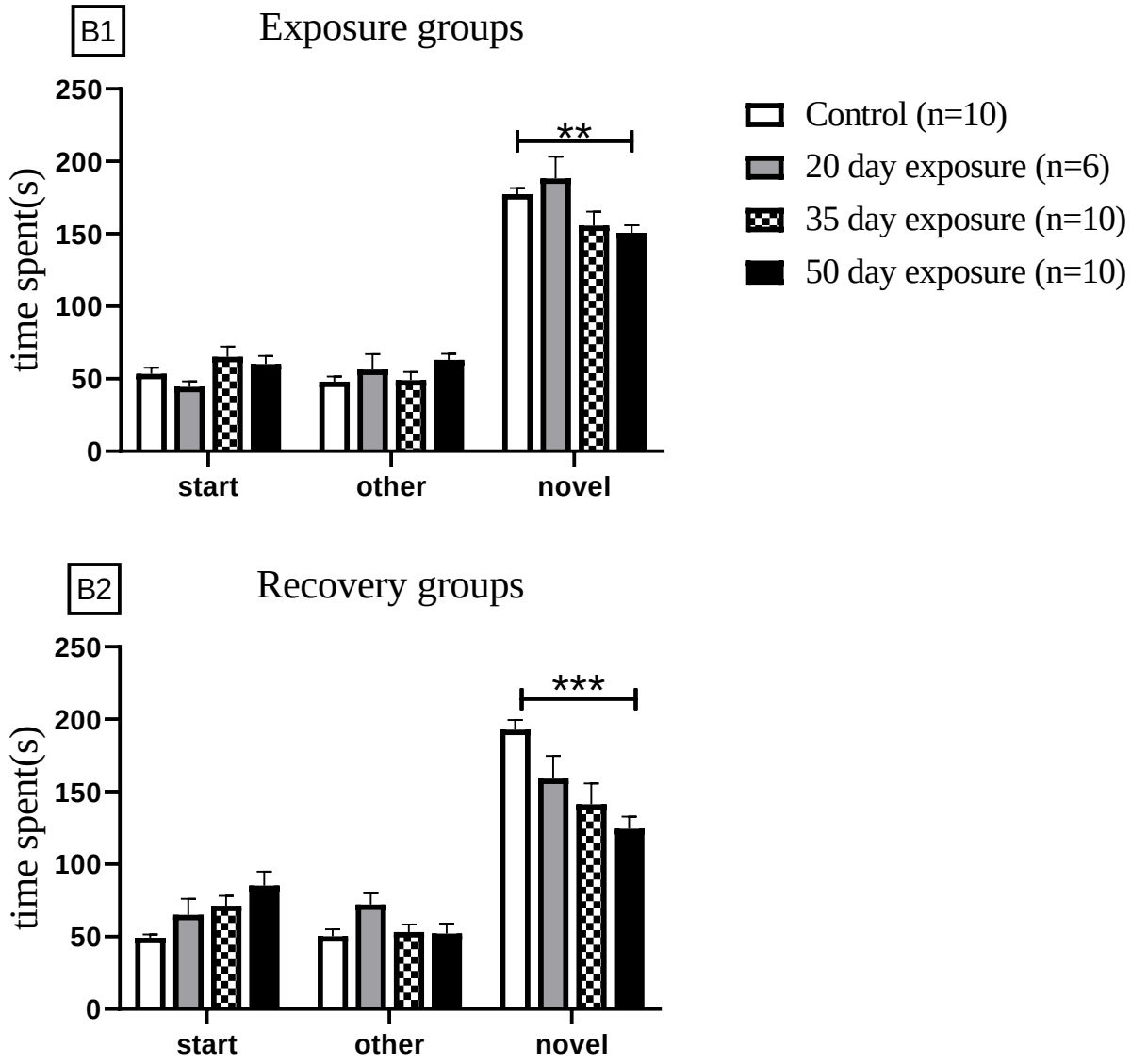


Figure 3.2.B: Time spent in each arm by animals in Y-Maze test. The graphs show the time spent in each arm (s) by control, 20 day exposure, 35 day exposure and their respective recovery groups. * is used for significant difference between control and Al treated groups. # is used for significance among Al treated groups. Error bars are represented as mean± SEM for two-way ANOVA, followed by Bonferroni's multiple comparison test with ** = $p< 0.01$, *** = $p< 0.001$ are the significance values.. s = seconds.

Spontaneous alternations performance and Alternate Arm Repeats (%) were calculated to assess impairment in spatial memory. Spontaneous alternation performance showed memory deficit in Aluminum treated groups. Highest impairment was shown by 35 day exposure group (50.15 ± 2.73) and 50 day exposure group (53.67 ±2.85) as compared to Control group (67.52 ± 1.41) and 20 day exposure group (61.94 ± 3.89). After recovery time all the groups showed minor improvement in spatial memory except 50 day recovery group (53.021 ± 1.48) which showed decreased spontaneous alternation than 50 day exposure group (53.61 ± 2.58) and Control group (69.30 ±1.45) (Figure 3.2 C).Short term memory impairment was observed by calculating Alternate arm repeats (AAR) and same arm repeats (SAR). 20 day exposure group (36.74 ±4.60), 30 day exposure group (31.82 ± 2.00) and 50 day exposure group (27.88 ± 2.57) showed higher arm repeats thus greater memory impairment as compared to control group (24.27 ± 1.30). Highest deficit was seen in 20 day exposure group (36.74 ±4.60) as compared to control group (24.27 ± 1.30). After completion of recovery period 50 day recovery group (32.21 ± 1.40) showed no improvement in comparison with 50 day exposure group (27.88 ±2.57). Highest improvement in spatial memory was seen in 20 day recovery group (29.15 ± 2.14) as compared to 20 day exposure group (36.74 ±4.60) (Figure 3.8: D). Control group (0.00 ± 0.00) showed no same arm repeats while 35 day exposure group (1.40 ±0.22) and 50 day exposure group (1.10 ± 0.31) showed spatial memory impairment. After recovery period, 20 day recovery group (0.00 ± 0.00) showed similar performance as that of control group (00.00 ± 00.00). Improvement in spatial memory was observed in 35 day recovery group (0.80 ± 0.24) and 50 day recovery group (0.50 ± 0.16) in comparison with their respective exposure groups (Figure 3.2 E).

(C)

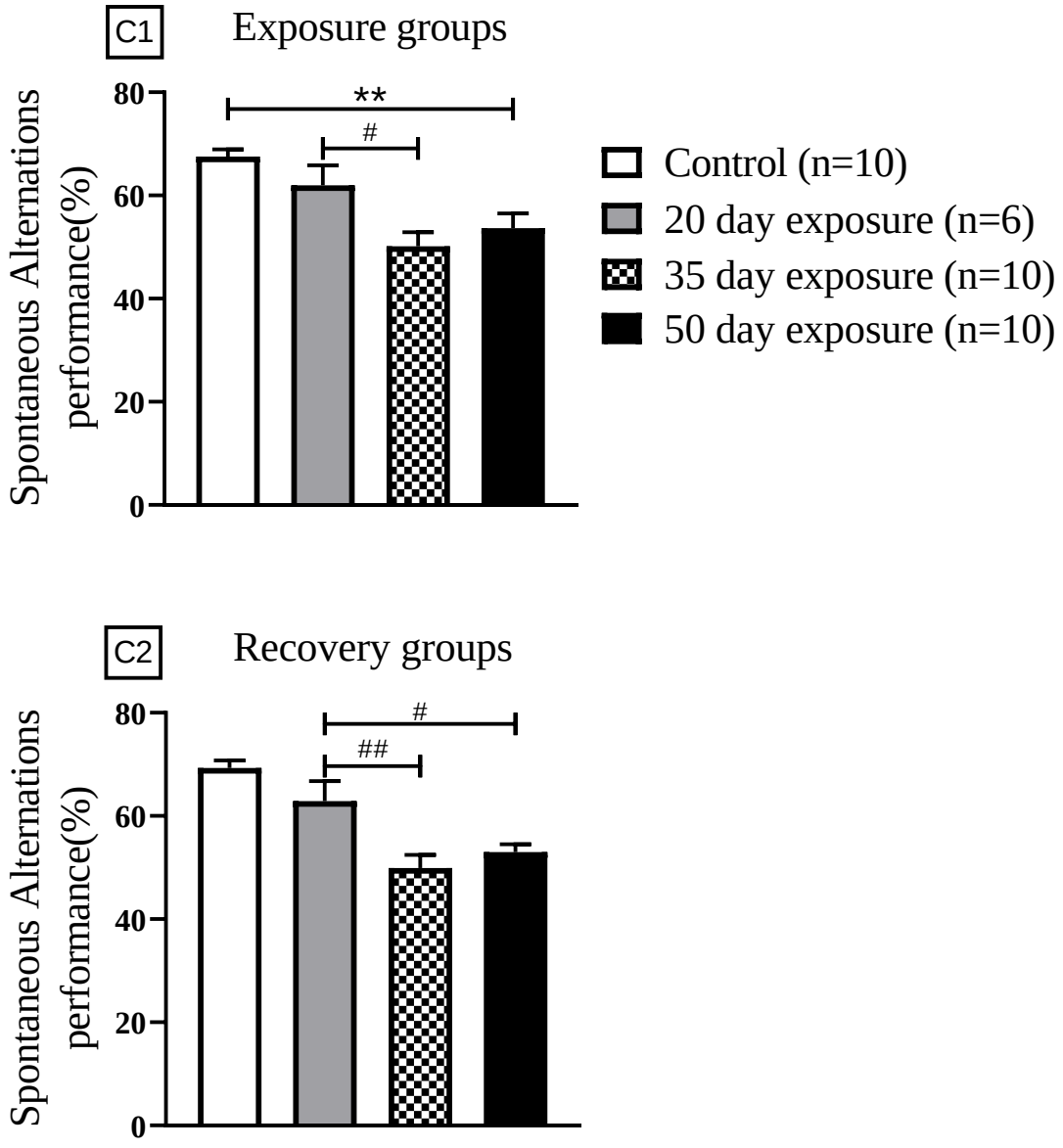


Figure 3.2 C: Effect of Aluminum on reference and working memory. Graph shows Spontaneous Alternation (%) in all groups. Error bars are represented as mean± SEM for two-way ANOVA, followed by Bonferroni's multiple comparison test with *=p<0.05, ** = p< 0.01 for exposure group and #=p<0.05 ## = p< 0.01, ### = p< 0.001 are the significance values for recovery group. s = seconds.

(D)

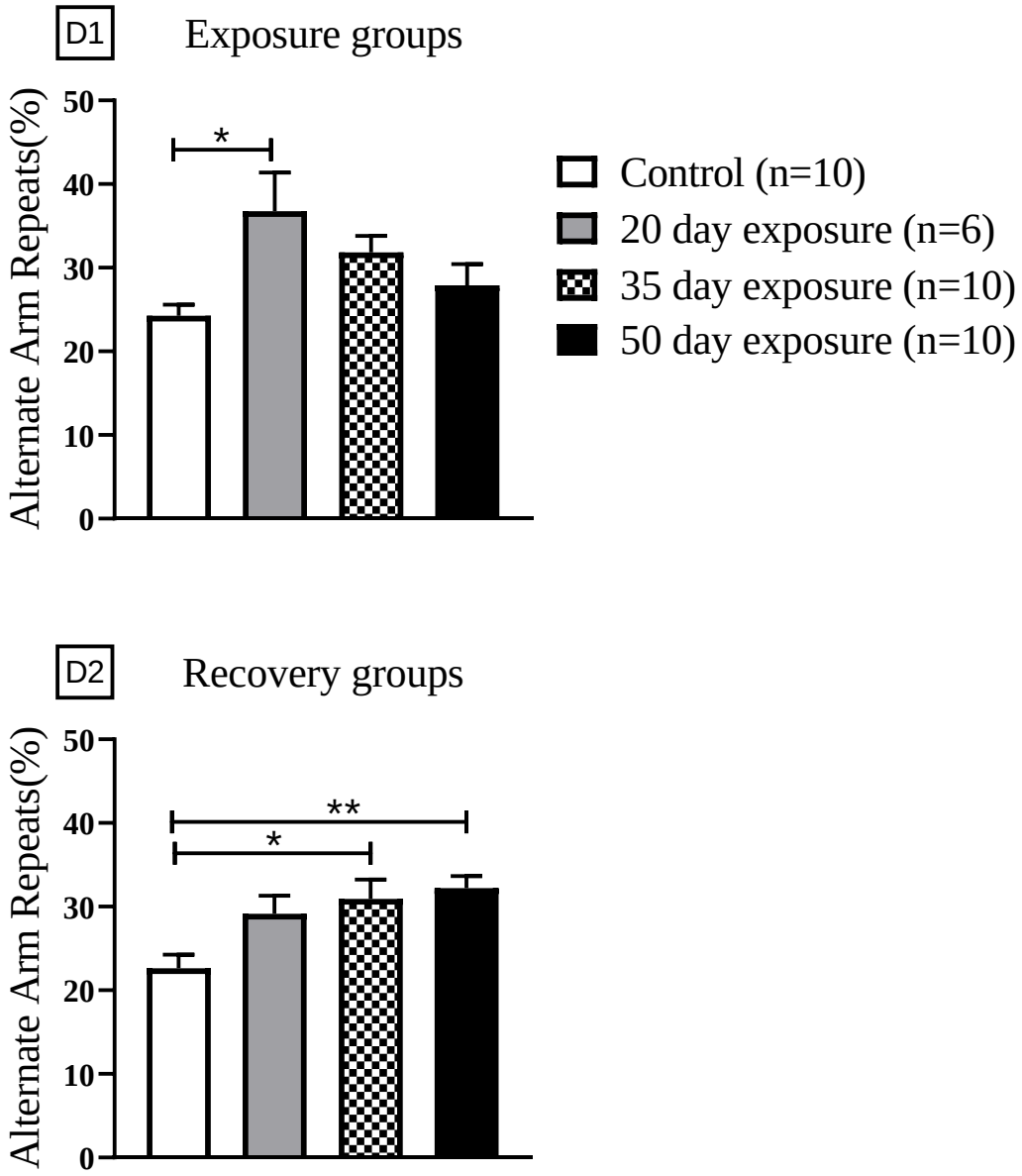


Figure 3.2 D: Effect of Aluminum on working memory. Graph shows the Alternate arm repeats (%) by all groups. Error bars are represented as mean± SEM for two-way ANOVA, followed by Bonferroni's multiple comparison test with *=p<0.05, ** = p< 0.01, *** = p< 0.001 for exposure group and #=p<0.05 ## = p< 0.01, ### = p< 0.001 are the significance values for recovery group. s = seconds.

(E)

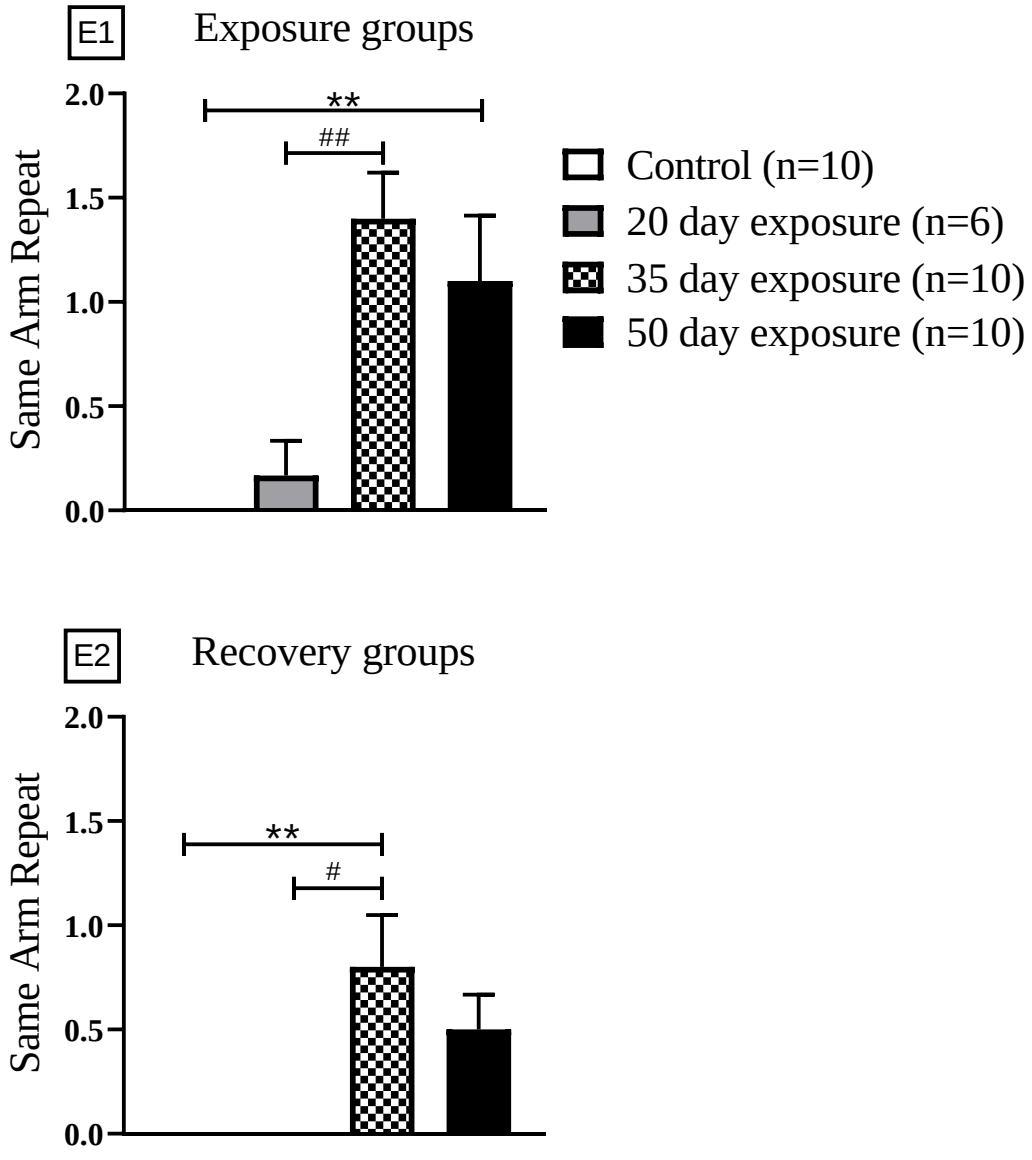


Figure 3.2 E: Same arm repeats; Y maze test. Graph shows the same arm repeats, by exposure and recovery groups. Error bars are represented as mean± SEM for two-way ANOVA, followed by Bonferroni's multiple comparison test with *=p<0.05, ** = p< 0.01, *** = p< 0.001 for exposure group and #=p<0.05 ## = p< 0.01, ### = p< 0.001 are the significance values for recovery group. s = seconds.

3.3 Social Preference and Novelty Test:

Sociability and social preference was evaluated in session i of test and session ii determined social novelty preference. interaction time of animal with empty cage, mouse 1 and mouse 2 was analyzed as well as total time spent in three chambers i.e. mouse 1, mouse 2 and center.

In session I, all the groups showed higher preference for mouse 1 as compared to Empty cage. However, aluminum exposed 20 day exposure group (82.83 ± 9.56), 35 day exposure group (77.60 ± 5.66) and 50 day exposure group (65.20 ± 4.31) showed low social preference for mouse 1 as compared to control group(186.40 ± 10.68). 50 day exposure group (65.20 ± 4.31) presented least interaction time with mouse 1 as compared to other exposure groups. After recovery period, 20 day recovery group (141.00 ± 12.73) showed improved sociability in comparison with 20 day exposure group (82.83 ± 9.56). However, 35 day recovery group(44.80 ±4.63) and 50 day recovery group (51.50 ±8.00) did not show any improvement in comparison to 35 day exposure group (77.60 ± 5.66) and 50 day exposure group (65.20 ± 4.31) respectively (Figure 3.3 A). In session II all the groups showed higher social novelty preference i.e. more interaction time with mouse 2 as compared to mouse 1. But in comparison with control group (138.30 ±11.99) social novelty preference was low in 20 day exposure group (100.83 ± 4.92), 35 day exposure group (43.30 ±5.78) and 50 day exposure group (64.90 ±9.88) with least in 35 day exposure group (43.30 ±5.78). After recovery time, social novelty preference was enhanced showing higher interaction with Mouse 2 in control group(153.00 ±13.58), 20 day group (116.66 ±18.78) and 35 day group(58.50 ± 8.43) as compared to their respective exposure groups. 50 day

recovery group(51.90 ± 5.47) showed less social novelty preference as compared to 50 day exposure group (64.90 ± 9.88) (Figure 3.3 B)

(A)

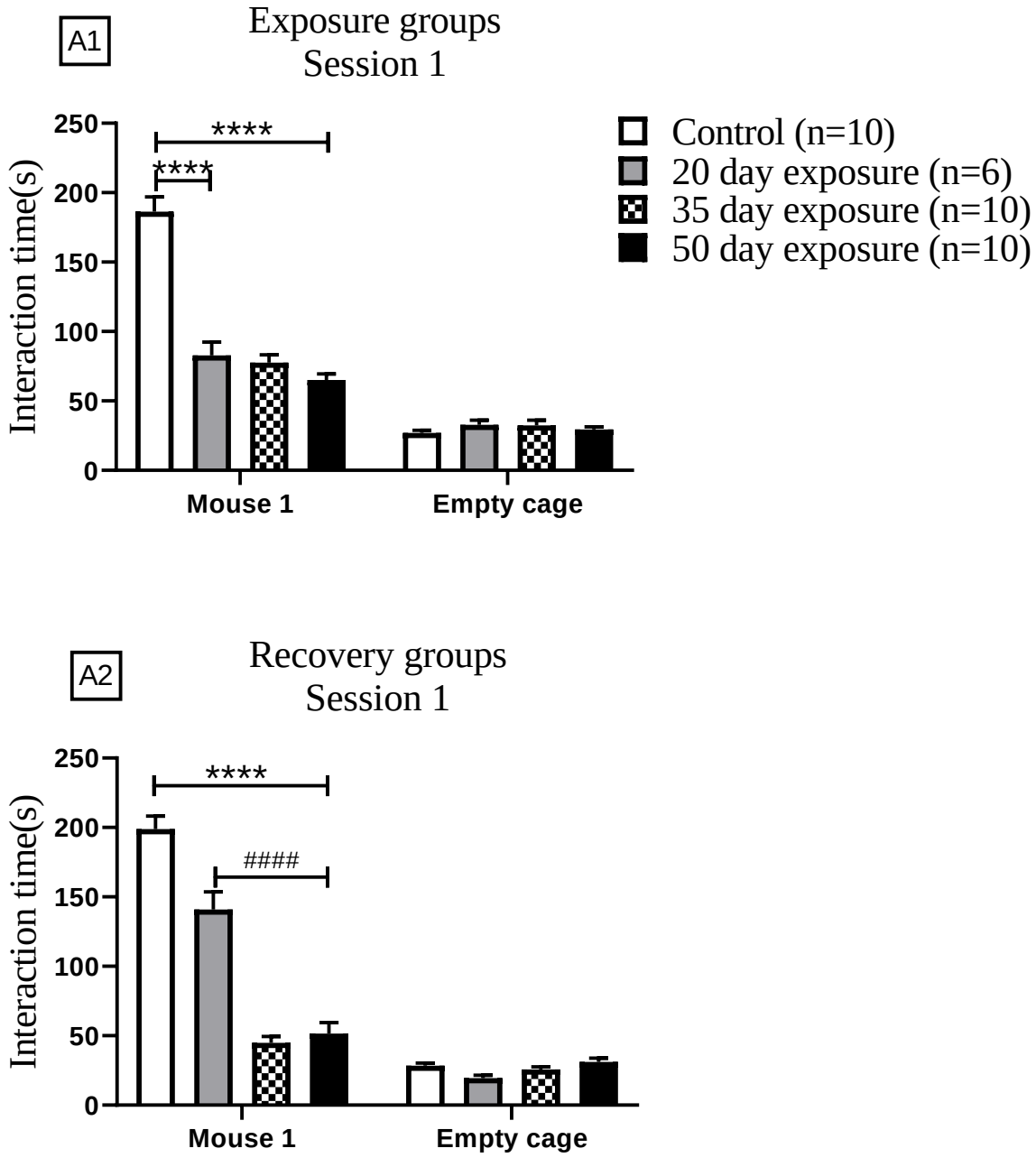


Figure 3.3 A: Effect of Temporal exposure of aluminum on Sociability behavior (Session-I): Graph shows interaction time during session I (s) by the Control, 20 day exposure, 35 day exposure,

50 day exposure and their respective recovery groups. ****=p<0.0001, show significance between control and Al treated groups and #### = p< 0.0001 among Al treated groups.

(B)

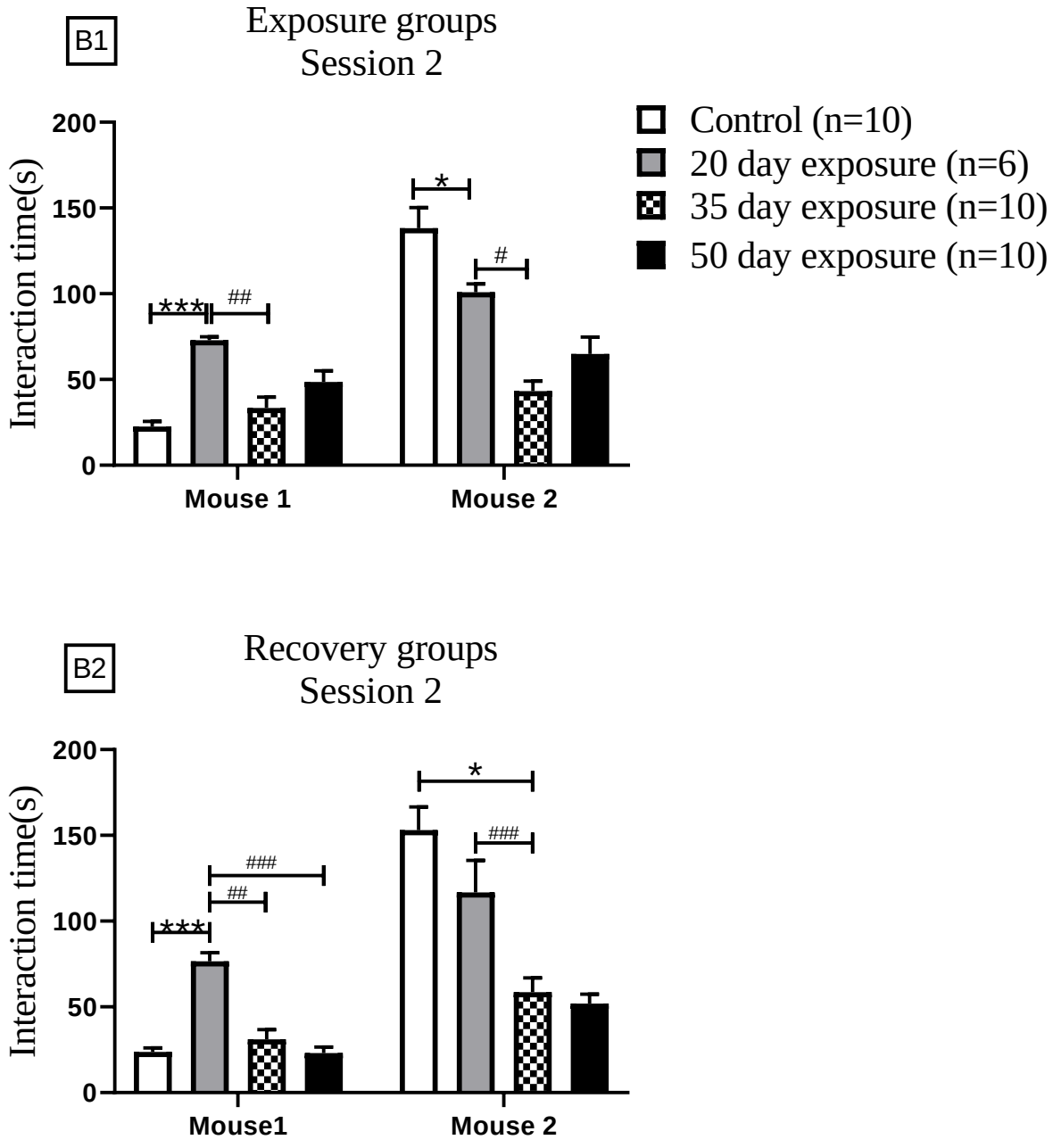


Figure 3.3 B: Effect of Temporal exposure of aluminum on Sociability behavior (Session II): Graph shows interaction time during session II (s) by the Control, 20 day exposure, 35 day exposure,

50 day exposure and their respective recovery groups. *=p<0.05,***=p<0.001 show significance between control and Al treated groups, ## = p< 0.01, ### = p< 0.001.

Similar trends were observed in sociability assessment by calculating time spent in mouse 1, mouse 2 and center chamber. In Session I it was observed that all the groups, control (343.4 ± 11.28), 20 day exposure group (295.16 ± 9.35), 35 day exposure group (274.80 ± 13.35) and 50 day exposure group (271.20 ± 13.14) spent higher time in Mouse 1 chamber as compared to center and empty cage chamber. 50 day exposure group (271.20 ± 13.14) spent least time with mouse 1 as compared to Control group (343.4 ± 11.28). After recovery period, 35 day recovery group (298.20 ±17.62) showed improved sociability as compared to 35 day exposure group (274.80 ± 13.35). 20 day recovery group (293.50 ± 9.53) and 50 day recovery group (229 ± 14.97) did not show any improvement in sociability after recovery (Figure 3.3 C). In session II control group(337.60 ± 15.27), 20 day exposure group (286.16 ± 4.04) and 30 day exposure group (243.90 ±33.03) showed higher social novelty preference. In comparison with control group, all Aluminum exposed group showed less social novelty preference. After recovery period, it was observed that 20 day recovery group (300.66 ± 14.88) and 35 day recovery group (302.00 ± 14.59) spent more time with mouse 2 as compared to their respective exposure groups. Though, 50 day recovery group (203.30 ± 14.47) showed less social novelty preference as compared to 50 day exposure group (234.70 ± 23.749) by spending less time in mouse 2 chamber (Figure 3.3 D). Percentage discrimination index clearly shows that all Aluminum exposure groups, 20 day exposure group (57.93 ± 1.33), 35 day exposure group (58.15 ± 2.86) and 50 day exposure group (57.41 ± 3.49) interacted less with novel mouse (mouse 2) as compared to control group (85.23 ± 2.30). 20 day exposure group showed least preference for novelty. After recovery, moderate improvement in performance was observed in 35 day

recovery group (66.39 ± 5.90) and 50 day recovery group (69.39 ± 2.89). Very little progress was observed in 20 day recovery group (58.66 ± 3.11) (Figure 3.3 E).

(C)

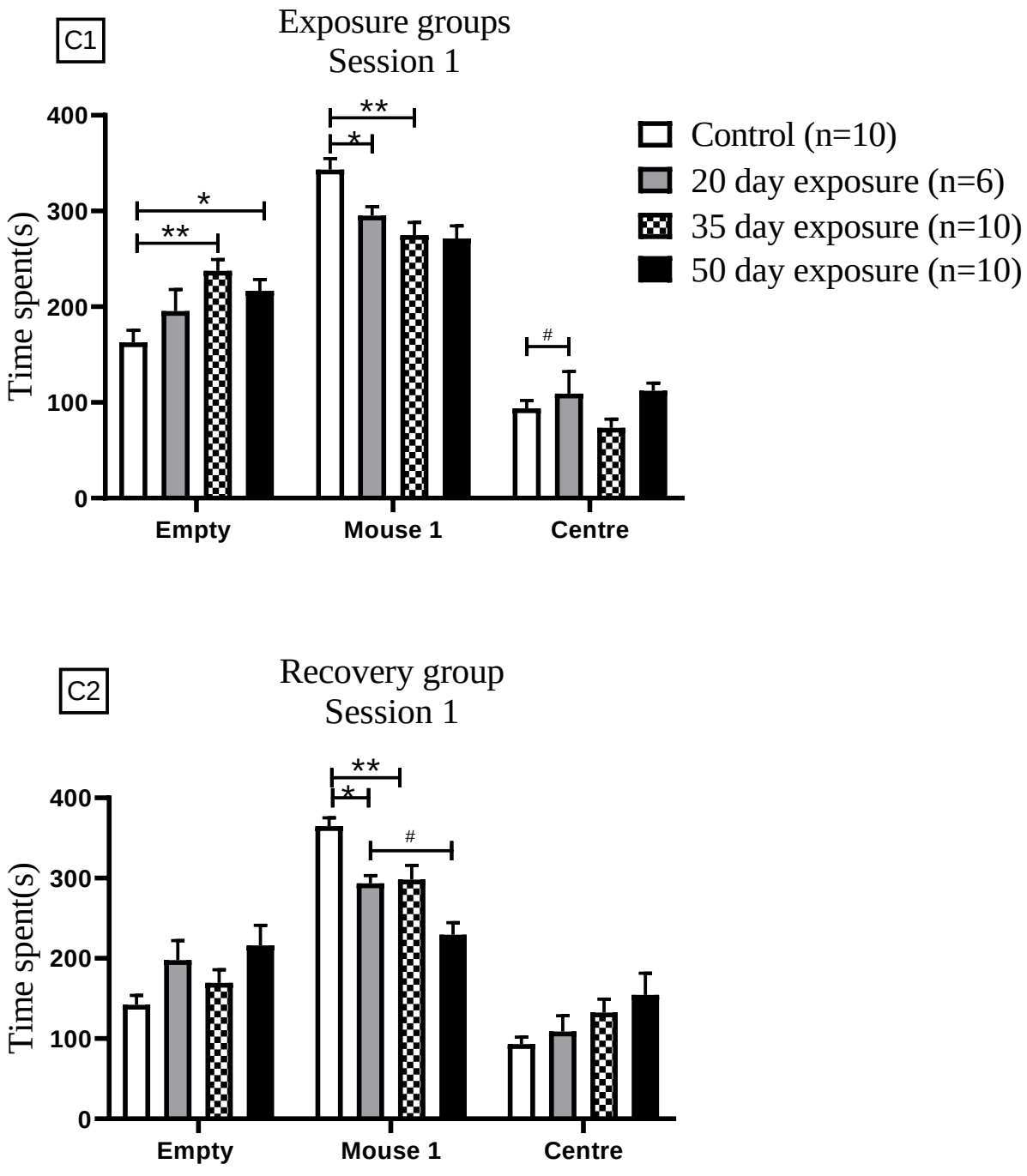


Figure 3.3 C: Social Novelty preference (Session-I): Graph shows time spent (s) in each chamber

during session I all groups. *=p<0.05, **=p<0.01 show significance between control and Al treated groups and # = p< 0.05 are the significance values among Al treated groups.

(D)

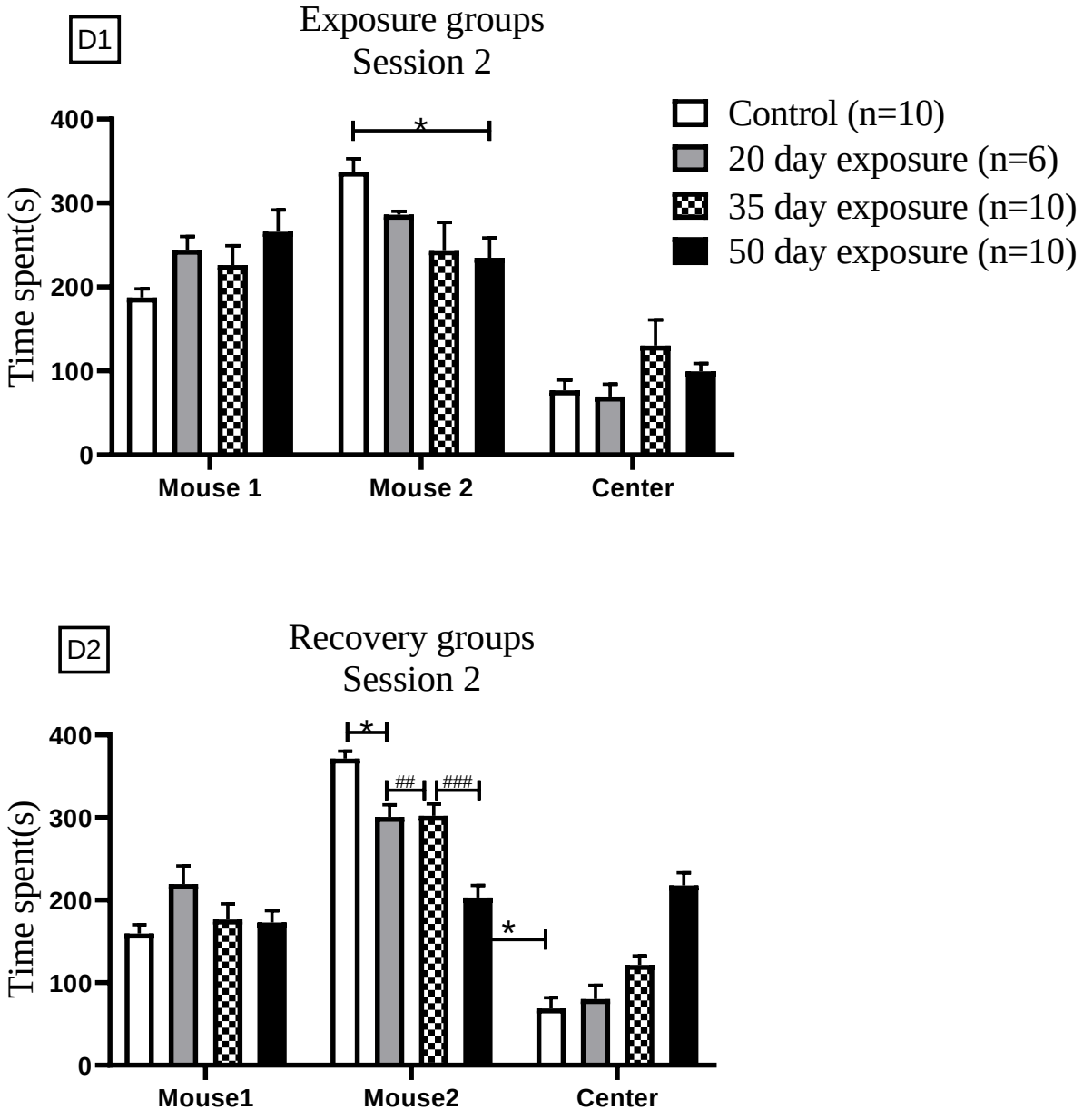


Figure 3.3 D: Social Novelty preference (Session-II): Graph shows time spent (s) in each chamber during session II by the Control, 20 day exposure, 35 day exposure, 50 day exposure and recovery groups.

*=p<0.05 show significance between control and Al treated groups, ## = p< 0.01, ### = p< 0.001 are the significance values among Al treated groups.

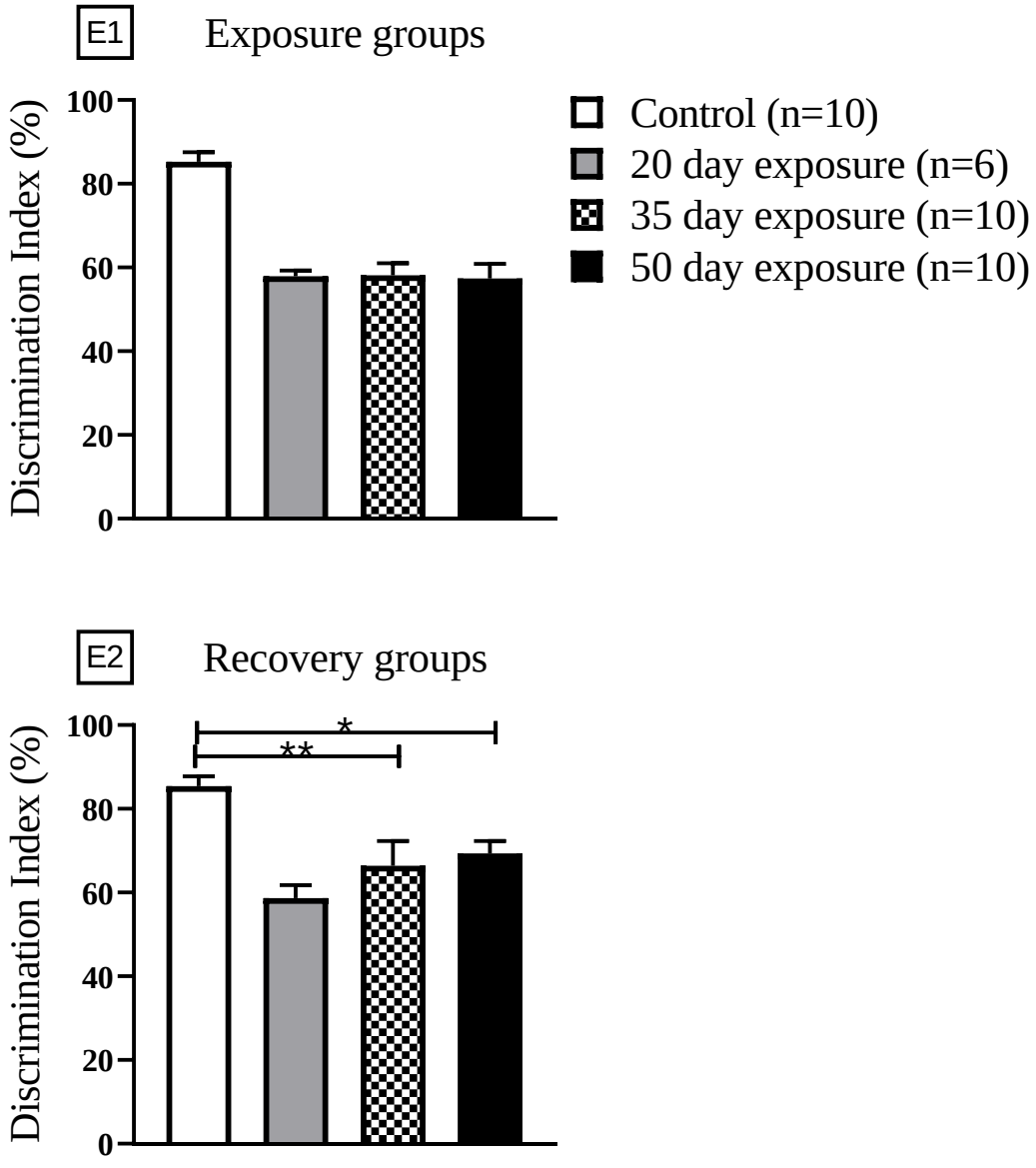


Figure 3.3 E: Outcome of Aluminum on social novelty test: Graph present percentage Discrimination Index during session II by the Control, 20 day exposure, 35 day exposure, 50 day

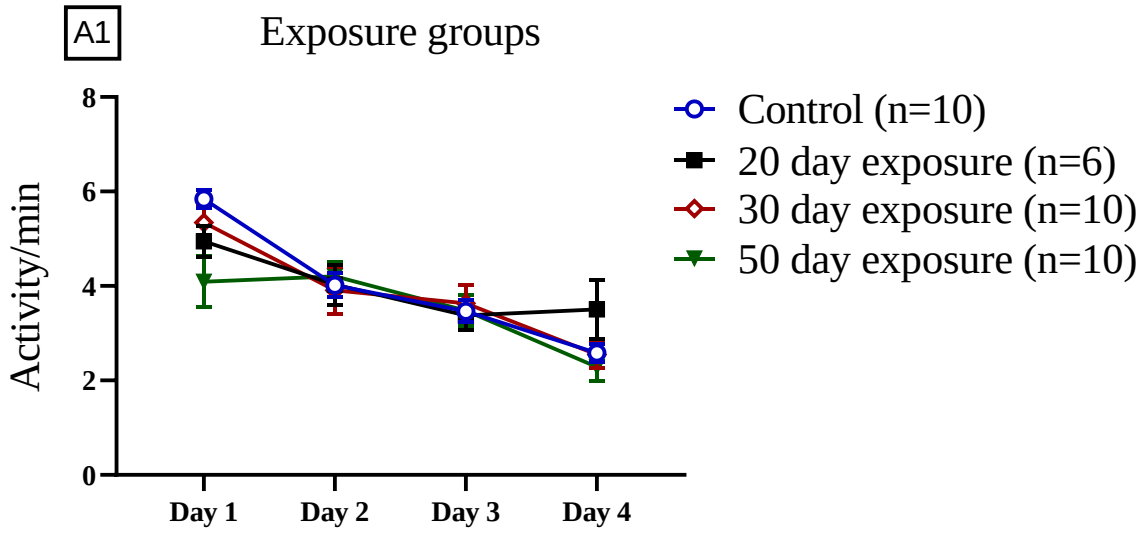
exposure and recovery groups. *=p<0.05, ** = p< 0.01 show significance between control and Al treated groups through One way ANOVA and post hoc Bonferroni's test.

3.4 Hole Board Test:

Hole board test was used in order to evaluate the effect of Aluminum exposure on long term memory and short term memory in different temporal groups. Locomotion activity of mice was also assessed by calculating Activity/ min in hole board from day 1 to day 4. On day 1, 20 day exposure group (4.94 ± 0.82), 30 day exposure group (5.34 ± 1.16) and 50 day exposure group (4.09 ±1.70) showed less locomotion than control group (5.84 ± 0.56). On day 4 similar trend was observed but overall locomotion activity was decreased in in control group (2.58 ± 0.59), 20 day exposure group (3.50 ± 1.51), 35 day exposure group (2.54 ± 0.90) and 50 day exposure group (2.28 ± 0.93). Least locomotion activity was observed in 50 day exposure group from day 1 to day 4. However, after recovery period 50 day recovery group (5.63 ±2.35) showed increased locomotion than 50 day exposure group (4.09 ±1.70) from day 1 to day 4 (Figure 3.4 A).Latency to visit the first hole (baited or un baited) was calculated to evaluate anxiety level in mice. On day 1 control group (9.8 ± 1.05) showed least latency thus less anxiety as compared to 20 day exposure group (13.33 ± 0.95), 35 day exposure group (12.44 ± 1.48) and 50 day exposure group (12 ± 1.30). Highest level of anxiety was observed in 20 day exposure group (13.33 ± 0.95). After recovery period performance of 35 day recovery group (11.55 ± 1.55) and 50 day recovery group (11.45 ± 1.23) was improved. While 20 day recovery group (13.37 ± 0.95) showed higher anxiety level as compared to 20 day exposure group (13.33 ± 0.95). Similar

trends were seen on day 4 with control group (6.17 ± 0.42) showing least anxiety level as compared to 20 day exposure group (8.66 ± 0.77), 35 day exposure group (6.47 ± 0.72) and 50 day exposure group (7.62 ± 0.95). After recovery period performance on day 4 was improved in all the groups with anxiety level lower in 20 day recovery group (7.50 ± 0.77) and 50 day recovery group (7.13 ± 0.98) (Figure 3.4 B).

(A)



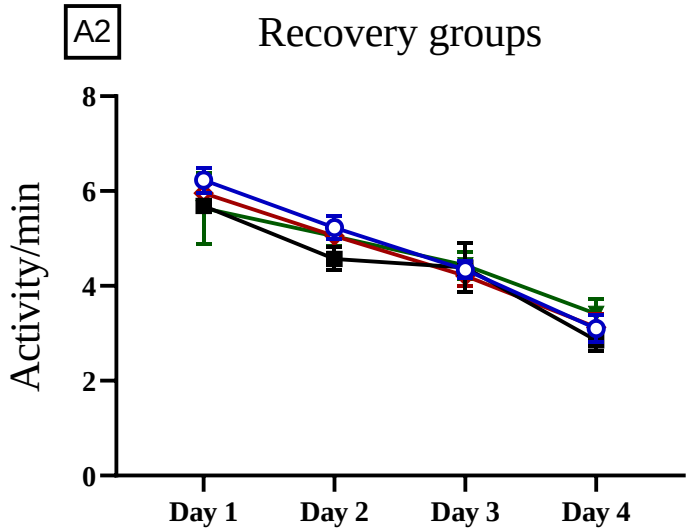
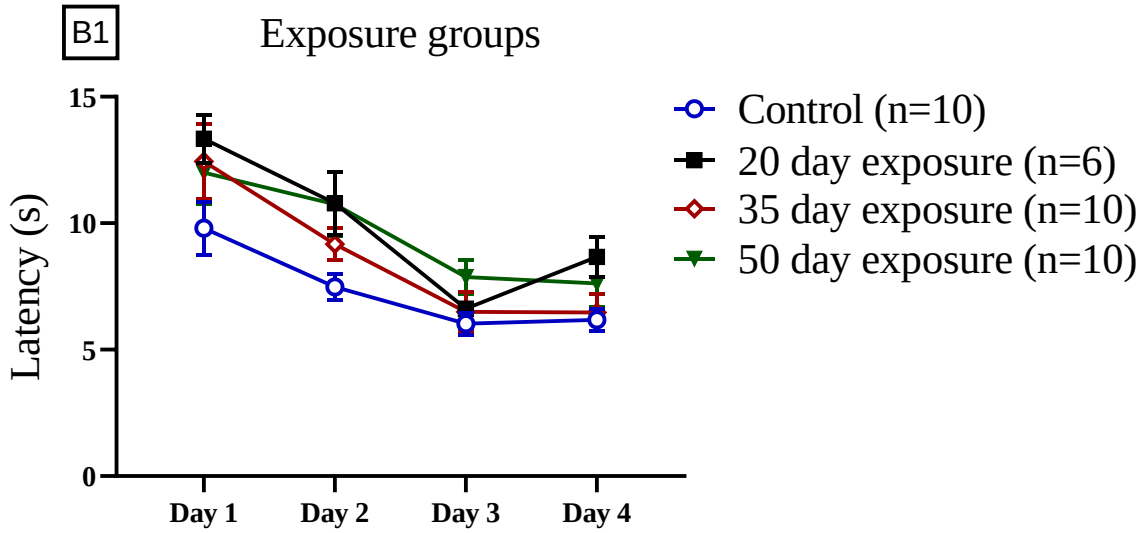


Figure 3.4 A: Effect of Aluminum exposure on locomotion activity. Graph shows activity per minute Control, 20 day exposure, 35 day exposure and 50 day exposure and their respective recovery groups. Error bars are represented as mean± SEM by 2 way ANOVA test. s= seconds.

(B)



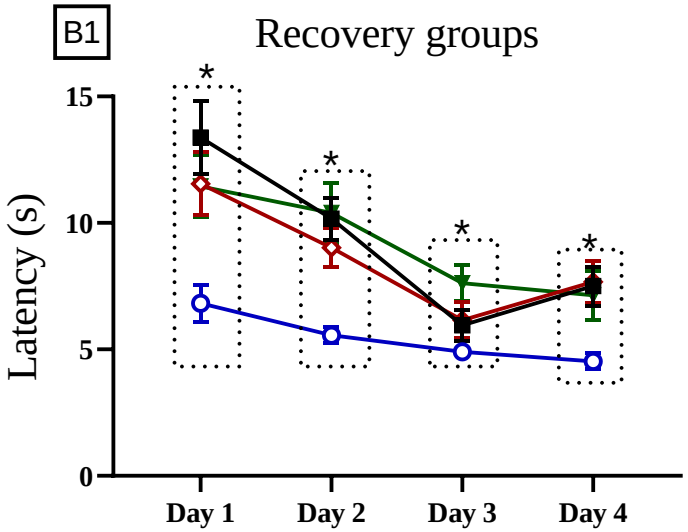


Figure 3.4 B: Effect of Aluminum exposure on anxiety levels. Graph shows the latency (s) for visit to first hole, by the groups Control, 20 day exposure, 35 day exposure and 50 day exposure and their respective recovery groups.*= $p < 0.05$, is the significant value between Control and Aluminum treated groups. Error bars are represented as mean± SEM by 2 way ANOVA test. s= seconds.

To evaluate reference memory throughout 4 days, Reference memory error (RME) was calculated. On day 1, 20 day exposure group (18.20 ± 1.98), 35 day exposure group (16.26 ±1.64) and 50 day exposure group (23.25 ± 1.40) showed higher impairment in referential memory as compared to control group (7.07 ± 0.43). Highest reference memory errors were observed in 50 day exposure group (23.25 ± 1.40). Reference memory errors were gradually decreased in all the groups from day 1 to day 4. After recovery period it was observed that control group (5.8 ± 0.85), 20 day recovery group (13.12 ± 1.22) and 35 day recovery group (13.11 ± 1.98)have improved reference memory as compared to 50 day recovery group (21.90 ± 1.21). Similar trend was observed at day 4, 50 day recovery group (11.87 ± 1.49) showed higher

number of reference memory error as compared to control (2.07 ± 0.32), 20 day recovery group (9.50 ± 0.28) and 35 day recovery group (9.37 ± 1.15) (Figure 3.4 C). For all 4 days Working memory error (WME) were also calculated to asses short term memory and learning through the test. On day 1 control group (2.37 ± 0.19) showed least working memory error as compared to 20 day exposure group (6.64 ± 0.61), 35 day exposure group (7.42 ± 0.91) and 50 day exposure group (9.45 ± 0.41). Highest impairment of short term memory was observed in 50 day exposure group (9.45 ± 0.41). WME were reduced in all the groups from day 1 to day 4. Highest number of working memory errors were seen in 20 day exposure group (4.67 ±0.36) at day 4. After recovery period working memory (short term memory) on day 1 was improved in 20 day recovery group (4.54 ± 0.50) and 35 day recovery group (5.62 ± 0.35). 50 day recovery group (10.92 ± 0.60) did not show any improvement as compared to 50 day exposure group (9.45 ± 0.41). On day 4 slight improvement was observed in 20 day recovery group (2.62 ± 0.27), 35 day recovery group (3.85 ± 0.26) and 50 day recovery group (4.25 ± 0.28) as compared to their respective exposure groups (Figure 3.4 D).

(C)

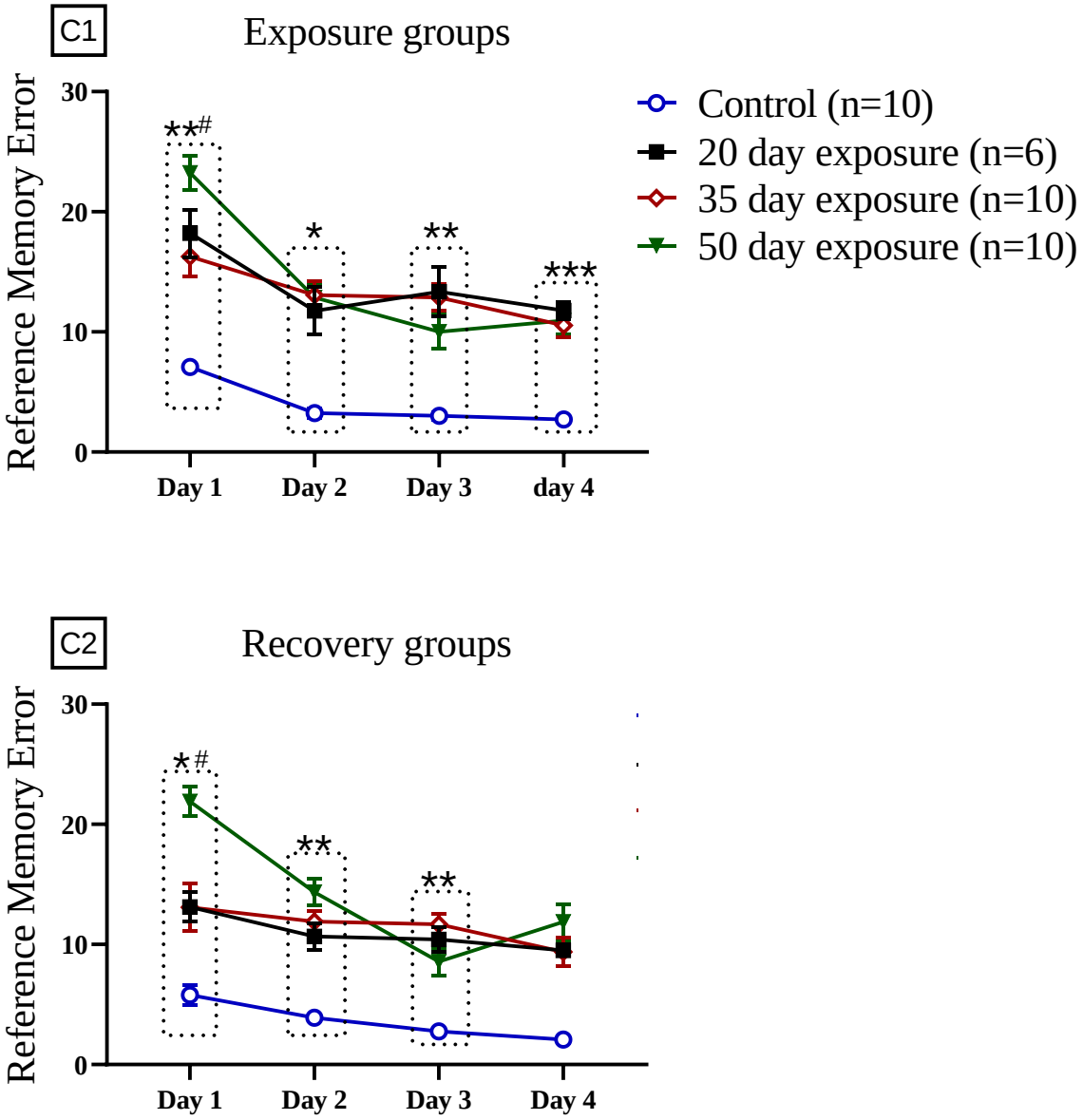


Figure 3.4 C: Effect of Aluminum temporal exposure on reference memory. Graph shows reference memory errors in Control, 20 day exposure, 35 day exposure and 50 day exposure and their respective recovery groups.*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$ is the significant value between Control and Aluminum treated groups and #= $p < 0.05$, ##= $p < 0.01$ among Aluminum treated groups. Error bars are represented as mean± SEM by 2 way ANOVA test.

(D)

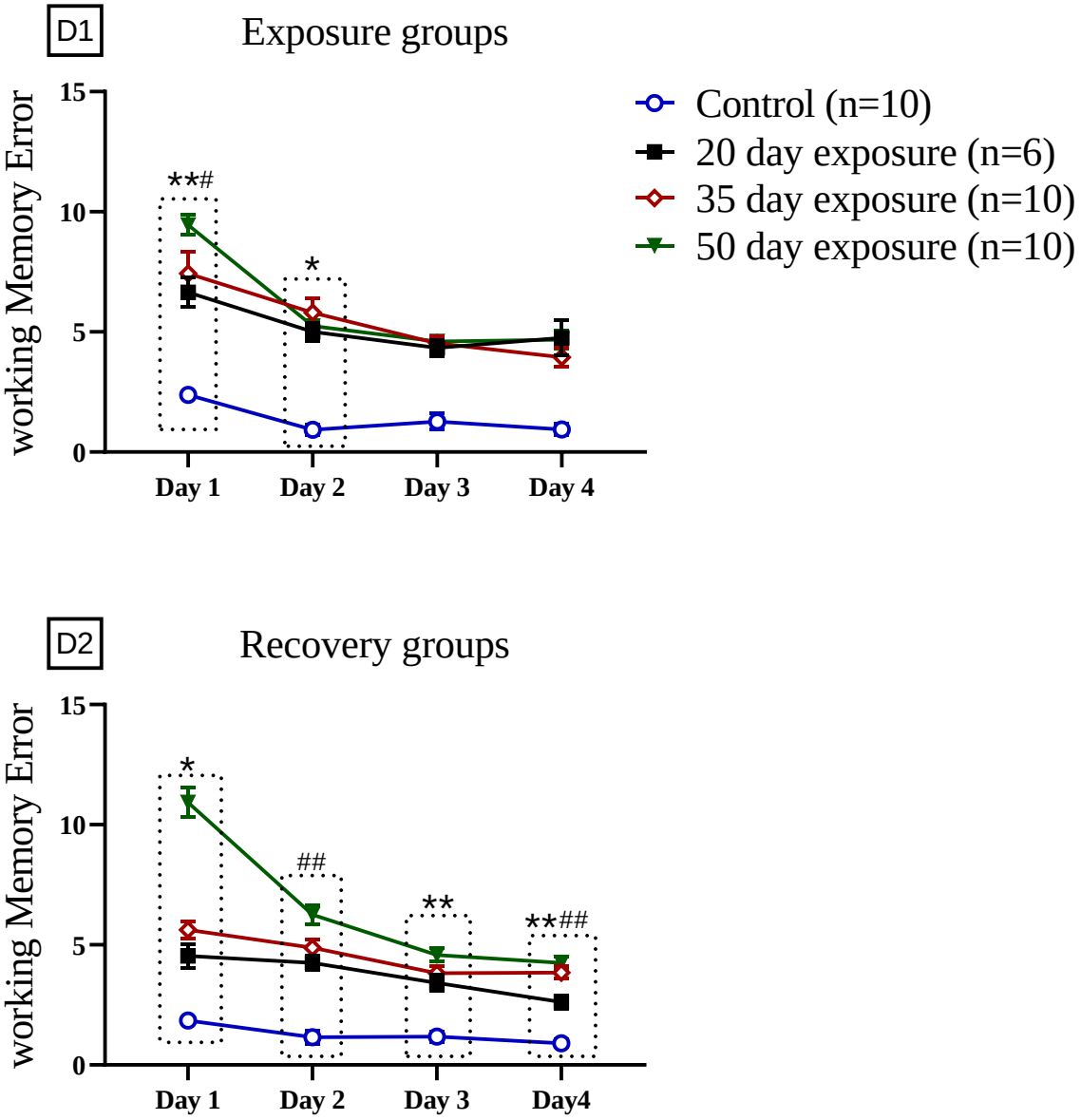


Figure 3.4 D: Effect of Aluminum temporal exposure on working memory. Graph shows working memory errors in Control, 20 day exposure, 35 day exposure and 50 day exposure and their respective recovery groups.*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$ is the significant value between Control and Aluminum treated groups and #= $p < 0.05$, ##= $p < 0.01$ among Aluminum treated groups. Error bars are represented as mean± SEM by 2 way ANOVA test.

3.5 Result Interpretation:

Table 8: Interpretation of results.

Test	Brain region involved	Behavior	Damage	Recovery
Y maze test	Hippocampus prefrontal cortex	spatial learning and memory	20 day exposure group >50 day exposure group > 35 day exposure group	35 day recovery group >20 day recovery group > 50 day recovery group
Morris water maze	Hippocampus	spatial learning and memory	50 day exposure group >20 day exposure group > 35 day exposure group	35 day recovery group >20 day recovery group > 50 day recovery group
Hole board test	Hippocampus Amygdala	exploratory behavior/anxiety	50 day exposure group >35 day exposure group > 20 day exposure group	35 day recovery group >50 day recovery group > 20 day recovery group
Three chamber assay	prefrontal cortex	sociability	50 day exposure group >20 day exposure group > 35 day exposure group	35 day recovery group >20 day recovery group > 50 day recovery group

CHAPTER 4

DISCUSSION

DISCUSSION

Modification of behavior by the process of acquiring new information called as learning and the remembrance of past experiences called as memory, are exceptional traits of higher organisms (Cassilhas et al., 2016). Short term memory and long term memory, spatial and referential learning and memory are associated deeply with the Hippocampal, prefrontal cortex and amygdala region of the brain (Noble & Kanoski, 2016). Any decline in learning and memory can result into various neurodegenerative diseases like Alzheimer's, Parkinson disease and dementia (Wang et al., 2017). Neuronal inflammation, oxidative stress, neurofibrillary tangles formation and tau protein accumulation are the major roots of decline in cognitive functions (learning and memory).

Aluminum, the most abundant metal in the Earth's crust due to its light weight and silvery-white appearance, its alloys are preferred in the manufacture of several contemporary utility items (Martin et al., 2013). Aluminum salts are used in water filtration, their main role is as a coagulant of organic matter; to reduce color, turbidity and the levels of micro-organisms. Even consumer products such as antacids, astringents buffered aspirin, food additives, and antiperspirants contain small quantities of aluminum compounds (Udeh & Udeh, 2004). The mobility levels of the metal have increased significantly in recent decades(McKain et al., 2015). Westberg et al have found that the air inside smelters, foundries and re melting plants can have considerable concentrations of Al oxides and Al compounds suspended in it (Westberg et al., 2001). Whereas in Antarctica air concentrations of Al can be as low as 0.0005μg/m 3, in industries aluminum levels up to 1μg/m 3 can be recorded (Udeh & Udeh, 2004).

In a study in Poland, researchers found, that in water with low pH, aluminum fluorides and sulfates prevailed, while at neutral pH, aluminum hydroxides and organics

predominated(Frankowski et al., 2011) Additionally, in treated water, aluminum coagulants are often used to remove microbes, organic matter, and color. Ohno et al. estimated that the average Japanese adult from any of six cities consumed almost 2.2% of their total dietary intake of aluminum from drinking water (Ohno et al., 2010) These concentrations are found to be consistent with data from the United States where aluminum concentrations in finished municipal drinking water amounted to 1% of the dietary intake of aluminum for an adult (Krewski et al., 2007). This value is less than the 4% value predicted by WHO (2010), while assuming water contained 0.1 mg Al/L.

Aluminum has been associated with neuronal death due to the formation of amyloid plaques (Exley et al., 1997). Aluminum is linked with the formation of neurofibrillary tangles in hippocampal region of Alzheimer disease patients (Wang et al., 2017). Aluminum has been reported at higher level in brain of Alzheimer's patients and plays major role in its progression (Nessa & Khan, 2015). Studies have also shown that exposure to aluminum can cause memory impairment in animal models (Walton & Wang, 2009). Chronic exposure to aluminum results in its accumulation specifically hippocampal region of mouse brain (De Jager et al., 2014). One of the animal study has shown significant impairment in memory formation after Aluminum exposure (Thenmozhi et al., 2015). Long term exposure to Aluminum has also been associated with the initiation and progression of dementia (Rani et al., 2015).

Current study is focused on how different temporal groups provided with same total exposure of Aluminum exhibit different level of learning and memory and impairment and whether the exposure caused permanent impairment or not in each group. Animals were divided into 4 groups i.e. Control, 20 day exposure (292.5 mg /kg/day), 35 day exposure (175mg/kg) and 50 day exposure (117mg/kg/day) groups given. After successful completion of exposure times

behavioral tests were performed on these animals. Further all these groups were given recovery period of 20 days in which normal water and feed was given. Behavioral tests were again performed after recovery period to assess recovery in learning and memory impairment in different time groups. Metal Detection Assay through Atomic absorption spectroscopy was also performed to evaluate aluminum deposition in hippocampus, prefrontal cortex and amygdala part of brain. Due to Covid-19 situation results are pending.

Morris Water Maze test (MWM) was performed in order to assess the effect of Aluminum on long term memory and spatial memory. MWM is the most widely accepted test to evaluate hippocampal functioning in spatial learning and long term memory in mice. Animal tries to find platform in round tub with equipped with spatial cues in training sessions when dropped from different directions (Vorhees & Williams, 2006). Previous study by Farhat et al had shown that mice treated with 250 mg/kg of $AlCl_3.6H_2O$ in drinking water for 42 days have shown reduced spatial reference memory as compared to Control group animals in MWM test (Mehpara Farhat et al., 2019). Our result also matched with this study as 20 day group (292.5 mg /kg/day) showed impaired spatial and reference memory in MWM escape latency task as well as platform crossings (probe trial). In training session task, 50 day exposure group (117mg/kg/day) showed highest deficit in spatial learning and memory as compared to Control group. 20 day exposure group (292.5mg /kg) showed slightly less impairment than 50 day exposure group (117mg/kg/). While 35 day exposure group (175mg/kg) has shown least impairment. Slight recovery was observed in performance of all recovery groups in comparison with their respective groups. This may indicate that long term exposure or high dose exposure to Aluminum cause higher impairment in learning and memory as compared to slow and low dose exposure. In probe trial highest impairment of long term memory and spatial learning was shown by 50 day exposure

group then 20 day exposure group and least by 35 day exposure group in comparison with Control group. Highest recovery in spatial memory and learning was observed in 35 day group and least or no prominent recovery was seen in 20 day exposure group. The results may indicate that long term exposure or high dose exposure to Aluminum can cause higher impairment in reference and spatial learning and long term memory as compared to slow and low dose exposure.

Animal natural exploratory behavior is recognized by Y-maze test. It is employed to evaluate both spatial working memory and reference memory. Intact working memory is associated with prefrontal cortex and spatial reference memory with hippocampal functioning (Kraeuter et al., 2019). Rodents are naturally inclined to visit unexplored novel arm more than start and other arm. To define reference memory impairment time spent in Novel arm and No. of entries in Novel arm was assessed. Result were consistent with MWM test result as 50 day exposure group and 20 day exposure group showed highest impairment than Control and 35 day exposure. Very low or no significant improvement in reference memory was observed in 20 day recovery and 50 day recovery group however improvement was observed in 35 day recovery group. Spontaneous alternations, alternate arm repeats and same arm repeats were calculated to assess short-term memory. Rodents employ their working memory and generally visit arm that is visited least recently i.e. alternative visits between three arms. Rodents generally tend to explore the arm visited least recently, and so, normally they are expected to alternatively visit the three arms (Wietrzych et al., 2005). Least number of spontaneous alternations was observed in 50 day exposure group implicating impaired working memory. Other than that no significant impairment or recovery was observed. Alternate arm repeats data showed highest impairment in 20 day group and 50 day group as compared to 35 day and Control group. After recovery 50 day

recovery group did not show any improvement in performance while 35 day recovery group presented improved working memory. Same arm repeat data result was consistent with alternate arm repeats result with 50 day exposure group showing highest impairment in working memory. Slight recovery was observed in 35 day recovery group and 20 day recovery group but no improvement observed in 50 day recovery group.

Three chamber sociability test was performed to assess sociability in three phases. First is habituation, second is Session I to assess sociability in animal and third is Session II to evaluate social novelty preference (Moy et al., 2004). Previous study has shown decrease sociability in aluminum (250mg/kg) 42 day exposure groups as compared to sociability level of Control and decreased social novelty preference (Farhat, Mahboob, Iqbal, et al., 2017). Our results were found to be consistent with previous study. In Session I 50 day exposure group spent least time in Mouse 1 chamber and also interacted more with empty cage as compared to Mouse 1 while all other groups interacted more with Mouse 1 than empty cage. Impairment in sociability was not recovered in 50 day recovery group after recovery period. But 35 day recovery presented better performance in both time in Mouse 1 chamber and interaction with Mouse 1. In Session II highest impairment of social novelty preference was seen in 50 day exposure group as it preferred to interact more with Mouse 1 than Mouse 2 as compared to Control group. 20 day exposure group and 35 day exposure group showed less social novelty preference than Control but still interacted and spent more time with Mouse 2 than Mouse 1. After recovery period performance of 35 day recovery group regarding interaction with Mouse 2 was significantly improved. But no improvement was observed in 50 day recovery group. These results indicate that long term exposure and high exposure of aluminum can cause deficit in sociability of mice and novel object recognition. Percentage discrimination results were also consistent with the

other parameters. 20 day exposure group showed least discrimination between novel mouse (Mouse 2) and Mouse 1 as compared to control with least recovery after recovery period.

Hole board test was employed to assess anxiety level and locomotion activity in rodents as well as to evaluate working memory and spatial reference memory. Anxiety was assessed by calculating the time taken by animal to poke the first hole whether baited or unbaited and locomotion performance was assessed by calculating activity/min. Throughout the 4 days least locomotion activity was presented by 50 day exposure group, then 20 day exposure and then 35 day exposure group. No significant improvement was seen in 50 day recovery group. However, slight improvement in performance was observed in 20 day recovery and 35 day recovery group. Our results of working memory error were consistent with the factor that anxiety interferes with the working memory by interfering with the tasks involving complex attention and coordination (Salthouse, 1996). It builds tension and nervousness which result in poor perception resulting in poor performance of working memory (Wetherell et al., 2002). Reference memory errors and working memory errors were calculated to assess reference memory (long term memory) and working memory (short term memory) respectively. RME were highest in 50 day exposure group as compared to control, 20 day exposure and 35 day exposure group. On day 4 highest RME were observed in 20 day exposure group then 50 day exposure group and least in 35 day exposure group. After recovery period slight improvement was seen in all groups except 50 day recovery group in which RME did not decrease. Working memory errors result was consistent with reference memory error result with highest WME in 50 day exposure group and 20 day exposure group on day 1 and day 2 respectively. Very low or non-significant improvement in WME was noted after completion of recovery time period. Hence, we can assume that long term exposure of Aluminum low dose (117 mg/kg/day) and short term exposure of aluminum high

dose (292.5mg/kg/day) both with same total exposure (5850mg/kg) results in impairment of hippocampal dependent learning and memory which cannot be recovered significantly on its own especially in long term exposure case. Summary of the whole research is illustrated in Figure 4.1.

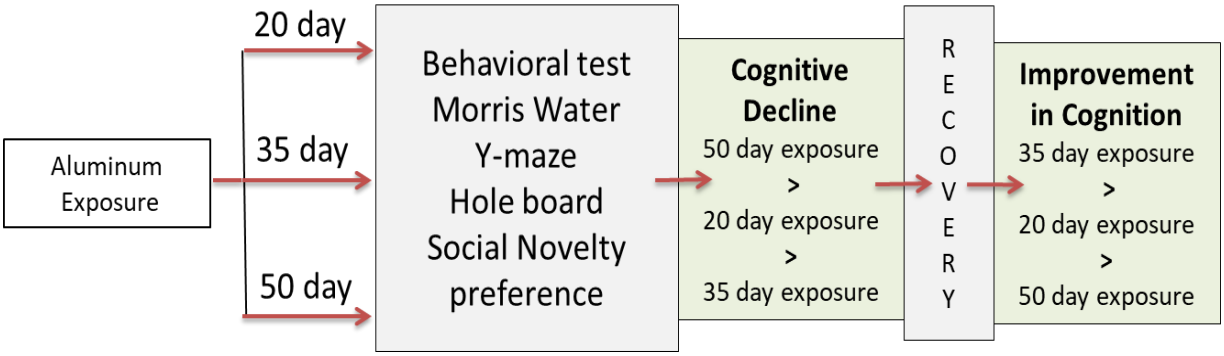


Figure 4.1: Illustration of experiment and results.

CHAPTER 5

CONCLUSION

CONCLUSION

Increased exposure to aluminum contaminated drinking water has led to impairment in learning and memory process. This study presents how different time dependent exposures of same total dose of Aluminum can cause differential impairment in hippocampal dependent learning and memory, which can be irreversible on its own. Results of the study clearly presents that long term exposure of Aluminum with low dose and short term exposure with high dose both can cause learning and memory impairment that cannot be reversed on its own without any treatment. However, moderate dose exposure of Aluminum for moderate period of time cause low level of learning and memory impairment that can be reversed slightly on its own. Still further research is needed to establish the exact mechanism of temporal exposure of Aluminum on higher cognitive functions.

CHAPTER 6

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