Evaluation of Al Exposure on Empathetic Behavior in Rat Model

Master of Science

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Evaluation of Al Exposure on Empathetic Behavior in Rat Model

A thesis submitted in partial fulfilment of the requirement for the degree of Masters of Science

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This thesis is dedicated to my parents and brothers

for their endless support and encouragement

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Al	Aluminum
AD	Alzheimer's Disease
$AlCl_3 \cdot 6H_2O$	Al chloride hexahydrate
$AlCl_3$	Al chloride
ANOVA	Analysis of Variance
ACC	Anterior Cingulate Cortex
ACC-BLA	Anterior Cingulate Cortex- Basolateral nucleus of Amygdala
ACH	Acetylcholine
ALS	Amyloid Lateral Sclerosis
As	Arsenic
BBB	Blood Brain Barrier
BLA	Basolateral Amygdala
BNST	Bed Nucleus of the Stria Terminalis
Cd	Cadmium
Cr	Chromium
CNS	Central Nervous System
EBT	Empathy Behavior Test
EPM	Elevated Plus Maze
HI	Hazard Index
HPA	Hypothalamic-Pituitary-Adrenocortical
IFG	Inferior Frontal Gyrus

Pb	Lead
LC	Locus Coeruleus
mPFC	Medial Prefrontal Cortex
mAChRs	Muscarinic Acetylcholine Receptors
NA	Nucleus Accumbens
nAChRs	Nicotinic Acetylcholine Receptors
Ni	Nickel
SNS	Sympathetic Nervous System
SEM	Standard Error of Mean
SEL	Social-Emotional Learning
ToM	Theory of Mind
vmPFC	Ventromedial Prefrontal Gyrus

ABSTRACT

Empathy, a complex psychological attribute, is referred to as an increase in affiliation towards someone in distress with a desire to reprieve their travail. Though it is a common behavioral response in humans and other organism with advanced cognition, the underlying neural circuitry and involvement of several brain regions are not yet fully understood. The current study investigated the role of social isolation and aluminum (Al)-induced neurotoxicity on regulation of empathy. An 80-day protocol was designed to evaluate the effect of Al exposure (80 mg/kg of Al in drinking water) and isolation on empathy. Female Wistar rats (200-300g) were housed in plastic cages and acclimatized. The animals were divided into 4 groups i.e. Al exposed (n = 10), Control (n = 10), isolation only (n = 10), and Al + isolation (n = 10). The control and isolation only group were given distilled water. Behaviour tests were performed at two intervals i.e. 20 and 40 days to assess short term and long term effect of metal toxicity and isolation. The model for Empathy Behavior Test (EBT) implied use of restrainer stress method where a conspecific is trapped in a perforated container under observation by the experimental rats. Empathy response was measured through assessing the number of interactions, number of climbings, freezing behavior, and time spent in close proximity to subject rat. The EBT was performed prior to as well as after 24 h of self-experiencing the restrain stress to evaluate enhancement in empathy. Moreover, anxiety and stress levels were measured by Elevated Plus Maze (EPM) and grooming behavior (spray test). It was observed that the rats exposed to Al drinking water made significantly less number of interactions and climbings after long term exposure ($p < 0.0001$). However, short term Al exposure was less detrimental ($p < 0.01$). Whereas, in case of isolated rats the freezing behavior tremendously increased after long term isolation ($p < 0.0001$). Furthermore, though freezing behavior was observed after short term Al exposure, it was diminished after long term exposure. In particular, the empathetic response did not increase after self-experience of distress in both the groups as compared to their respective controls. Accompanied with these behavioral changes tremendous increase in anxiety and stress like symptoms was observed in Al-exposed and isolated animals. The Al exposure and Al + isolation groups made significantly less ($p < 0.0001$) number of entries in open arms of EPM. Similarly, the number of incorrect grooming bouts were significantly higher ($p < 0.0001$) in these groups. Latency to start grooming was also observed to be increased in isolated and Al exposed group signifying the stressed condition. This study provided the evidence that empathetic behaviour is influenced by metal exposure and social isolation and may have a direct association in disrupting the neural circuitry of empathy. Moreover, isolation have a peculiar relationship with empathy as some components of empathy were increased (affective) while others decreased (cognitive). This preliminary and first of its kind study highlights the significant impact of Al-induced neurotoxicity however determination of the exact neural circuitry behind subcomponents of empathy is warranted.

Chapter 1

INTRODUCTION

Empathy is a multifaceted psychological trait with various cognitive and behavioural components. Clinically, empathy is defined as the ability to understand and show emotional response towards the perceived difficulty of another person (Sharma et al., 2021). Empathy is mainly categorized into two types: the affective empathy and cognitive empathy. Unlike emotional empathy which can be observed in many animal species, cognitive empathy is more predominantly observed in humans, primates, and rodents (Elsegood et al., 2010). The affective empathy (also known as emotional empathy) involves the capacity to understand and feel the perspective, opinions or discomfort of others. The cognitive empathy, on the other hand, engages higher cognitive system of brain to adopt another person's psychological point of view. It is known as an advanced form of perspective-taking system. Shamey G. et al. described cognitive empathy in terms of Theory of Mind (ToM) that in simpler terms can be expressed as the ability to put oneself into someone else's shoes. It is considered as a higher-level process, involving cognitive processes and is a form of altruistic behaviors (Shamey et al., 2011).

Empathy is one of the most significant element of social interaction. Exhibiting an empathetic nature is essential to understand the mental state of people encountered on daily basis. It serves to be a ground for establishing an interactive system at work place, academia, medical assistance, psychotherapy sessions, gaming grounds and many more. Correspondingly, from the neuroscience perspective, further evaluation of empathetic behavior can help better understand the links between emotion and cognition (Ben et al., 2011).

Metal toxicity is an emerging environmental and health hazards especially in underdeveloped areas of Pakistan. The adverse outcomes are not only faced by humans, but also agricultural plants, farm animals, and all other living creatures. All together the hazardous circumstances increase the concentration of toxic elements in acid rains and flooding water. These increasing environmental challenges create an imbalance in nature leading to contaminated water, food and soil (Liaquat et al., 2019).

Al is the 3rd most abundant element found in the earth crust and is widely used as a food additive and a coagulant in purification process of drinking water. Unlike other essential trace elements like iron, magnesium, zinc, copper and selenium; Al is not essential for mammalian body. Previously Al^{3+} salts were not considered detrimental, because it forms monomerichydroxy compounds in solution. These compounds tend to form insoluble colloidal and polymeric complexes that are not absorbed by our body. However, environemental hazards like acid rains may cause release of Al salts that can get deposited into bodily tissues if ingested (Walton et al., 2007).

Al on entering the gastrointestinal tract, is absorbed at the rate of 0.22%, and up to 90% of Al from blood binds the transferrin protein. In this way the carrier protein transfers the metal to brain through receptor-mediated endocytosis via blood brain barrier (BBB). After absorption through the gastrointestinal tract Al moves to the liver, where the majority of it is eliminated from bloodstream. If remained in body, it can cause detrimental outcomes. Long term exposure to Al contamination in drinking water leads to cognitive and morphological changes in the central nervous system (CNS) (Bondy et al., 2010). Hence, the current study investigated a yet unknown aspect i.e. effect of Al neurotoxicity by water on empathetic response in rats through different behavioural tests. Further the combined impact of Al exposure and social isolation was evaluated to demonstrate its correlation with empathy.

Chronic social isolation lead to deteriorating health damage especially neurodegeneration causing depression, stress and anxiety. The central nervous system (CNS) have established a complex relation with reward, pleasure or any form of deviations from all the behaviors in our surroundings that are repetitive. Persons who remain isolated for a long period of time start losing the ability to evaluate an incoming stimulus, categorize it, and analyze it by critical thinking based on past, current or future goals (Matsumoto a et al., 2006).

Impairments in empathy behavior is not only a crucial characteristic of various neurodevelopmental disorders, but also an acquired trait that greatly depends on environmental conditions, social attributes, parenting and childhood brought up etc. The empathy level have a peculiar relation with isolation. Despite of having a strong desire to interact, the socially isolated people still tend to have difficulty on facing others and display less empathy. On the other hand, it has been also observed that such people are more motivated to reconnect with others and are in search of social interaction (Gambin et al., 2018).

Al induced-toxicity and social isolation are among the emerging issues of underdeveloped countries and also some developed areas. A large number of population consume contaminated water because of unavailability of clean water, lack of education and poverty. On the other hand, numerous groups of society are trapped in fears of social interaction and disapproval complex that leads to depression, anxiety and stress (Cech et al., 2000).

Such an environment creates a barrier to attain a healthy, protective and caring society on the whole. Lowered empathetic nature in general population day by day can have terrible consequences. Thus, the current study speculated the empathy behavior in socially isolated rats for short as well as long time period. Moreover, it focussed to determine variation in empathetic response by experimental rats after Al exposure in group as well as isolation.

1.1. Aims and Objectives

The aims and objectives of this study were:

1. To examine the effect of Al exposure and isolation on anxiety and stress.
2. To evaluate the short term and long term effect of Al exposure on empathetic behavior.
3. To determine the combined effect of Al exposure and isolation on empathetic behavior.
4. To analyze difference in empathetic behavior before and after self-experience of distress.

Chapter 2

LITERATURE REVIEW

2.1 Empathy

Earlier in 1923, Buber classified empathy as one's capability to discriminate between the "I-you," where one is concerned of another person's thoughts and feelings, and the "I-it," where one is considering the person as an object. Later on, the empathetic trait was further categorized into two dimensions: affective and cognitive process (Zank et al., 2004).

A recent research in 2021 have proposed an empirical study to assess the influence of empathy on cooperative learning. From their perspective of sociology, cooperative learning is a skill that improves learning motivation, interpersonal skills, attitude, cognitive and non-cognitive qualities. This learning process not only enhances self-confidence but also reduces social anxiety (Smith et al., 2005).

Others have found that empathy is a major component of social-emotional learning (SEL) curriculum which means that it can be learned and acquired. A study conducted by Han et al. reported that there are numerous advantages of empathy development program in children just before entering the youth stage that have shown profound personality differences later in life to have empathetic nature towards everyone (Han et al., 2020). The data was also supported by another study that found a better academic performance of such students as compared to those who were under random self-learning groups (Wei et al., 2020).

2.1.1. Subcomponents of Empathy

The emotional empathy ('I feel what you feel') is considered as a primitive behavior i.e. an instinctive attitude to show empathy on seeing somebody in pain (Shamay et al., 2011). Whereas, cognitive empathy is explained by a study that evaluated the door opening tendency of an observer rat to rescue a cage mate in distress. They found that rats did not care to open the door if there was no cage mate trapped in the cage. However, the door opening tendency increased on observing distressed cagemate. Further evaluation showed that the rats preferred sharing a treat (food) with distressed conspecific instead of having it on its own. It was also found that empathetic response was higher in female rats as compared to the male rats (Mellanie et al., 2019).

Another study have also proved a differential relation of affective and cognitive empathy with emotional regulation. According to this study, the strategies that our brain opts for regulation of emotions greatly determine the explicated empathy. It was observed that increasing affective empathy elicits a fear response that can decrease the helping attitude through cognitional empathy (Thompson et al., 2022).

Various developmental studies indicate that the most basic form of empathy is the built in contagious crying in young age indicating a shared pain aroused by another person's emotional state. Similar data in literature supports episodes of fear and immobility in rodents on observing a cage mate in distress via emotional contagion. According to studies in past years, it was believed that complex cognitive form of empathy only exist in primates like humans, monkeys, apes, etc. However, later on studies have also investigated helping behaviour in rodents as a model of cognitive empathy (Mellanie et al., 2019; Ewelina et al., 2010).

In the world of behavioral neuroscience, rodents have been administered to different prosocial helping behaviors to evaluate their relationship with the conspecific. This involve their decision making ability as a momentary cost to personal gain, rather than an instinctive stereotypic response (Spreng et al., 2009).

2.1.2. Attributes of Empathy

Previous research have established certain attributes of empathy that are: emotional contagion, emotion comprehension, sensitive behavior, sympathetic physiological arousal, altruistic behaviour and prosocial helping behaviour (Decety et al., 2012). Emotional contagion occurs when a person's emotions involve a spontaneous convergence towards similar emotions in others (Neves et al., 2018). As described earlier, the observational fear and episodes of immobility in rodents on observing a cage mate in distress are a form of emotional contagion (Ben et al., 2011).

Emotional comprehension is described as the ability to identify another person's emotional state by facial and body expressions. (Göbel et al., 2016). Whereas, the sensitive behaviors helps to assess that how deeply a person perceives things, whether positive or negative. Furthermore, sympathetic physiological arousal occurs upon activation of the sympathetic nervous system that prepares the body for a physical reaction in flight or flee moments (Pijeira et al., 2019; Sathaporn et al., 2022).

Empathy in terms of asceticism is an attribute closely linked with egotism and altruism. Egotism is based on the intention to gain personal benefit out of any condition whereas altruism involves acts of gratitude and benefits for others. Empathy serves as a protective agent against anxiety and aggressive behaviour as well as a precursor to various social-emotional abilities. However, few studies have also investigated prosocial helping behaviour in rodents as a model of cognitive empathy involving critical thinking (Xiang et al., 2021).

2.2 Brain regions involved in regulation of empathy

The three major components of brain are cerebrum, cerebellum, and brainstem. Cerebrum, divided into the right and left hemisphere, is the largest part of brain that regulates critical thinking and cognitive processes. Whereas, the brain stem acts as a relay centre to connect cerebrum to the spinal cord and also performs various autonomic functions. Moreover, the emotional component of brain is regulated by the amygdala of limbic system in brain stem (Meyerhoff et al., 2021).

Empathy being a multifactorial trait is regulated by different brain areas (Figure 2.1), environmental factors, age group, social interaction etc. The empathetic response is presumed to be an emotional manifestation after analysing and critical thinking through cortex. According to the neural circuitry of empathy explored so far, the emotional and cognitive components of empathy are regulated by separate brain regions. The oxytocingeric system and inferior frontal gyrus (IFG) specifically control the emotional contagion and emotion recognition. While defective dopaminergic system and ventromedial prefrontal cortex (vmPFC) lead to impaired cognitive empathy. Moreover, the empathy for both observed and felt pain is controlled by network of anterior cingulate cortex (ACC). The Inferior frontal gyrus and Inferior parietal lobule were found to be active in vicarious pain and inactive in self-pain (Shamay et al., 2011)

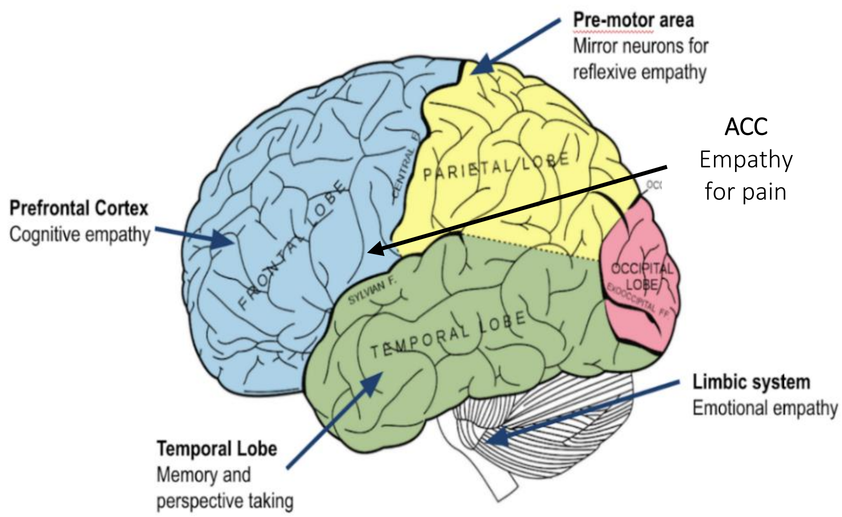


Figure 2.1: Brain regions involved in regulation of different components of empathy (Modified from Johannes et al., 2012).

2.3 Models for Empathy Behavior

Previously it was believed that cognitive empathy only exists in higher order living beings like humans and other primates. Whereas rodents only express emotional empathy as a fear response. However, later on many other studies demonstrated cognitive form of empathy in mice and rats through behavior testing (Mellanie et al., 2019). The findings of various studies are depicted in Table 2.1.

Table 2.1: Models for empathy behavior testing.

S No.	Empathy Behavior Model	Description	Reference
1	Restrainer door opening tendency for reward vs trapped conspecific	A cagemate is trapped in cage with a door opener. An observer rat after certain trials learns to open the door. Now if a cagemate is trapped in one cage and a reward (food treat) is placed in another cage, the observer rat rescues the cagemate first and share the treat together instead of opening the door for treat.	(Mellanie et al., 2019)
2	Socially transferred fear conditioning defensive behavior	Behavior of an observer is evaluated when transferred with a recently fear conditioned partner (by another aggressive conspecific). The observers shows more social exploratory and freezing behavior as an adaptation to defensive behavior in case of danger.	(Ewelina et al., 2010)
3	Perception Action Model in Water chamber	A cagemate is trapped in a one of the chambers of a multi- chambered box that is filled with water. An observer on the other dry chamber rescues the trapped animal on observing water/foot shock stress on the cagemate.	(Asli et al., 2018)
4	Separation- Reunion consolation test	Animals housed together for several days are separated and one of them is exposed to foot shock stress. On reunion, the naïve observer rat exhibit a natural empathetic response by licking and allogrooming behavior towards stressed rat.	(James et al., 2016)

2.4 Environmental and Health Hazards of Metal Exposure

A retrospective review by Waseem A. evaluated the pollution status of heavy metals in water, vegetables and soil of Pakistan. According to that report, it has been observed that industrial by-products have been release into the water streams and rivers of Pakistan. The estimated content of Cadmium (Cd), Chromium (Cr), Nickel (Ni), Lead (Pb), and Arsenic (As) in ground water of different areas of Pakistan is extremely high in the fresh water resources (Figure 2.2). Though a number of health and neurological cases have been reported because of Al toxicity, not enough data was available about content of Al toxicated water in Pakistan (Waseem et al., 2014).

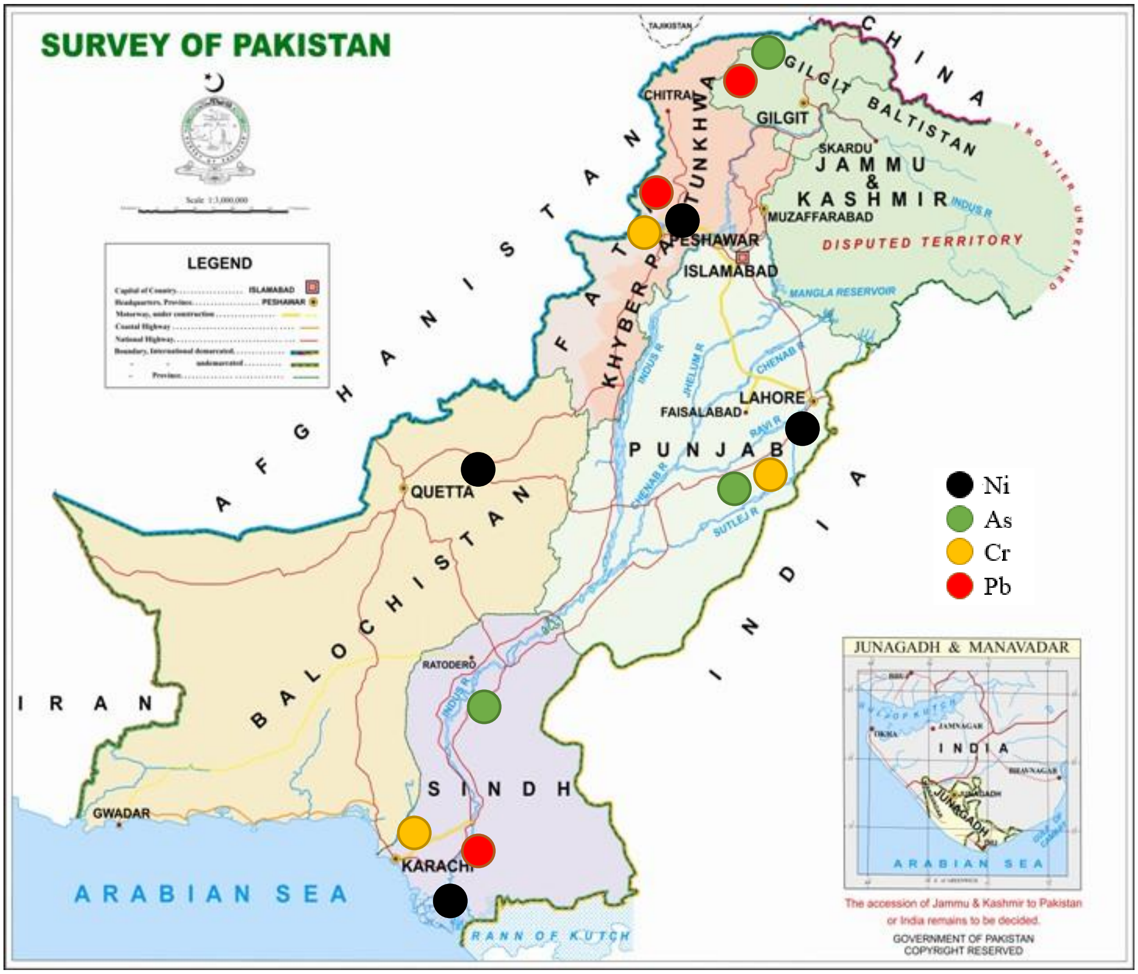


Figure 2.2: High incidence of metal toxicity through ground water in different regions of Pakistan (Modified from Waseem et al., 2014).

2.5 Prevalence of Al Toxicity

With the increase in prevalence of acid rains and acidification of soils, the bioavailability of Al is raised by release of Al^{3+} salts from insoluble minerals (WHO Report, 2022). The condition is even more toxicated by anthropogenic activities in Al industries that especially have adverse outcomes for animals that are exposed to various sources of dirty water and waste (Hardisson et al., 2017). Al salts are included in drinking water to clarify it and act as a coagulant to remove turbidity, bacteria, and organic waste. Although effective, this treatment may raise the concentration of Al at the time of ingestion. Additionally, high residual concentrations could lead to Al deposits in the distribution system, which could lead to a rise in Al content in tap water (WHO Report, 2022). Considering the mentioned scenario, the developing countries are at an emerging risk of Al toxicity in these circumstances.

2.5.1. Al absorption and toxicity

The World Health Organization (WHO) has established a tolerable daily intake of Al as 1 mg/kg of body weight (Alasfar et al., 2021). It has also been reported that if concentration of Al^{3+} in plasma raises beyond 0.4 μM, the Al salts starts depositing in bodily tissues including brain, kidneys, bones and liver. Henceforth, the toxicity of Al depends greatly on its exposure route and solubility rate in body (Bondy et al., 2010).

The clearance half-life of Al is predicted to be less than a year at the time. The results of the study's pharmacokinetic research revealed that the terminal clearance half-life of Al in blood was seven days and that it was 170 days throughout the whole body. Due to sample size considerations, another study found biliary excretion accounts for less than 1% of Al excretion while Al excretion via urine was found to be approximately 9–17% within two weeks, less than 2% of the Al was found in the stools and more than 80% of it was eliminated in the urine (Riihimäki et al., 2012; Xu et al., 1992).

2.5.2. Al exposure and neurodegeneration

Al neurotoxicity can adversely affect cholinergic neurotransmission. It is found to interrupt Acetylcholine (ACH) synthesis and binding and then degrading it. ACH is a primary neurotransmitter of the parasympathetic nervous system and that is required at the neuromuscular junction. The Al hinders the binding step of acetylcholine with the muscarinic acetylcholine receptors (mAChRs) and nicotinic acetylcholine receptors (nAChRs). This can critically affect learning and memory process in hippocampus, a hallmark of AD (Mehpara et al., 2021).

Another study facilitated that Al promotes the formation of β-amyloid aggregations. The accumulation of senile plaques in neurons causes dementia and AD. They investigated the role of Al in transport of Acetyl-CoA from mitochondria. It was suggested that Acetylcholine synthesis greatly depends on the Acetyl-CoA from mitochondria in terminal nerve endings. Thus Al, by inhibiting the entry of Calcium reduces transport of Acetyl-CoA and indirectly the Ach synthesis people (Szutowicz et al., 2001).

2.6. Social Isolation

According to literature, it has been recognized as one of the leading cause of mortality by morbidity or even suicide. Social relationships (spouse, parent-child, siblings etc) are usually perceived as symbol of care and protection. The neural circuitry is specialized to transform all such emotional perceptions through different brain regions that stimulate hormonal or neurotransmitter release.

2.6.1. Neural basis of social isolation

It is predicted that a higher association of hypothalamic-pituitary-adrenocortical (HPA) activation with social isolation and threats are faced by lonely people. The HPA axes was thought to be regulated by prefrontal cortex and limbic system (Matsumoto a et al., 2006).

The neural network of prefrontal cortex and limbic system is activated upon environmental stresses like loneliness, social defeat and social isolation. Studies have predicted that medial and central nuclei of amygdala along with bed nucleus of the stria terminalis (BNST) are further connected to paraventricular nucleus of hypothalamus and brain stem mediating autonomic system. This cascade of signals activates the sympathetic nervous system (SNS) through sympathetic nerve fibers. This results in release of catecholamine neurotransmitter norepinephrine into the blood stream (Figure 2.3). Moreover, the isolation stress also increase the release of acetylcholine and dopamine in cortex to regulate anxiety. However, a prolonged isolation leads to chronic brain damage and the body fails to respond to stressors. Such people face extreme fear and anxiety if their isolation is reverted (Jacobson 2014; Matsumoto a et al., 2006).

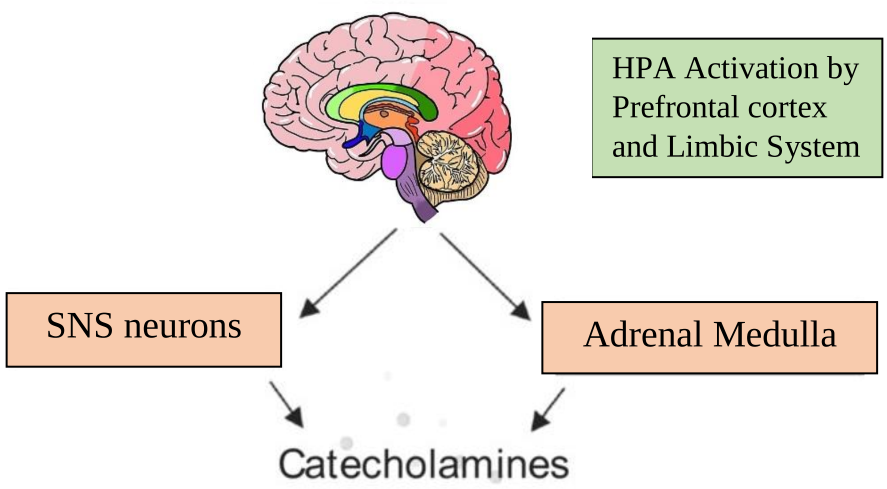


Figure 2.3: Social isolation triggering the release of catecholamines by activation of sympathetic nervous system neurons (Modified from Matsumoto a et al., 2006).

2.7. Impaired Empathy can be a consequence of Al exposure and social isolation

A number of findings suggest that impaired empathy is frequently seen in autistic, schizophrenic and psychiatric patients ((Ben a et al., 2011; de Waal a et al., 2017). At the same time, it has been seen that as a child enters the adolescence phase, a decrease in time spent with parents' results in increased parent–child conflicts.

Thus an increased aggressive behaviour is found in youth making it essential to assess their attitude towards their surrounded people in any form of pain or discomfort (Beelmann et al., 2014). As seen in literature, Al neurotoxicity causes serious damage to brain, it is hypothesized that this will also result in empathy impairment by damage to empathy related brain regions.

Similarly, short term as well as long term social isolation, as observed in the post Covid period, resulted in anxiety like behaviours in people (Guadagni et al., 2020). A study found a peculiar relation of empathetic behavior with anxiety and depression such that high levels of affective empathy and low levels of cognitive empathy were associated with social anxiety. Different findings suggest that lower levels of empathy are greatly linked to aggression, criminal acts and antisocial behaviours (Morrissette et al., 2021).

Another key finding about isolation proposed that loneliness and social avoidance is a major contributor to cognitive decline. According to a longitudinal study the elderly people who feel lonely are at double the risk to develop AD as compared to those who were socially interactive. Another study found that people who have a larger social network have better cognitive functioning including memory and learning (Gambin et al., 2018).

A recent review (2022) suggested a differential relationship between cognitive and affective empathy with emotional regulation. In conclusive, increasing affective empathy beyond an extent develops a fear of helping others as in case of social isolation. Such lonely people have more emotional attachment with others but fail to express it by prosocial helping behavior with a fear of rejection. They try to engage themselves in a protective and avoidance behaviours which can ultimately results in lowered empathetic response. However, it was suggested that to eliminate this fear of social disapproval, lonely and isolation must be brought back to life with complemented with any kind of social reward. This can motivate them to improve their social abilities, cognition, emotional and empathy regulation (Thompson et al., 2022).

Chapter 3

MATERIALS AND METHODS

3.1 Ethical Statement

The laboratory animal house of Atta ur Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST) housed the animals under regulated environment. Animals were kept under standard housing conditions by providing feed, distilled water and 12- hour light/dark cycle. The rat room had controlled temperature of 25 ± 2 ºC. Animals were fed regular food made up of 4% crude fibre, 9% crude fat 30% crude protein and 10% moisture. This study has been approved by Institute Review Board at ASAB, NUST (IRB# 135). Each experiment was conducted in accordance with the principles established by the Institute of Laboratory Animal Research, Division on Earth and Life sciences, National Institute of Health, USA (National Research Council Committee for the Update of the Guide for the & Use of Laboratory, 2011).

3.2 Chemicals

Al chloride hexahydrate ($AlCl_3$•$6H_2O$) purchased from Sigma Aldrich Company.

3.3 Study Design

An 80 days protocol was designed to evaluate the effect of Al exposure and isolation on empathy. The body weight of 6-8 weeks rats was maintained at 200-

300mg and water uptake at a steady rate before the start of experiment. In phase I, the animals were divided into 2 groups: The experimental group was given $AlCl_3$ in drinking water whereas the control group was given distilled water. Behaviour tests were performed at two time intervals i.e. 20 days and 40 to access short term and long term effect of metal toxicity. Similar protocol was followed for phase II in which the effect of isolation was simultaneously tested with metal exposure. The animals were divided into 2 groups: rats in experimental group kept in isolation and given Al water. Similarly, the control for isolation group were given distilled water.

3.4 In vivo Aluminum Chloride administration

Female Wistar rats of 7-10 weeks (n=40; weight 180-220 gm) were used in this study. Each animal cage (40cm×25 cm×15 cm), containing 5 animals were used for assessing effect of Al exposure on empathetic behavior. The rats were divided into following 4 groups. The control and subject groups were given distilled water. Whereas the metal exposed groups were given 80 mg/kg dose of Al dissolved in distilled water. The dose of 80 mg/kg means that for every kilogram weight of animal, the amount of Al intake should be 80 mg/Kg. Prior to the initiation of experiment, daily water intake and weights of rats was measured in order to calculate dose. The detail of treatment and animal groups is provided in Table 3.1 and Figure 3.1 and 3.2.

Table 3.1: Description and housing condition of experimental groups.

S. No	Group name	Description	Housing condition	Number of animals
1	Control	No $AlCl_3$ exposure for 40 days	Grouped	10
2	Al exposed	$AlCl_3$ (80mg/kg) given in drinking for 40 days	Grouped	10
3	Isolation only	No $AlCl_3$ exposure for 40 days	Isolation	10
4	Al exposed + isolation	$AlCl_3$ + isolation for 40 days	Isolation	10
5	Subject	Subject rats were enclosed in restrainer to evaluate empathy score of experimental and control animals in EBT.		

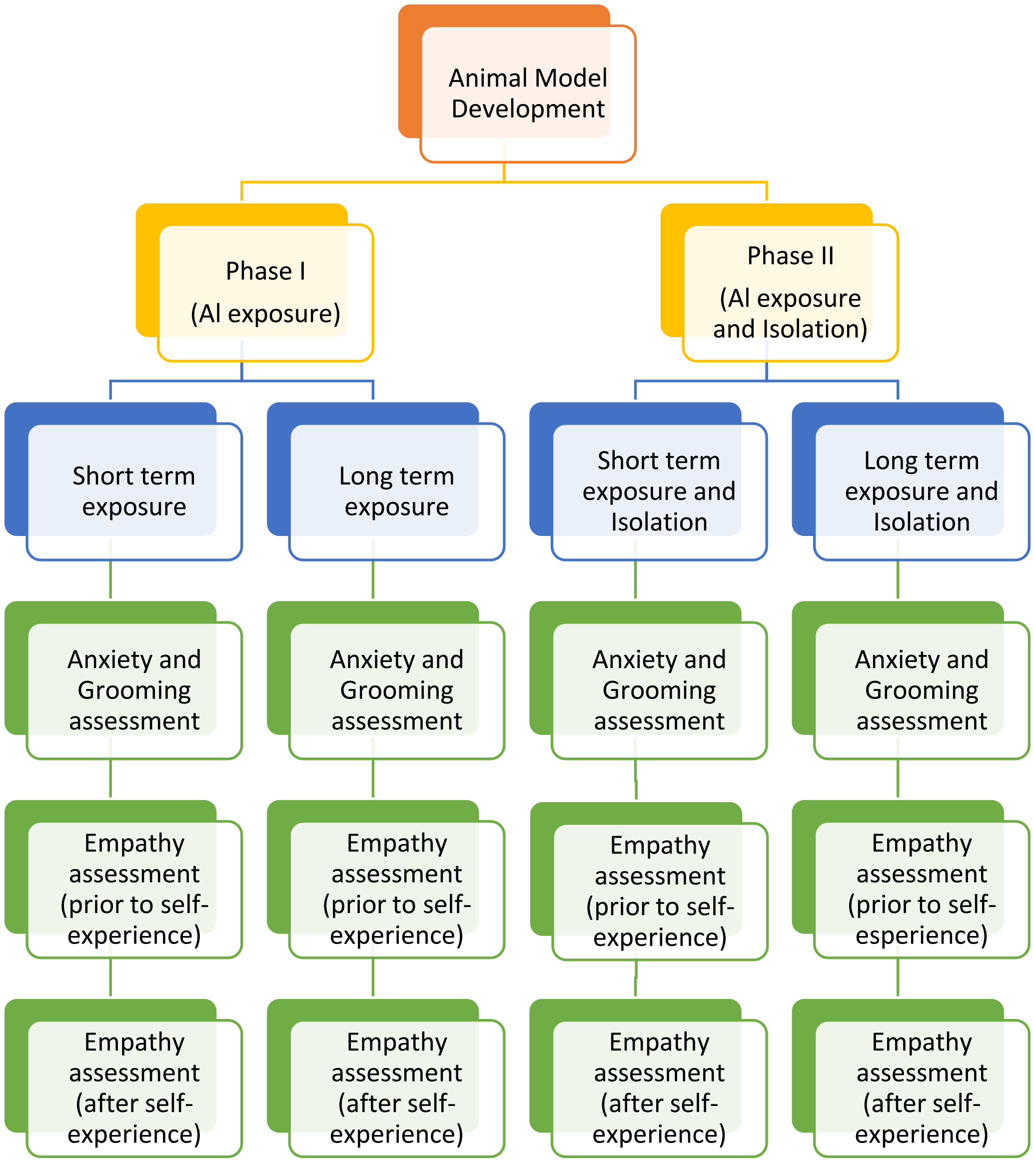


Figure 3.1: Workflow of behavioral tests in phase I (Al exposure phase) and phase II (Al exposure + isolation phase).

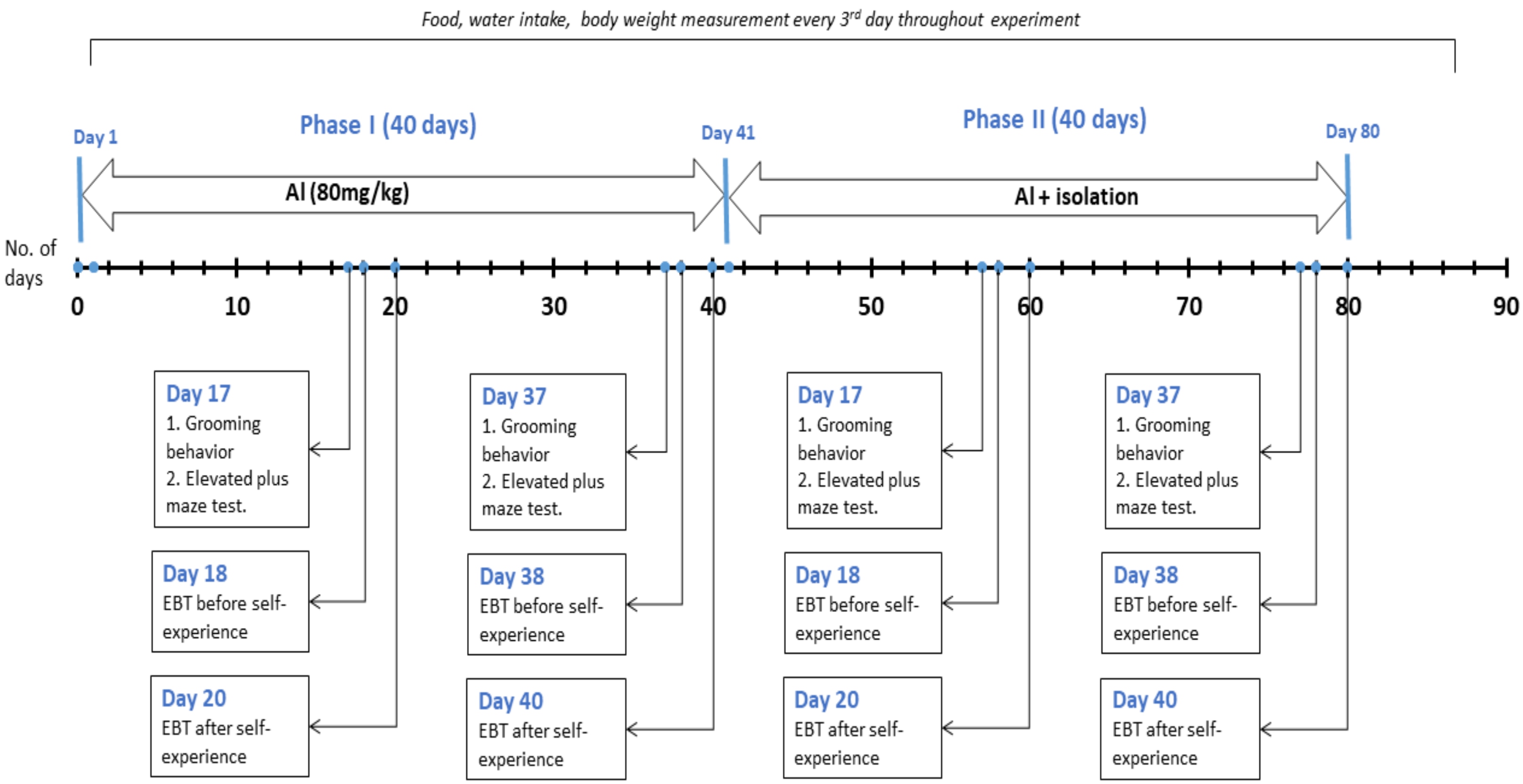


Figure 3.2: Timeline of behavioral tests in phase I (Al exposure phase) and phase II (Al exposure + isolation phase).

3.5 Body weight and water intake measurements

The body weight was measured twice a week for the entire treatment duration. Similarly, water intake was monitored on daily basis in order to regulate the metal dosage according to the variations.

3.6 Behavioral tests

Behavioural tests were carried out during the light cycle of rats i.e. between 9am to 6pm, in order to avoid any variability caused of circadian rhythm. The animals were habituated in a separate behaviour room that was well lighted and was regulated at 22 ± 2 ºC. Environmental disturbance or human interference like noise disruption were kept to minimum. Each behaviour test was either performed on separate days or at least by a time gap of 2 hours to avoid any biased results.

3.6.1. Anxiety assessment behavior

The EPM is extensively used to analyse degree of anxiety in rodents. The primary aim is to determine the avoidance behaviour towards open spaces (Figure 3.3). Rodents tend to remain in closed spaces and avoid open arms when in state of anxiety. The protocol was same as explained previously (Arendash et al., 2004), however few modifications were made. The apparatus used in this test consisted of plus shaped maze made of alloy. It consisted of two open arms (50.5 × 10cm) and two closed arms (50.5 × 10 x 49.5 cm) which were connected with central platform (10 ×10cm). The apparatus was 75.5cm high from the ground. Each rat was placed on the centre arena with its head facing to one of the closed arms and provided free access to all arms. The rat was then allowed to explore the apparatus for 5 mins. The behavior was recorded on camera and analysed later to calculate the following parameters:

a. Number of entries into the open arm.

b. The time spent in open arm.

EPM was performed on day 17 and day 37 of Phase I and II both. A single arm entry was counted if two of the animal's paws and more than half of its body were in the respective arm. To minimize skewed results due to olfactory cues, the apparatus was cleaned with 70% ethanol after each trial.

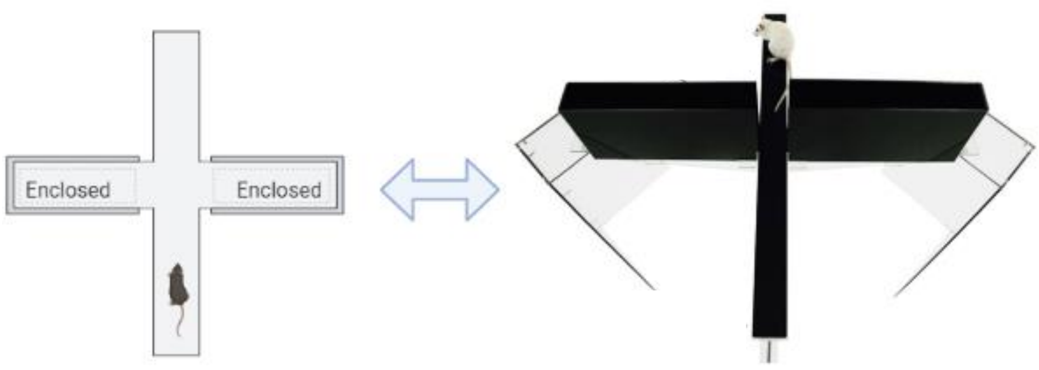


Figure 3.3: Diagrammatic representation of elevated plus maze.

3.6.2. Grooming assessment behaviour

Grooming behaviour is widely used in ethological studies to assess animal stress, anxiety, and depression as the grooming pattern and duration is highly sensitive to stress conditions. The spray test was used to artificially induce grooming in rats (Figure 3.4). For performing spray test, same protocol was used as explained previously by (Shiota et al., 2016), however few modifications were made. The rat was placed in square opaque iron alloy box (40 × 40 × 40 cm) and allowed to habituate for 10 mins. A spray bottle was filled with distilled water (room temperature). The spray nozzle was then directed 20–30 cm away toward the rat and sprayed from above eight times to adequately coat the rat's dorsal surface with mist. Grooming behaviour was recorded for 10 min using a video camera. The following parameters were assessed:

a. Number of correct grooming bouts.
b. Duration of correct grooming bouts.
c. Number of incorrect grooming bouts.
d. Duration of incorrect grooming bouts.
e. Latency to start grooming.

Grooming bout refers to an uninterrupted grooming within which the animal is grooming the body region in a defined and specific pattern in four phases (i.e. the nose, the face, and the head, followed by body licking) (Shiota et al., 2016).

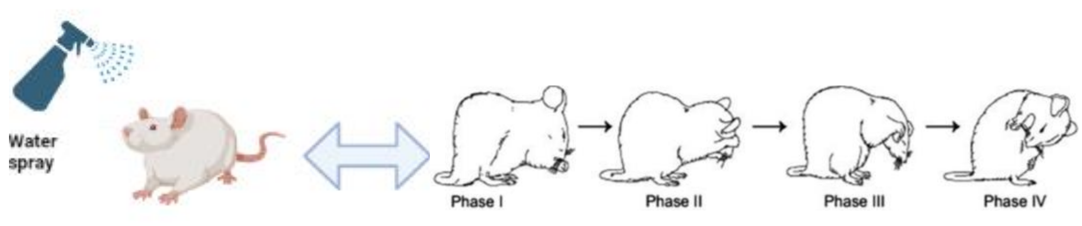


Figure 3.4: Diagrammatic representation of spray test and syntactic pattern of grooming behavior in rats.

3.6.3. Empathy assessment behaviour

The empathy behaviour test was used to investigate empathetic behaviour in rodents on observing conspecific in distress. A modified procedure of protocol used by Mellanie et al. was carried out to measure empathy response (Mellanie et al., 2019). The empathy scores were measured based on a 3 day protocol at two time intervals.

a. Day 18/38: Empathy behaviour testing before self-experience of restraint stress.

b. Day 19/39: Observer rats subjected to restraint stress for 2 hours for self-experience.

c. Day 20/40: Empathy behaviour testing 24 hours after self-experience of restraint stress.

3.6.3.1. Conspecific under restrainer stress

A conspecific was subjected to restraint stress in a transparent plastic container (20 cm length x 6.5 cm diameter) with 1cm holes at short gaps. The subject rat remained in restraint stress for 20 min prior to testing phase.

3.6.3.2. Habituation

A square shaped arena (40 x 40 x 40cm) was used for empathy behaviour test. It was divided into proximal and distal area by drawing a boundary. The observer rat was allowed to explore the box for 20 min. Its locomotor activity was accessed by calculating the time spent in central arena as compared to the distal ends of the box. The time spent in central area during 1st and last five mins of the habituation phase was noted.

3.6.3.3. Testing phase

The restraining bottle having the subject rat was placed in the centre of the arena with the observer rat for next 20 min (Figure 3.5). The subject rats restrained in the perforated plastic container were new to the observer rats. Empathy behaviour was monitored using a video recording. The following parameters were assessed throughout the testing phase:

a. **Number of interaction:** Interactions through holes to help subject rat in distress. (An interaction was counted when the observer rat tried to jab the hole with hands or mouth).
b. **Number of climbings:** Number of attempts to climb the bottle to rescue conspecific in restrainer. (A climbing was counted if at least half of the body including forelimbs of the observer rat was above the perforated restrainer).
c. **Freezing behavior:** Number and duration of freezing to evaluate fear response in rats after observing a subject in distress. (A freezing was counted as an episode of immobility for more than 5 seconds).
d. **Time spent in close proximity:** Time spent in close proximity to subject rat (during first 5 and last 5 mins of both phases of EBT). Moreover, the locomotor activity was compared to that of the habituation phase when no conspecific under stress was placed in the box.

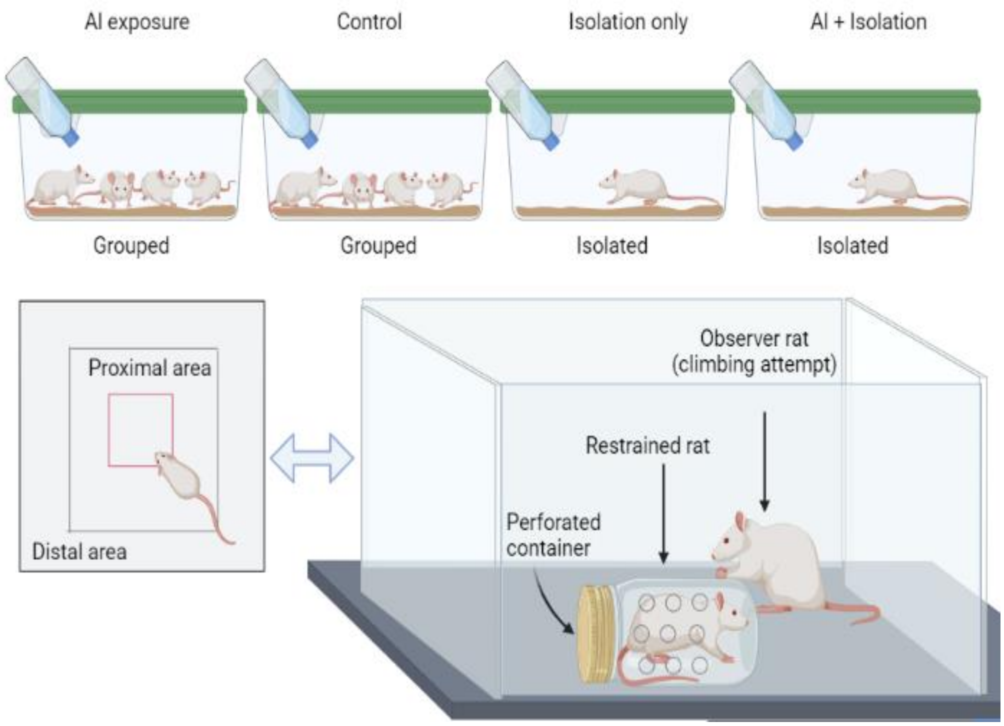


Figure 3.5: Diagrammatic representation of empathy behavior test.

3.7 Statistical Analysis

GraphPad Prism (Version 8.0) was used to conduct statistical analysis. The statistical tests applied to analyze the data were, Two-Way ANOVA followed by Bonferroni's multiple comparison test. It was noted that the significant p-value was less than 0.05. Data was presented as mean ± standard error of mean (SEM).

Chapter 4

RESULTS

4.1 Effect of Al exposure on water intake

The water intake was monitored regularly on daily basis and Al dosage was adjusted accordingly (Figure 4.1). It was observed that during the 1st two weeks of experimental trial, the water intake of Al exposed rats did not increase. However, the animals got habituated to Al water soon after that and a gradual increase in water intake was observed till the last week of experimental trial. Overall, the water intake of animals on distilled water was more than that of Al exposed.

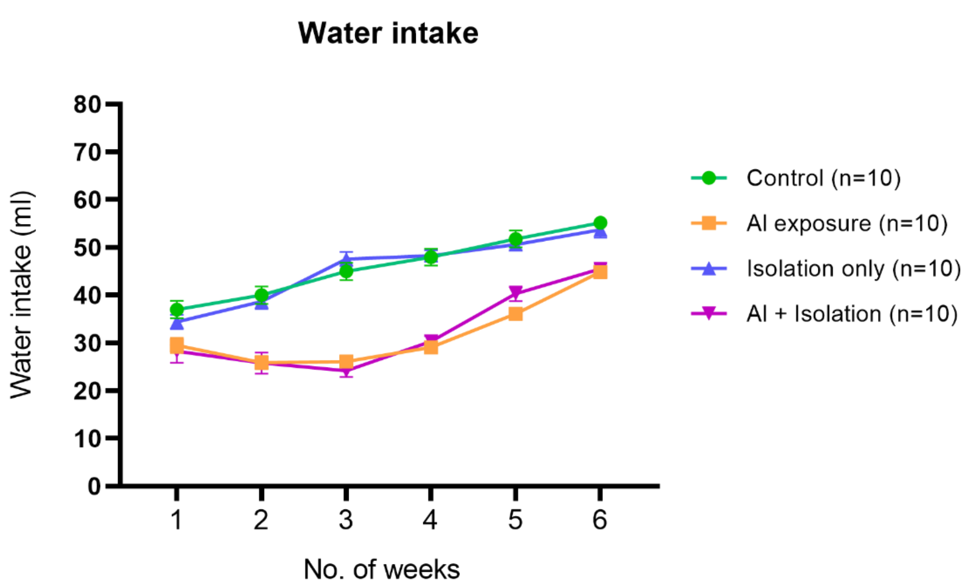
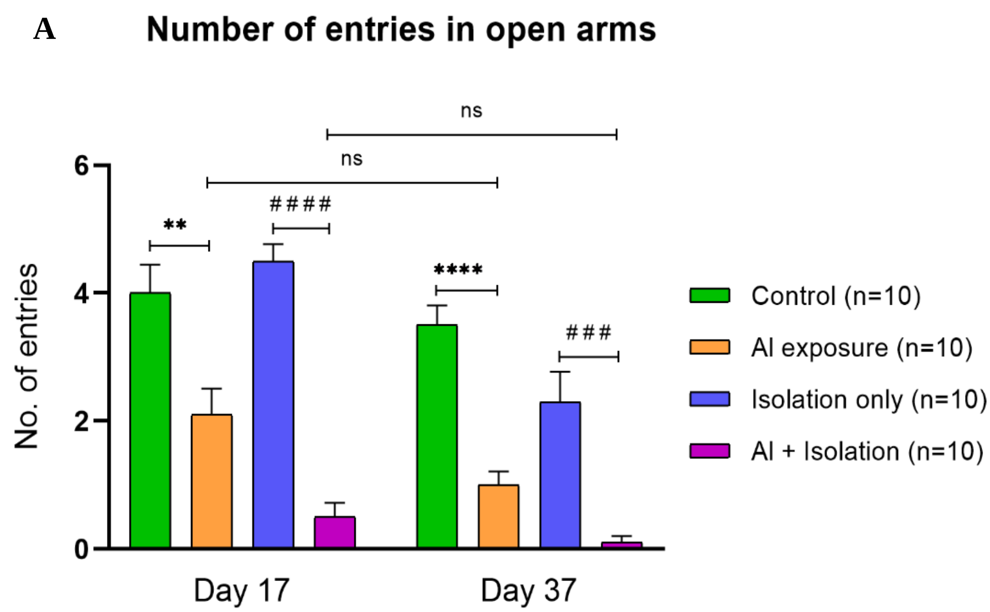


Figure 4.1: Effect of Al exposure on water intake. The water intake of Al exposed animals was less than that on distilled water. A steady increase in water intake was observed after 2 weeks of adjustment to Al water.

4.2 Al exposure and social isolation increase anxiety

The EPM was performed to evaluate the anxiety levels in observer rats and establish a trend with empathetic behavior. The animals in state of anxiety tend to spend more time in closed arms of EPM. As depicted by figure 4.2A, the Al exposed group (2.10 ± 0.40) made significantly less ($p < 0.01$) number of entries in open arm on day 17 as compared to the Control (4 ± 0.44). Similarly after long term Al exposure, the difference between these groups is significantly high ($p < 0.0001$). On the other hand the isolation only group (4.50 ± 0.26) made significantly more number of entries than the Al + isolation (0.20 ± 0.22) group on day 17 ($p < 0.0001$) as well as day 37 ($p < 0.001$). Further, the difference between time spent (Figure 4.2B) by isolation only group (31.600 ± 0.80) was highly significant ($p < 0.0001$) as compared to Al + isolation group (3.20 ± 1.34) on day 17. Similar results were observed between Al exposed group (3.60 ± 0.99) and Control (21.20 ± 2.03) on day 37. Overall, the results indicate that anxiety levels increases in case of both Al exposure and isolation.

A



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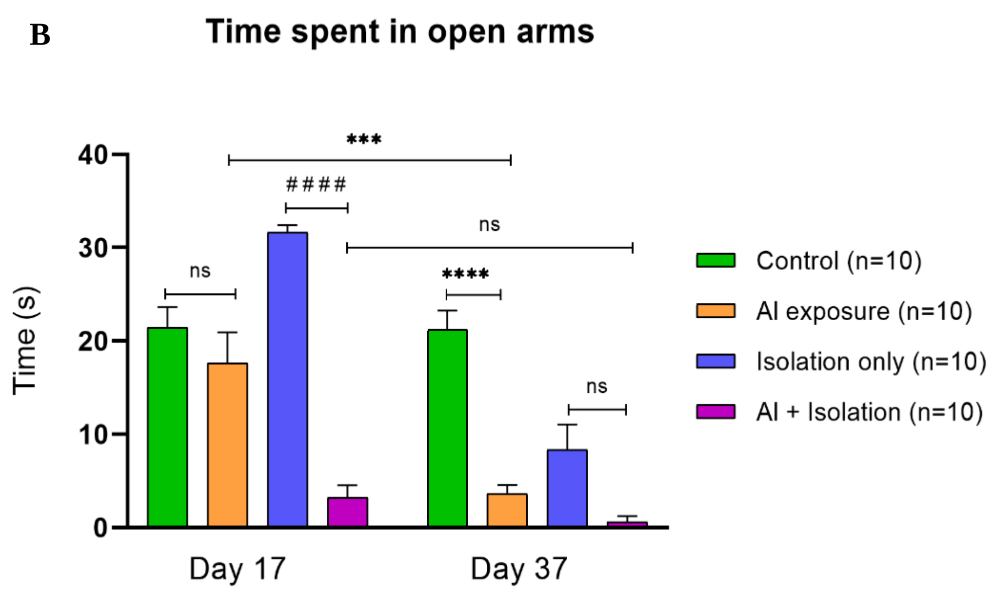

Figure 4.2: The effect of Al exposure and isolation on (A) The number of entries in open arm of EPM. (B) The time spent in open arm of EPM. Error bars are represented as mean ± SEM, for Two- way ANOVA, followed by Bonferroni's multiple comparison test using Graphpad Prism. n.s = p> 0.05, *=p<0.05, **= p< 0.01, ***=p< 0.001 and ****= p< 0.0001 are the significance values for Al exposure group and # =p<0.05, # #= p< 0.01, # # # =p< 0.001 and # # # #= p< 0.0001 represents p value for Al + isolation group.

4.3 Al exposure and social isolation increases stress like symptoms

Like anxiety, stress condition was also clearly observed to have a direct link with Al exposure and isolation. Animals under stress showed delayed and interrupted grooming. The Figure 4.3A indicate that the difference of correct grooming bouts between Al exposed (6.40 ± 0.45) and Al + isolation groups (2.80 ± 0.41) was nonsignificant with their respective controls on day 17. However, on day 37, the Control (7.60 ± 0.60) made significantly more ($p < 0.0001$) number of correct grooming bouts as compared to Al exposed group (3.20 ± 0.29). The significant difference ($p < 0.01$) difference between Al exposed group on day 17 and day 37 also indicate that long term exposure to Al metal causes elevated stress like symptoms. Next the duration of correct grooming bouts was evaluated and similar result were observed (Figure 4.3B). The difference between time spent correct grooming by Al exposed group was significantly high ($p < 0.0001$) on day 37 (47.80 ± 1.96) as compared to that on day 17 (139.30 ± 4.91) and Control on day 37 (152.60 ± 8.67). Moreover, the Al + isolation group (12.90 ± 4.15) also spent significantly less time in correct grooming as compared to isolation only (43.90 ± 3.53) on day 17 ($p < 0.001$). Similar results were obtained on day 37 ($p < 0.01$). The interrupted grooming was considered as the one that did not follow the correct grooming pattern. Figure 4.3C and figure 4.3D shows that overall the number and duration of incorrect grooming was high in all the 3 groups except Control. On day 17, the number of incorrect grooming bouts were significantly high ($p < 0.01$) in Al exposed group (2.10 ± 0.23) as compared to the Control (0.20 ± 0.13). Moreover, the significant difference further increased on day 37 (p<

0.0001). Similarly, in case of Al + isolation group, the number of incorrect grooming bouts were significantly ($p < 0.0001$) high on day 37 (5.70 ± 0.36) than that on day 17 (2.50 ± 0.34). The exactly same pattern of significance was observed when time taken for each of the incorrect grooming bouts was added to calculate the total incorrect grooming duration (Figure 4.3D).

Finally, the latency to start grooming (Figure 4.3E) just after the water spray was evaluated as an indicator to stress response. Later the animal starts grooming, greater is the stress level. It was observed that the Al exposed group (170.50 ± 16.02) started grooming significantly late ($p < 0.0001$) as compared to Control (18 ± 7.68) on day 17. Similar results were obtained day 37 ($p < 0.0001$). The latency to start grooming was also significantly different ($p < 0.0001$) between Al + isolation group (342 ± 8.13) and isolation only (135.50 ± 7.28) on day 17. Whereas, on day 37 the significant difference was ($p < 0.001$) and latency by isolation only group (284 ± 12.22) has further increased signifying that isolated animals were in under high stress. Long term Al exposure also increases stress level significantly ($p < 0.01$).

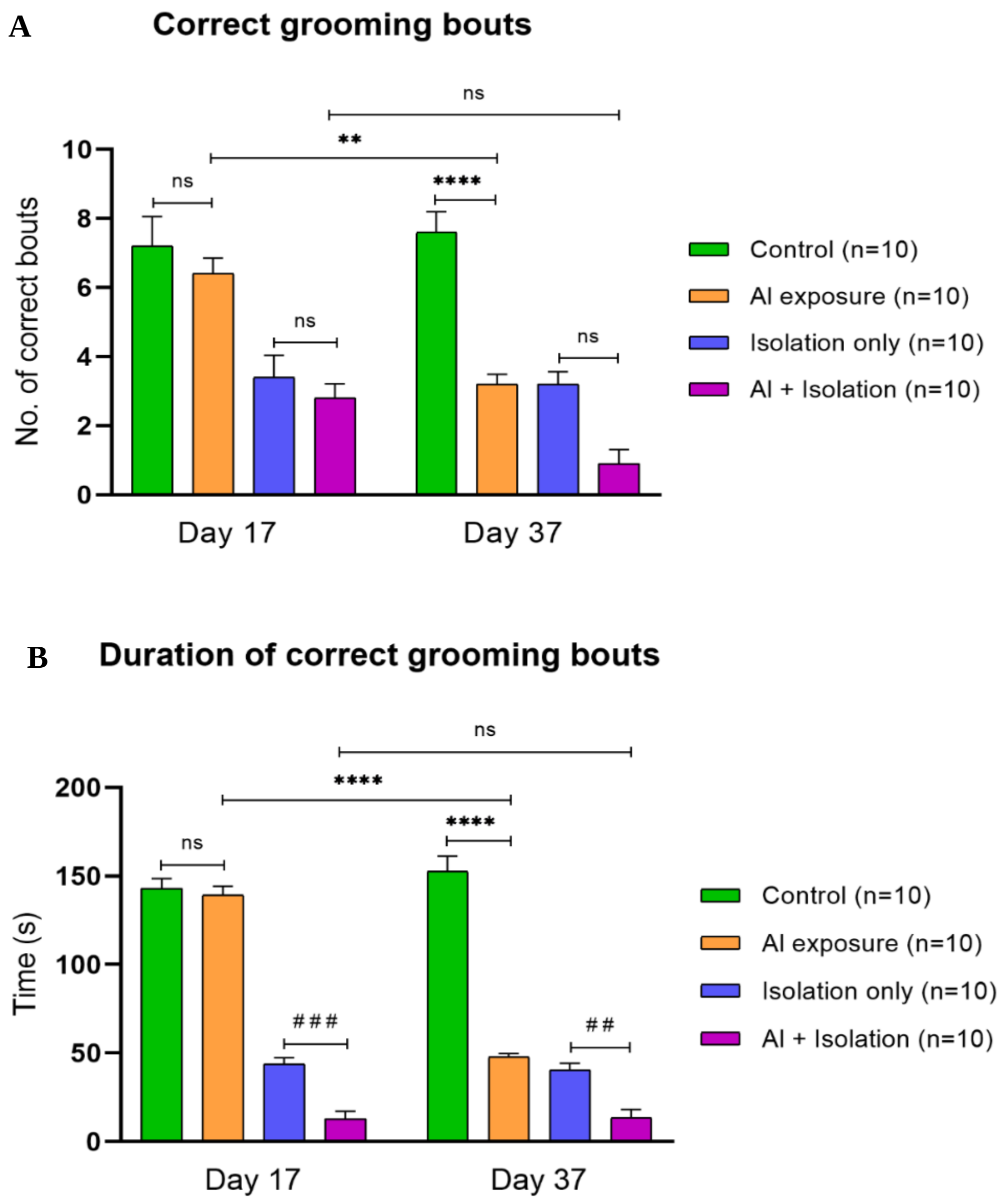

Figure 4.3: Effect of Al exposure on stress. (A) Number of correct grooming bouts (B) Duration of correct grooming bouts. Error bars are represented as mean ± SEM, for Two- way ANOVA, followed by Bonferroni's multiple comparison test using Graphpad Prism. n.s = p> 0.05, *=p<0.05, **= p< 0.01, ***=p< 0.001 and ****= p< 0.0001 are the significance values for Al exposure group and # =p<0.05, # #= p< 0.01, # # # =p< 0.001 and # # # # = p< 0.0001 for Al+ Isolation group.

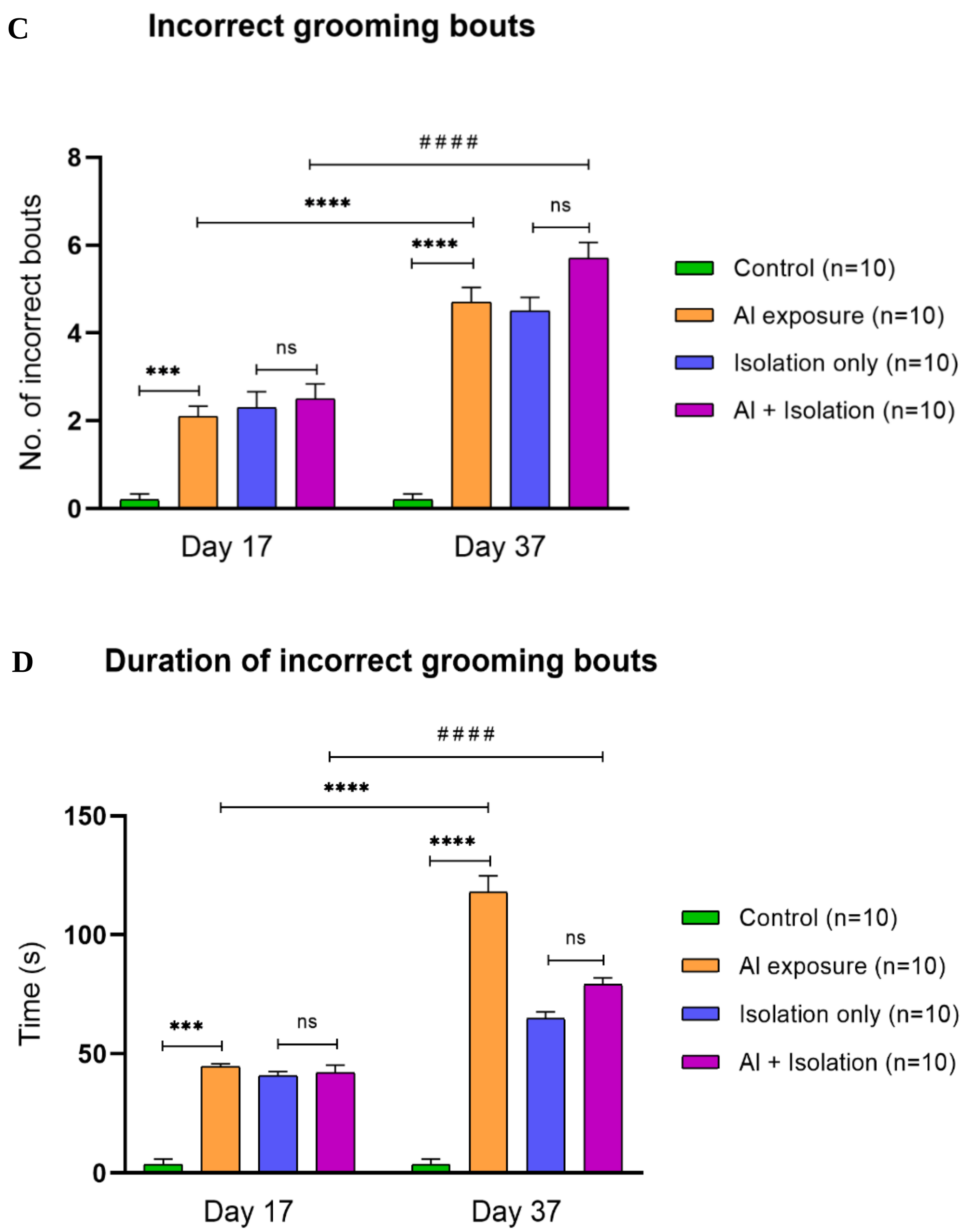

Figure 4.3: Effect of Al exposure on stress. (C) Number of incorrect grooming bouts (D) Duration of incorrect grooming bouts. Error bars are represented as mean ± SEM, for Two- way ANOVA, followed by Bonferroni's multiple comparison test using Graphpad Prism. n.s = p> 0.05, *=p<0.05, **= p< 0.01, ***=p< 0.001 and ****= p< 0.0001 are the significance values for Al exposure group and # =p<0.05, # #= p< 0.01, # # # =p< 0.001 and # # # # = p< 0.0001 for Al+ Isolation group.

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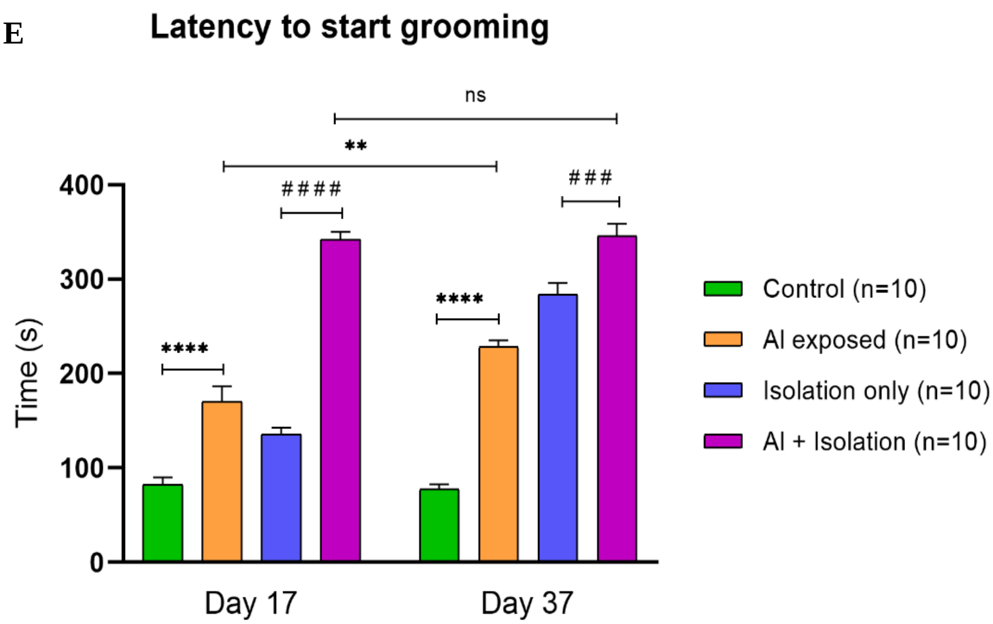


Figure 4.3 Effect of Al exposure and isolation on stress: (E) Latency to start grooming. Error bars are represented as mean ± SEM, for Two- way ANOVA, followed by Bonferroni's multiple comparison test using Graphpad Prism. n.s = p> 0.05, *=p<0.05, **= p< 0.01, ***=p< 0.001 and ****= p< 0.0001 are the significance values for Al exposure group and # =p<0.05, # #= p< 0.01, # # # =p< 0.001 and # # # # = p< 0.0001 for Al+ Isolation group.

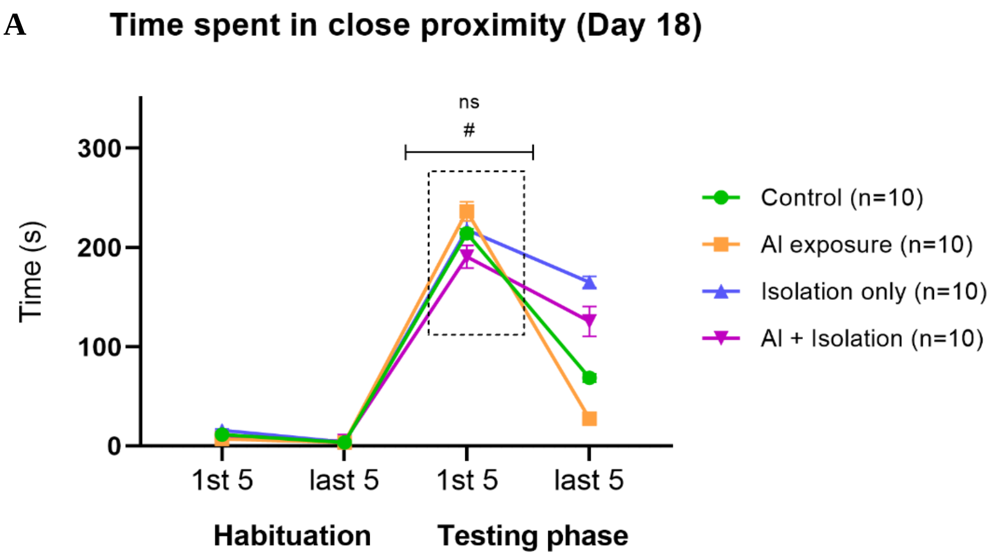
4.4 Al intoxication induce less empathetic nature

The animals exposed to Al in drinking water expressed fairly less empathy on observing conspecific in distress. All the animals spent more time in central arena of the box during testing phase but not habituation. The empathetic response was observed to be highest during the first five mins of testing phase. As depicted by figure 4.4A, the Al + isolation group (190.60 ± 11.33) spent significantly less ($p<0.05$) time in close proximity to the subject rat in distress as compared to the isolation only group (217.800 ± 8.880) on day 18. Similarly, on day 38 (Figure 4.4B) the time spent by Al exposed group (176.90 ± 7.27) was significantly less ($p<0.05$) than that of Control (213 ± 6.48).

For further evaluation, the number of interactions through holes made by observer rat were calculated. As shown by Figure 4.4C the Al exposed group (28.80 ± 2.42) made less number of interactions than Control (51.40 ± 1.37) on day 18. However, there was not a significant difference. But on day 38 the interactions by Al exposed group (21.40 ± 0.61) were significantly reduced ($p< 0.0001$) as compared to the Control (48.20 ± 2.59). These findings suggest that short term exposure to Al is less destructive but long term exposure lead to substantial decline of empathy. Whereas, the difference between Al + isolation group and isolation only was non-significant, suggesting that isolation did not decrease empathetic behavior.

Next the number of attempts by observer rat to climb the bottle were estimated. As demonstrated by Figure 4.4D, overall the Control group made highest number of climbing with an aim to rescue the conspecific in distress. The climbings of Al exposed group (4.60 ± 0.68) was significantly less ($p < 0.01$) as compared to Control (11.20 ± 1.54) on day 18. Moreover, it further reduced on day 38 ($p < 0.0001$). The number of climbing by Al + isolation group (11.40 ± 0.90) was less than that of isolation only (15 ± 1.63) on day 18. However, there was no significant difference suggesting that isolated animals almost equally climbed the bottle to rescue the animal in distress.

A



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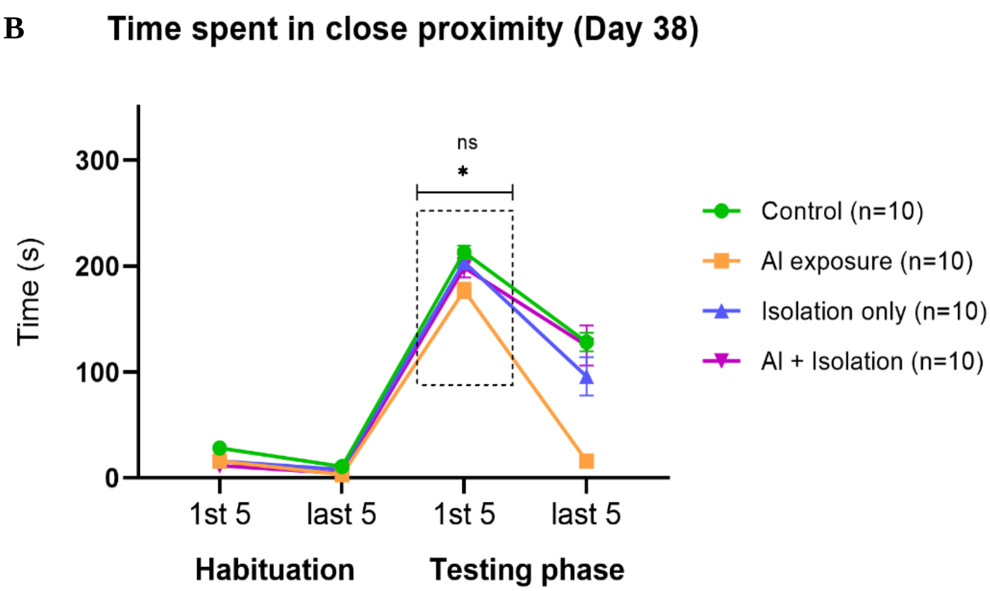

Figure 4.4 Short term and long term effect of Al exposure and isolation on empathy behavior: (A) Time spent in close proximity on day 18 of exposure. (B) Time spent in close proximity on day 38 of exposure. Error bars are represented as mean ± SEM, for Two- way ANOVA, followed by Bonferroni's multiple comparison test using Graphpad Prism. n.s = p> 0.05, *=p<0.05, **= p< 0.01, ***=p< 0.001 and ****= p< 0.0001 are the significance values for Al exposure group and # =p<0.05, # #= p< 0.01, # # # =p< 0.001 and # # # # = p< 0.0001 for Al+ Isolation group.

C

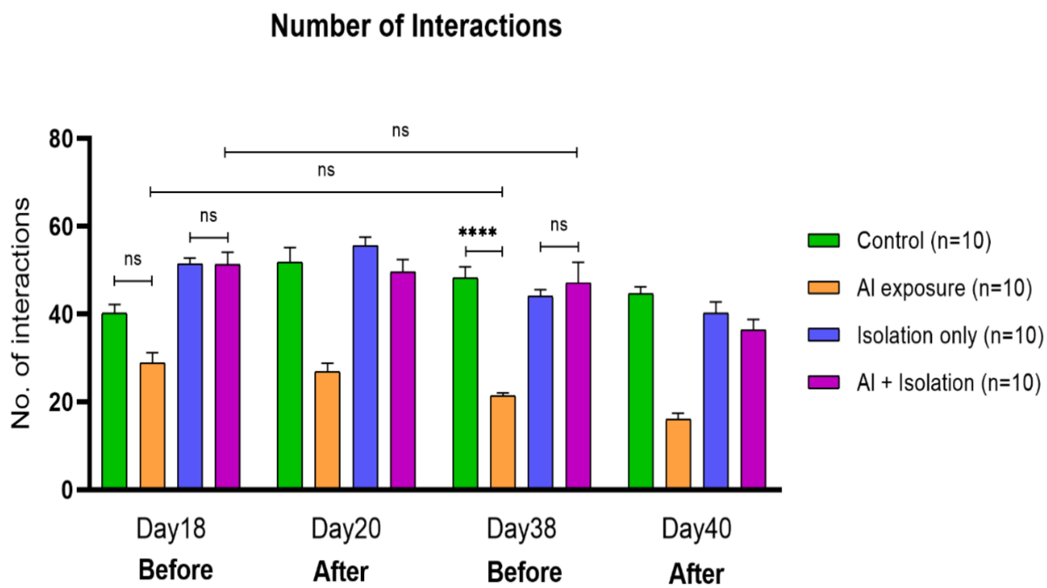

Figure 4.4 Short term and long term effect of Al exposure and isolation on empathy behavior: (C) Number of interactions before self- experience of distress. Error bars are represented as mean ± SEM, for Two- way ANOVA, followed by Bonferroni's multiple comparison test using Graphpad Prism. n.s = p> 0.05, *=p<0.05, **= p< 0.01, ***=p< 0.001 and ****= p< 0.0001 are the significance values for Al exposure group and # =p<0.05, # #= p< 0.01, # # # =p< 0.001 and # # # # = p< 0.0001 for Al+ Isolation group.

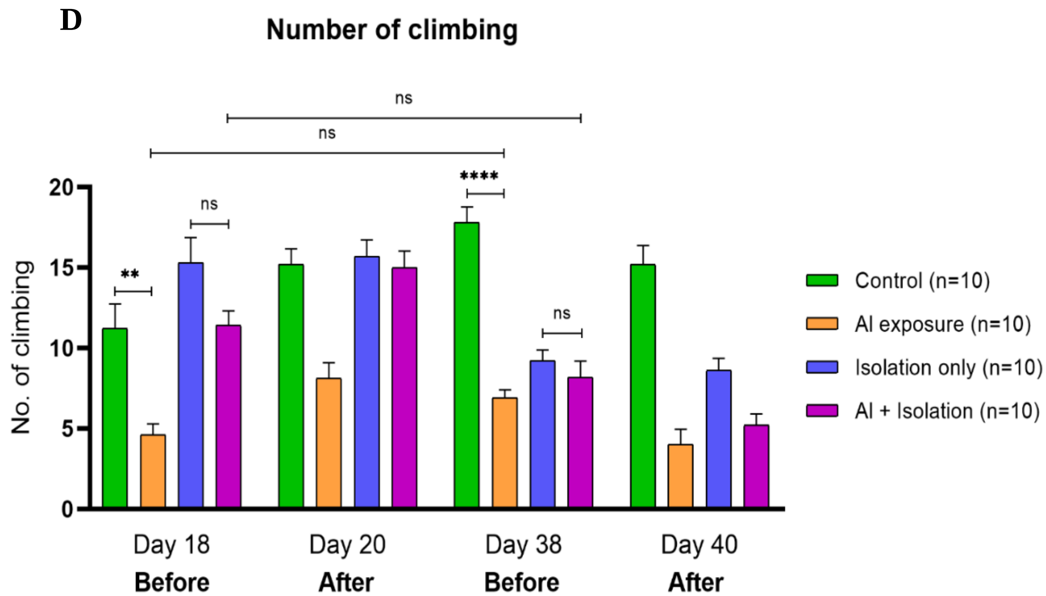

Figure 4.4 Short term and long term effect of Al exposure and isolation on empathy behavior: (D) Number of climbing before self- experience of distress. Error bars are represented as mean ± SEM, for Two- way ANOVA, followed by Bonferroni's multiple comparison test using Graphpad Prism. n.s = p> 0.05, *=p<0.05, **= p< 0.01, ***=p< 0.001 and ****= p< 0.0001 are the significance values for Al exposure group and # =p<0.05, # #= p< 0.01, # # # =p< 0.001 and # # # # = p< 0.0001 for Al+ Isolation group.

4.5 Empathy increases after self-experience of distress in controls but not in Al exposed and isolated animals

Empathy being an acquired trait, was assumed to be increased after self-experience of similar pain. Henceforth, all the groups were exposed to restrain stress 24-hours prior to next experimental trial. To evaluate empathetic behavior after passing through distress, EBT was again performed the next day respectively. As depicted by figure 4.5A, the difference between time spent in close proximity to subject rat by Al exposed group (181.80 ± 4.70) was significantly high ($p < 0.0001$) as compared to the Control (249.30 ± 11.62) on day 20. Similar results were obtained on day 40 ($p < 0.0001$). However, there was no significant difference between time spent by Al + isolation group and isolation only. This supports the fact that empathy improves after experiencing similar form of pain normally but not in case Al exposure.

The figure 4.5C clearly depicts that the difference between number of interactions made by Al exposed group (26.90 ± 1.92) was highly significant ($p < 0.0001$) than that of Control (55.60 ± 1.98) on day 20. Similarly, on day 40 ($p < 0.0001$) the Control (40.20 ± 2.56) made higher number of interactions with the subject rat after passing through a similar experience, but there was no increase in interactions of Al exposed group (16 ± 1.45). Another interesting finding was that though isolated groups expressed more empathy previously; there was no improvement in empathy after experience. The graph shows that there is no significant difference in Al + isolation group on day 18 (51.30 ± 2.82) vs day 20

(49.60 ±2.83) and day 38 (47.10 ± 4.73) vs day 40 (36.30 ± 2.44). Further evaluation of number of climbings (Figure 4.5D) revealed that there was a significant difference ($p < 0.01$) between Al exposed group (8.10 ± 0.99) and Control (15.20 ± 0.97) on day 20 and a highly significant difference ($p < 0.0001$) on day 40. Similarly, the Al + isolation group (5.20 ± 0.71) made significantly less ($p < 0.0001$) number of climbing as compared to isolation only group (8.60 ± 0.76) on day 40.

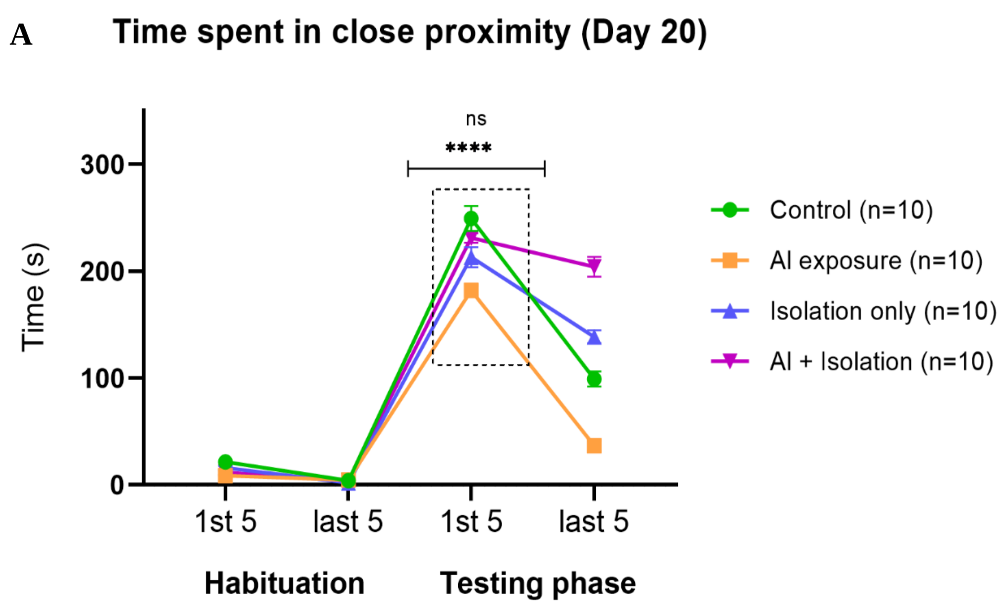

Figure 4.5 Effect of self-experience of distress on empathy behavior: (A) Time spent in close proximity on day 20 of exposure. Error bars are represented as mean ± SEM, for Two- way ANOVA, followed by Bonferroni's multiple comparison test using Graphpad Prism. n.s = p> 0.05, *=p<0.05, **= p< 0.01, ***=p< 0.001 and ****= p< 0.0001 are the significance values for Al exposure group and # =p<0.05, # #= p< 0.01, # # # =p< 0.001 and # # # # = p< 0.0001 for Al+ Isolation group.

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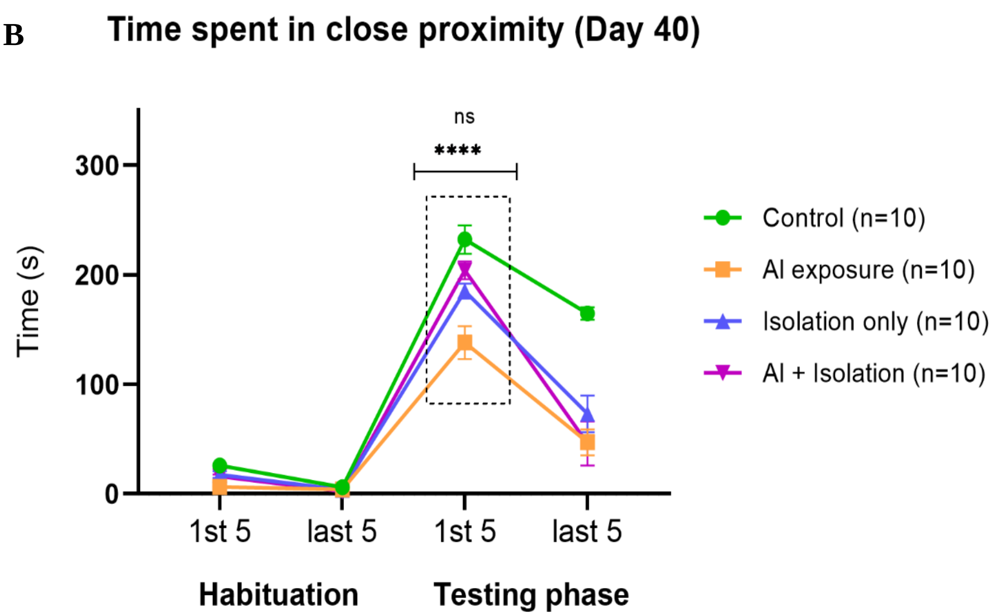

Figure 4.5 Effect of self-experience of distress on empathy behavior: (B) Time spent in close proximity on day 40 of exposure. Error bars are represented as mean ± SEM, for Two- way ANOVA, followed by Bonferroni's multiple comparison test using Graphpad Prism. n.s = p> 0.05, *=p<0.05, **= p< 0.01, ***=p< 0.001 and ****= p< 0.0001 are the significance values for Al exposure group and # =p<0.05, # #= p< 0.01, # # # =p< 0.001 and # # # # = p< 0.0001 for Al+ Isolation group.

C

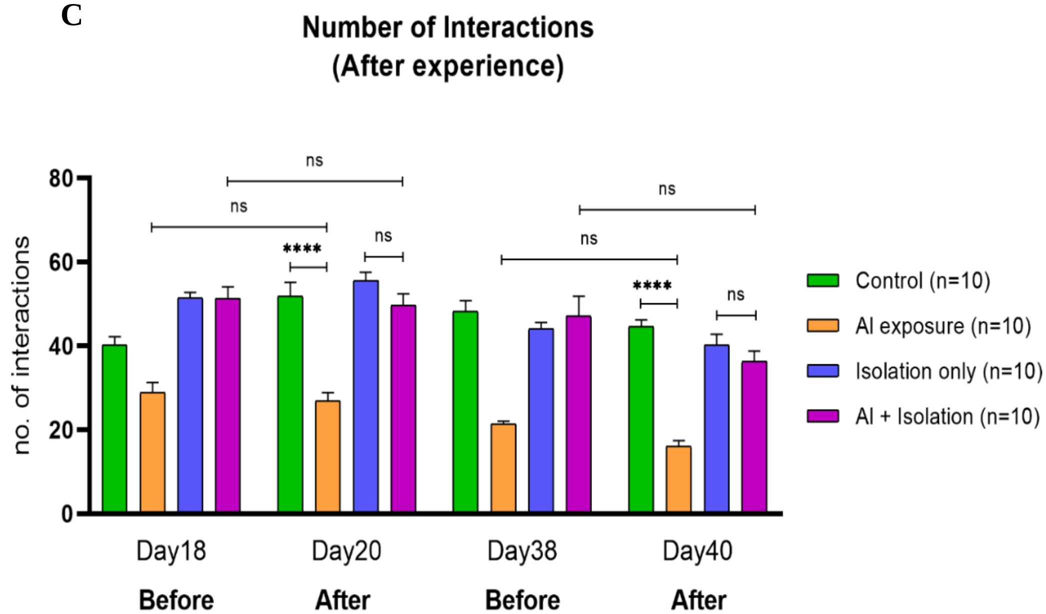

Figure 4.5 Effect of self-experience of distress on empathy behavior: (C) Number of interactions after self- experience of distress. Error bars are represented as mean ± SEM, for Two- way ANOVA, followed by Bonferroni's multiple comparison test using Graphpad Prism. n.s = p> 0.05, *=p<0.05, **= p< 0.01, ***=p< 0.001 and ****= p< 0.0001 are the significance values for Al exposure group and # =p<0.05, # #= p< 0.01, # # # =p< 0.001 and # # # # = p< 0.0001 for Al+ Isolation group.

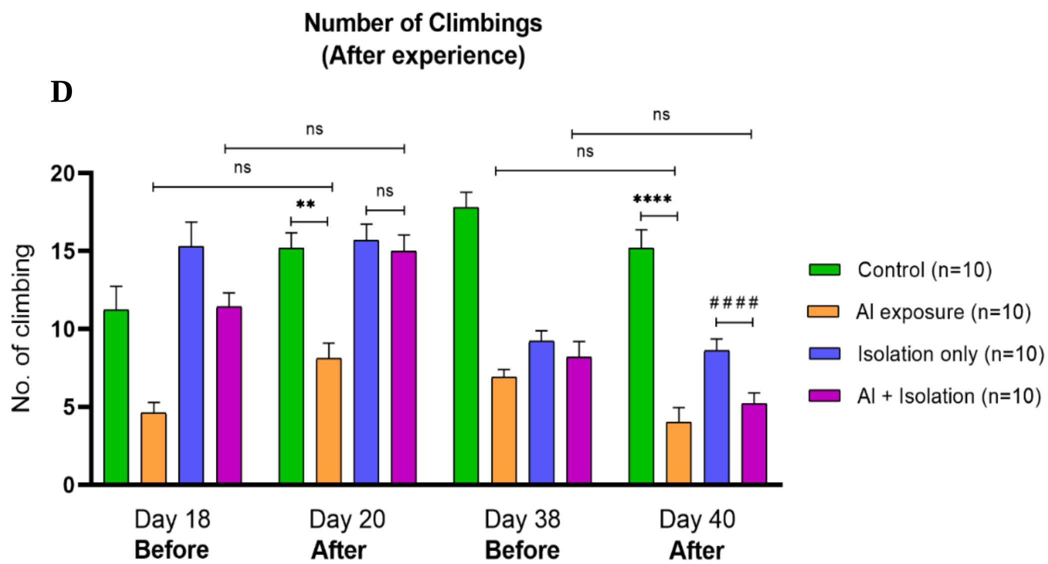

Figure 4.5 Effect of self-experience of distress on empathy behavior: (D) Number of climbing after self- experience of distress. Error bars are represented as mean ± SEM, for Two- way ANOVA, followed by Bonferroni's multiple comparison test using Graphpad Prism. n.s = p> 0.05, *=p<0.05, **= p< 0.01, ***=p< 0.001 and ****= p< 0.0001 are the significance values for Al exposure group and # =p<0.05, # #= p< 0.01, # # # =p< 0.001 and # # # # = p< 0.0001 for Al+ Isolation group.

4.6 Short term and long term Al exposure reduces fear response but long term isolation triggers excessive fear in rats

Freezing behavior was analyzed during the Empathy Behavior Test as a measure of fear response in rats on observing the conspecific in discomfort. The time and number of freezing were considered as a consequence of emotional empathy. Figure 4.6 depicts number of freezing before (A) and after (B) the self-experience to restrain stress. The difference between Al exposed group (0.90 ± 0.39) and Control (6.50 ± 0.40) before self-experience was highly significant ($p < 0.0001$) on day 18 as well as day 38. However, there was no significant difference among the isolated groups before experiencing self- pain. According to graph (B), the number of freezing increases significantly ($p<0.05$) after experience in long term isolation and but decreases ($p<0.05$) in short term Al exposure from their respective controls. Similarly, on day 40 the difference between Al exposed (0.60 ± 0.26) and Control (8.00 ± 0.63) is highly significant ($p<0.0001$).

The duration of each freezing episode was summed up to calculated freezing duration throughout the testing phase. Overall, the long term isolation only group spent the highest time in freezed condition. As shown in figure 4.6C, the difference between Al exposed (6 ± 2.56) vs Control (41.60 ± 0.49) was significantly high ($p<0.0001$) on day 18. Similar results were obtained on day 38. Similarly, the Al + isolation group (7.50 ± 2.14) spent significantly more ($p<0.0001$) time in freezing condition than isolation only (31.70 ± 2.41) on day 18 but not on day 38. The significant difference observed between Al + isolation group on day 18 (7.50 ± 2.14) and day 38 (23 ± 2.26) strongly validate the inverse relation of freezing between Al exposure and isolation.

The freezing duration after passing through similar distress increases in all the groups except that of long term Al exposure (Figure 4.6D). The difference between isolation only (46 ± 3.31) group and Al + isolation group (26 ± 2.76) was highly significant ($p < 0.0001$) in case of short term isolation after experience. Similar results were obtained in case of long term isolation ($p < 0.0001$). There was no significant difference between Control and Al + Control on day 20. However, the freezing duration of Al exposed group (5 ± 2.10) was significantly low as compared to Control (63 ± 1.70) on day 40 suggesting an extremely lowered emotional empathy in case of long term Al exposure. These findings also suggest that fear response tremendously raises in long term isolation that can be an indication of emotional contagion but at the same time may result in inadequate helping behavior.

A

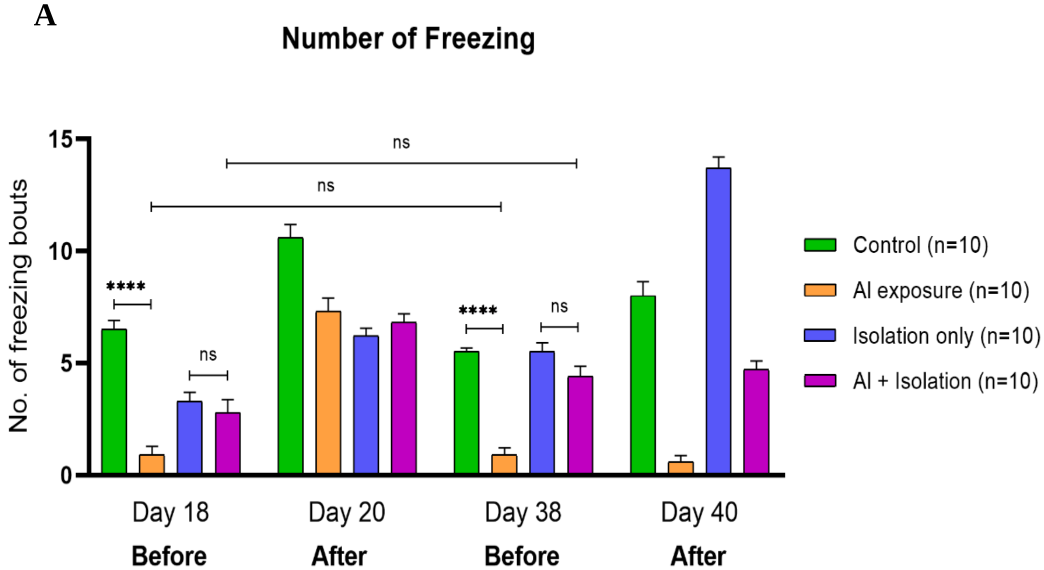

Figure 4.6 Effect of short term, long term and self-experience on freezing behavior. (A) Number of freezing before self- experience of distress. Error bars are represented as mean ± SEM, for Two- way ANOVA, followed by Bonferroni's multiple comparison test using Graphpad Prism. n.s = p> 0.05, *=p<0.05, **= p< 0.01, ***=p< 0.001 and ****= p< 0.0001 are the significance values for Al exposure group and # =p<0.05, # #= p< 0.01, # # # =p< 0.001 and # # # # = p< 0.0001 for Al+ Isolation group.

B

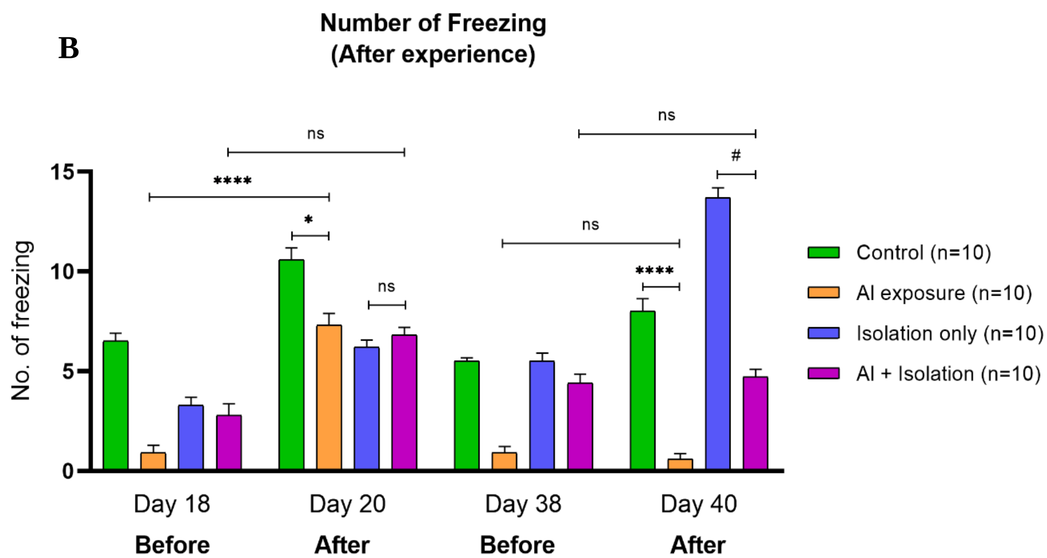

Figure 4.6 Effect of short term, long term and self-experience on freezing behavior. (B) Number of freezing after self- experience of distress. Error bars are represented as mean ± SEM, for Two- way ANOVA, followed by Bonferroni's multiple comparison test using Graphpad Prism. n.s = p> 0.05, *=p<0.05, **= p< 0.01, ***=p< 0.001 and ****= p< 0.0001 are the significance values for Al exposure group and # =p<0.05, # #= p< 0.01, # # # =p< 0.001 and # # # # = p< 0.0001 for Al+ Isolation group.

C

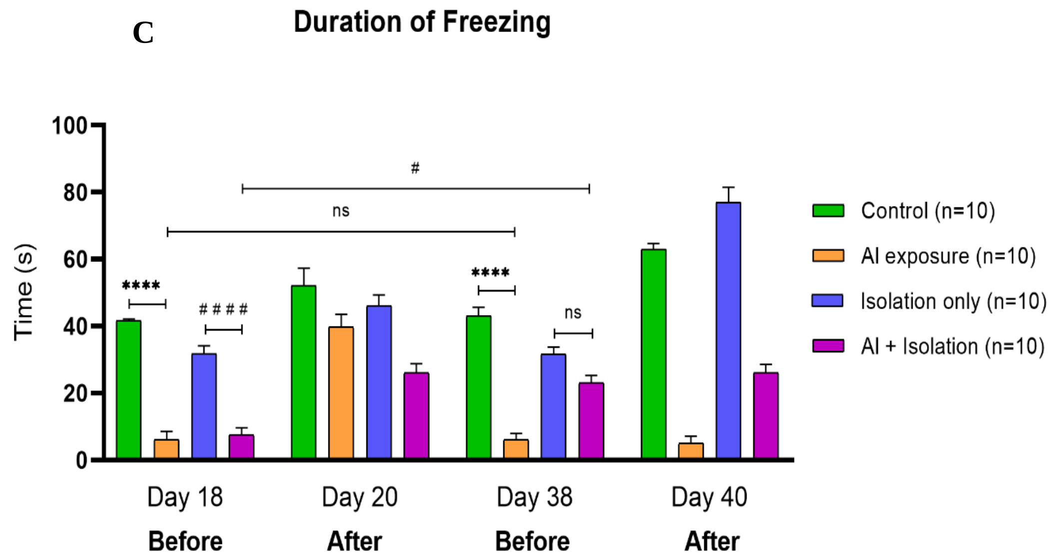

Figure 4.6 Effect of short term, long term and self-experience on freezing behavior. (C) Duration of freezing before self- experience of distress. Error bars are represented as mean ± SEM, for Two- way ANOVA, followed by Bonferroni's multiple comparison test using Graphpad Prism. n.s = p> 0.05, *=p<0.05, **= p< 0.01, ***=p< 0.001 and ****= p< 0.0001 are the significance values for Al exposure group and # =p<0.05, # #= p< 0.01, # # # =p< 0.001 and # # # # = p< 0.0001 for Al+ Isolation group.

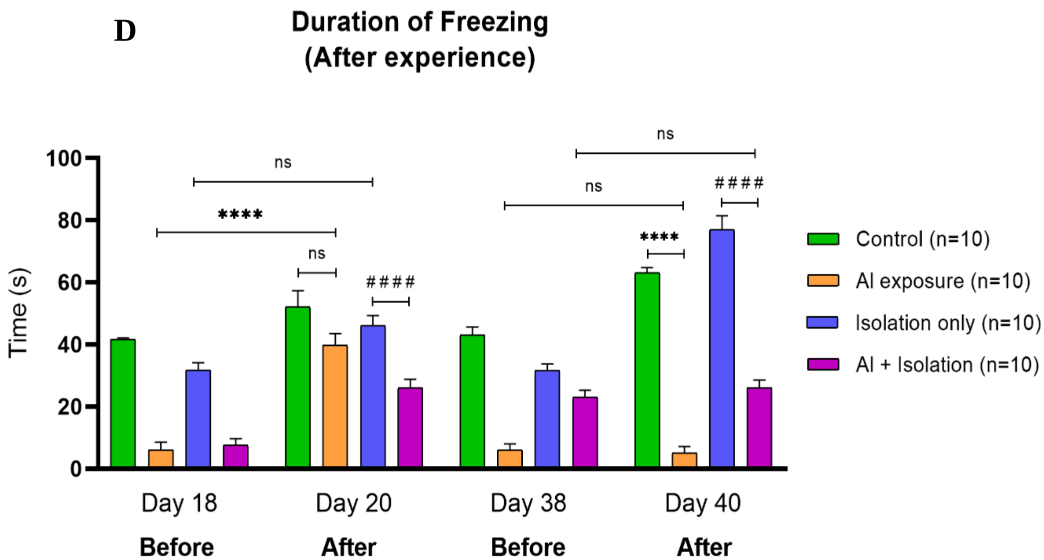

Figure 4.6 Effect of short term, long term and self-experience on freezing behavior. (D) Duration of freezing after self- experience of distress. Error bars are represented as mean ± SEM, for Two- way ANOVA, followed by Bonferroni's multiple comparison test using Graphpad Prism. n.s = p> 0.05, *=p<0.05, **= p< 0.01, ***=p< 0.001 and ****= p< 0.0001 are the significance values for Al exposure group and # =p<0.05, # #= p< 0.01, # # # =p< 0.001 and # # # # = p< 0.0001 for Al+ Isolation group.

Chapter 5

DISCUSSION

In present study, the role of social isolation, metal (Al) exposure on regulation of empathy behavior were investigated. The novel findings include: (1) Rodents exposed to Al toxicated drinking water exhibit less empathetic nature after a long-term exposure. (2) Cognitive empathy increases after passing through similar form of discomfort under normal conditions but not in case of Al exposure and isolation. (3) Isolation have a peculiar relation with empathy i.e. increases affective empathy but decreases cognitive empathy. (4) Al water and isolation tremendously increase anxiety and stress like symptoms.

According to studies in past years, it was believed that complex cognitive form of empathy only exist in primates like humans, monkeys, apes, etc. However, various recent studies have established cognition like consolation and prosocial helping behavior in different species of rodents including prairie vole (*Microtus ochrogaster*), Wister rat etc (Fontes et al., 2019; Knapska et al., 2009). Thus rodents are now considered as a reliable model to study empathetic behavior. The current study features attributes of emotional as well as cognitive empathy by an observer rat towards a conspecific under restraint stress. The findings were highly consistent with multiple other studies that employed electric footstock stressor method (Burkett et al., 2016; Cox et al., 2020). Other studies used the 3-chambered water tank apparatus to measure helping behavior (Virk et al., 2015). One of the strengths of this study was the use of restrainer stress model. It not only imitates the natural intricacy faced by rodents as compared to artificial stressor methods mentioned above, but also gives a better visual manifestation of being distressed to the observer rat. The observer maintained interaction with the trapped conspecific regardless of the fact that the rescue was unattainable. Furthermore, the study proves that the behavioural response was solely empathy based by eliminating the factor of exploratory activity, the behavioural trial was performed prior to as well as following self-experience to restrainer stress. It was evident that empathy level was profoundly increased after self-experience of distress in grouped animals on distilled water.

A growing body of evidence against Al salts causing neurodegeneration have been established so far. A comprehensive survey of literature on 13 reports was found significant by a meta-analysis proving cognitive deficits and increasing number of AD cases in areas where Al concentration in the municipal drinking water was high (Allsop et al., 2018). Though the exact neural mechanism of empathy in yet not clear, various studies have supported its link with amygdala, prefrontal cortex and Anterior Cingulate Cortex (ACC). A study proposed that ACC- Basolateral nucleus of amygdala (ACC-BLA) circuits are essentially related with observational learning and social interaction (Liu et al., 2020). This study found reduced empathetic response after long term Al exposure. Whereas the helping behavior like interactions through holes and climbing attempts further diminished after self-experience of distress. Intriguingly, it was also observed that though Al exposed animals were anxious and stressed, their fear response expressed by freezing behavior was minimal. All these findings suggest that Al toxicity has adversely affected brain regions controlling various sub-components of empathy. The results were in parallel with another study that identified oxidative stress and dopaminergic neurotoxicity induced by Nano-Al_2O_3 as impaired dopaminergic system is also one of the hallmarks of impaired empathy (Goes et al., 2018).

In EPM, anxiolytic behavior is observed by increased number of entries and increased activity in the open arm. Al exposure increases anxiety in rats thus decreases number of entries and time spent in open arms. In case of isolated rats, Al exposure further increased anxiety level comparable to that of its control group. In a review, Arakawa (2018) addressed some studies discovered that socially reared rats are less active and anxious than isolated rats (Arakawa et al., 2018; Juczewski et al., 2020). However, varying isolation methods, age of the rodents, sex differences, test durations, environmental changes, and technique employed can lead to contradiction in data about anxiety.

Grooming behavior has been suggested useful in the behavioral studies of stress (Bibancos et al., 2007). Rodents enter an emergency state in reaction to a threat to its homeostasis, which can be caused by a real or imagined threat (stressor) (Kalueff et al., 2004). In rodents, grooming is an evolutionary significant action that serves to maintain the physiological equilibrium, comfort, and appearance of the mouse. Additionally, mice exhibit this behavior when they are anxious since grooming reduces stress (Chrousos et al., 2009). In this study, grooming behavior was analyzed according to Smolinsky et al. criteria, which indicates that grooming in rats follows a cephalocaudal pattern and any interruption in this grooming pattern is caused by anxiety (Smolinsky et al., 2009). In grooming behavior test, number and duration of correct and incorrect grooming bouts were measured. Various studies after the emergence of Covid-19 have examined the relationship between depression, stress and anxiety levels in socially isolated individuals (Kalkan et al., 2021). The results of current study were in accordance with these studies as the isolated rats expressed extremely stressed and anxious grooming response.

Moreover, the rodents have a natural tendency to start grooming within a short time if drenched in water as seen by the control rats. On the other hand, all the isolated and Al exposed rats, showed an extended latency to start grooming further highlighting their stress symptom. Even though stress and anxiety share same neural circuitry (brain regions including the basolateral amygdala (BLA), medial prefrontal cortex (mPFC), locus coeruleus (LC), as well as reward processing areas such as the nucleus accumbens (NA), appear to be affected in animal models of both stress disorders and anxiety disorders) but difference in environmental condition may have influence such difference in behavior (Shimizu et al., 2016).

During empathy behavior test, different parameters were used that analyse helping tendency to aid the subject in distress. Empathy level was observed to elevate in case of isolation as the number of interactions and climbings on the restrainer containing subject was improved in both grouped and isolated rats. By this outcome it is conceivable that the motive for lonely persons to reduce their prosocial acts is their desire to avoid social rejection and a bad emotional outcome. However, lonely persons will be encouraged to perform more prosocially when prosocial acts are accompanied with social incentives (Hu et al., 2020). A recent study conducted on Canadian population evaluated empathy, sleep quality and mood changes in Covid 19 isolation phase. They proposed that in isolation, as anxiety develops, empathy increases predominantly in females than male participants (Guadagni et al., 2020). The results were quite similar with findings of current study in rodents.

Despite of the fact that empathy increased after short term isolation, yet another interesting result was observed after long term isolation. There was a significant increase in affective empathy in terms of observational fear defined by freezing behavior. Whereas, at the same time, the cognitive empathy was not improved after self- experience of distress. It is comprehended that the animals in state of extreme fear condition have more emotional contagion for the conspecific that narrowed their effort to help them. These results were supported by a recent review (2022) that differentially related cognitive and affective empathy with emotional regulation. In conclusive, increasing affective empathy beyond an extent develops a fear of helping others as in case of social isolation! (Zaki et al., 2012; Thompson et al., 2022).

It was also observed that the empathy behavior of the rats in this study increased after experiencing distress in both types of housing conditions. This goes parallel with a recent study in which the ability to get in touch with and assist other animal in distress was positively correlated with posttraumatic stress symptoms brought on by the unpleasant event (Vezzali et al., 2016). Fear is a subjective emotion that manifests as behavioral and physiological reactions to ominous environmental cues. Direct exposure to dangerous stimuli, such as an electric shock, sets off fear responses. Surprising anatomical, neurochemical, and behavioral connections exist between human emotional empathy and rodent observational terror (Kim et al., 2019). In this study, freezing behavior was increased after experience of distress which is supported by previous studies (Kim et al., 2019; Preis et al., 2012). Surprisingly the freezing response in isolated rats after experience decreased which might be because of the reason that long-term (24-h post conditioning) compared to short-term (15-min post conditioning) memory, vicarious terror memories were stronger in socially reared mice than in isolate mice according to a study (Panksepp et al., 2016).

According to a toxic metals collective hazard index (HI) analysis, the public in most locations of Pakistan is at high risk of heavy metals consumption through contamination of drinking water and air. The areas affected most by heavy metal contaminated water in Pakistan include the Central region of Khyber Pakhtunkhwa, the North and Central areas of Punjab, and the Southern area of Sindh. This signifies that others areas within the country are also at risk. Likewise, reports in some developed areas in US, Canada, Japan and Europe have also associated Al in tap water causing a number of health problems, particularly neuro-skeletal disorders and cognitive impairments (Cech et al., 2000).

This study proves that if Al contaminated water intake is prolonged beyond HI limits of the human body, a lack of empathetic nature would become a hallmark of society. The brain areas contributing to emotional response can be disrupted leading to neurodegenerative disorders where individuals are least socially interactive and compassionate towards each other. Thus, eliminating metal contamination from all the primary sources is essential for public health and a prosperous society.

CONCLUSION

The present study shows a strong association between Al contamination, isolation and empathetic behavior. The results of EPM, EBT and spray test indicate hyperactivity of ACC and inactivity of prefrontal cortex in isolated rats. Similarly, the results of Al exposed rats indicate inactivity of both ACC and prefrontal cortex. Altogether, Al contaminated water can cause increased anxiety, stress, and decreased empathetic concerns towards others hence decreasing life's comfort. Such an environment creates a barrier to attain a healthy and protective society. Therefore detailed investigation of the molecular, biochemical, and neurological patterns of both Al exposure and isolation is required to explore the complex phenomenon of empathy and how it is affected by metal toxicity that may lead to multifaceted neurological disorders in humans.

Chapter 6

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Evaluation of Al Exposure on Empathetic Behavior In Rat Model

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