Evaluation of Social Deficits in Initial Stages of Alzheimer's Disease using $AlCl_3$ Mouse Model

BY

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Dedicated to

My beloved parents,

Brother, Dr. Shoaib bin Shakeel, Sisters, Namra, Nabia, Sadia and nieces Eeesha, Amna and Aisha.

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LIST OF ACRONYMS

%	Percentage
°C	Degree Celsius
AD	Alzheimer’s disease
EPM	Elevated plus maze
OA	Open arms
CA	Closed arms
$AlCl_3$	Aluminium trichloride
IP	Intraperitoneally
APP	Amyloid Precursor Protein
Pb	Lead
ADEAR	Alzheimer’s Disease Education And Referral Center

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ABSTRACT

Alzheimer's disease is a progressive neurodegenerative disease of the brain that ultimately leads to dementia. It is characterized by distinct behavioral and cognitive deficits that differ from those observed in normal aging. The objective of the study was to investigate the effects of intensive treatment of $AlCl_3$ on cognitive abilities in Alzheimer's disease in particular to the effect on social interactions, anxiety responses, aggressive behavior and social dominance. Chemically induced Alzheimer's disease mouse model was developed by treating 11 months old BALB/c mice with $AlCl_3$ intraperitoneally for a period of 14 days. On day 14, $AlCl_3$ treated mice were subjected to a battery of tests including elevated plus maze, social novelty and tube dominance test to assess cognitive abilities and behavioral responses. Elevated plus maze was used to assess exploratory behavior and anxiety responses. Time spent in closed arms by $AlCl_3$ treated mice was significantly higher ($p=0.001$) as compared to control mice indicating increase in anxiety response. The number of entries into open arms was significantly ($p=0.0005$) lower in $AlCl_3$ treated mice compared to control, demonstrating reduced exploratory activity. Assessment of sociability and interest in social novelty was done by social novelty test. A significant deficit in sociability was observed in $AlCl_3$ treated mice ($p=0.0003$). Tube dominance test was performed to assess the aggressive tendencies and dominant/submissive behavior in $AlCl_3$ treated mice, however a significant difference in aggressive tendencies of control and $AlCl_3$ treated mice was not observed ($p>0.05$). Results indicated that $AlCl_3$ induced mouse model of Alzheimer's disease depicts cognitive impairment like the development of AD in the initial stages and its relation to disease progression.

Understanding the behavioral and cognitive manifestations that occur in this model may aid in the early diagnosis and appropriate treatment of the disease.

Chapter 1

INTRODUCTION

Alzheimer's disease (AD), also known as Alzheimer disease in medical literature is a progressive neurodegenerative disease of the brain that ultimately leads to dementia (http://www.ncbi.nlm.nih.gov). At present, AD is progressive and irreversible. It is characterized by pathological changes in the brain that start interfering with many aspects of brain function and are translated into clinical signs such as; (i) decline in cognitive abilities (loss of memory, inability to concentrate, disorientation), (ii) functional abilities (getting dressed, language difficulties, eating, driving), (iii) mood and behavior (depression, anxiety, introversion) and (iv) physical changes (muscle stiffness, inability to walk or even smile) (www.med.nyu.edu). Individuals progress from mild Alzheimer's disease to moderate and severe disease at different rates and the time course of the disease varies with individual ranging from 5 to 20 years (www.alzinfo.org). AD patients in the final stages of the disease lose their ability to communicate, fail to recognize loved ones, become bed-bound and are dependent on care around the clock. In last stages, AD patients are more vulnerable to infections, including pneumonia (infection of the lungs). AD is ultimately fatal, and in most of the cases AD-related pneumonia is a contributing factor (Alzheimer's Association, 2012).

1.1 Epidemiology and risk factors

1.1.1 Prevalence

106 years after the first description by Dr. Alois Alzheimer (1906), AD is one of the most burdensome and disabling health conditions worldwide. According to World Health Organization (WHO, 2011), it is estimated that there are currently about 27-36 million people living worldwide with AD because of the ageing of the baby boomers. By 2050, this figure will quadruple and every 1 in 85 persons will be living with this disease (Brookmeyer *et al*., 2007). Much of this increase will be in the developing countries and will be due to the ageing population. It is estimated that between 2010 and 2050, the number of older people living in the developing countries is projected to increase more than 250 percent compared with a 71 percent increase in the developed countries (WHO, 2011). For people over the age of 65, AD represents the eighth leading cause of death (Hoyert and Rosenberg, 1997). Unfortunately data regarding the prevalence of AD in Pakistan is sparse but it is estimated that in India and South Asia, 0.40 million cases of AD and other forms of dementia are seen per year. There will be a 98 percent increase in the number of people living with dementia in this region by 2020 (Ferri *et al.*, 2005). The projected costs for the treatment and care for people living with AD are daunting. It is estimated that worldwide cost of AD and other forms of dementia account for 1 percent (US $604 billion in 2010) of the global GDP (World Alzheimer Report, 2010).

1.1.2 Risk factors

Age represents the strongest predictor for the development and progression of AD (Ferri *et al*., 2005; Tanna, 2004). According to ADEAR (Alzheimer disease Education and Referral Center), the number of people with AD doubles every 5 years after the age of 65. Women are statistically more likely than men to develop AD and other forms of dementia. Moreover, 16 percent of women over 71 years old develop degenerative brain disorder, only 11 percent of men of the same age are affected. The average life expectancy in women is greater than men. As a result women are more prone to age-related diseases such as AD but changes in hormone levels may also play a factor (Gandy, 2010). Studies show that low physical and mental activity, low socio economic status and illiteracy are also found to be associated with AD (Lindsay *et al*., 2002; Hall *et al*., 2000; Stern *et al*., 1994). It is also believed that people who have head injury have an increased chance of developing AD (Diamond, 2011). The correlation between Aluminium and AD is still under debate in the scientific community. Some studies indicate that exposure to Aluminium in drinking water increases the risk of developing AD in individuals however any causal relationship has not been established (Khan, 2008).

1.2 Genetics of Alzheimer's disease

The cause or rather causes of AD are not yet known. However, most scientists agree that AD, like other common chronic diseases, develops as a result of multiple factors rather than a single cause. Research has identified four genes that influence AD development. Three of these genes are involved in early onset AD and one is involved in late onset AD.

1.2.1 Early onset Alzheimer's

Mutations in three genes, the amyloid precursor protein (APP) gene located on chromosome 21, the presenilin 1 (PS1) present on chromosome 14, and the presenilin 2 (PS2) on chromosome 1, have been found in families with an autosomal dominant AD with onset in their 30s or 40s. PS1 mutations are the most frequent cause of familial early-onset AD, occurring in 11 percent of patients referred for testing (Rogaeva *et al.*, 2001). However it must be noted that these mutations account for only a small percentage of patients with the disease.

1.2.2 Late onset and sporadic Alzheimer's

Late onset AD occurs after the age of 65 and is the most common form of the disease, accounting for more than 99 percent of the AD cases. A single allele of APOE-ε 4 increases the risk of AD twofold, whereas if the alleles are present in homozygous configuration, there is a fivefold increase in risk of developing AD. It has been estimated that in 20 percent of the cases, APOE-ε 4 is the attributing factor in the development of the disease, making it the single most important risk factor for AD (Slooter *et al.*, 1998).

1.3 Pathogenesis of Alzheimer's disease

AD is characterized by the presence of amyloid plaques and neurofibrillary tangles in the brain, which are formed by beta amyloid (Aβ) peptide formed after the proteolytic cleavage of the amyloid precursor protein (APP) and hyperphosphorylated tau proteins respectively (Hardy and Selkoe, 2002). Studies carried out over the last two decades show that three major factors underlie the

pathogenesis of AD; (i) in AD there is a progressive accumulation and aggregation of Aβ in the brain (Selkoe, 1996; Hardy, 1997), (ii) accumulation of Aβ is the final effect of a number of mechanisms that influence production and aggregation of APP (Carson and Turner, 2002) and (iii) the aggregates of Aβ consist in a mixture of many peptides that have different N- and C-termini dictating the physical properties of Aβ itself (Russo *et al*., 2002).

Neurofibrillary tangles in the brain are formed by abnormally hyperphosphorylated tau protein (Lee *et al.,* 2000). The hyperphosphorylation of tau protein results in loss of its function, aggregation into paired helical filaments and gain of toxic activity (Augustinack *et al.*, 2002; Perez *et al.,* 2002). Hence, hyperphosphorylation of tau protein also has a critical role in the pathogenesis of AD in addition to amyloid plaques.

1.4 Cognitive decline and Alzheimer's disease

Cognitive impairment and AD are one of the leading causes of morbidity and mortality worldwide and are considerably burdensome to the affected persons, their families and society in general. Cognition is defined as a combination of skills that include attention, memory, learning ability, language, visuo-spatial skills, and other functions such as decision making, planning, setting a goal and judgment. Cognitive decline ranges from mild cognitive impairment (MCI) and age-related cognitive decline to severe form of dementia such as AD. AD is characterized by progressive deterioration of cognitive abilities in several domains including memory and at least one additional area (learning, orientation, language, comprehension and judgment) that can be severe enough to interfere with everyday

life (National Institute of Health, 2010). Surprisingly enough very little attention is given to the effect of AD on emotional control and social interaction (i.e. the ability to interact with others in society and handle emotions effectively). Although aberrant social behaviors have been observed among AD patients (Bozolla *et al*., 1992), awareness of these problems has not been put on focus.

1.5 Animal model for Alzheimer's disease

There are a number of animal models being used to study AD, but no single animal can be considered as a perfect or complete model to study this disease. Different models emphasize on different aspect(s) of the disease. In this study chemically induced AD mouse model was developed by treating the mice with Aluminium tri-chloride ($AlCl_3$) intraperitoneally. $AlCl_3$ has been used in a number of studies to induce AD in mouse models (Shati *et al*., 2011; Ding and Yang, 2010; Ouafa and Nour, 2008). The chemically induced mouse model was an important choice because of the following reasons:

i. BALB/c mice are easily available, simple and reproducible.

ii. It accurately exhibits cognitive changes associated with the clinical symptoms of AD.

iii. As a number of cognitive functions such as fear, anxiety, social interaction, social dominance and the establishment of social hierarchy are intrinsic behaviors in all animals, thus the results of this study can be extrapolated to humans.

iv. It is possible to evaluate decline in cognitive abilities quantitatively using this model using the following tests:

a) Elevated Plus Maze (EPM) test

b) Social novelty test

c) Tube dominance test

Therefore, chemically induced mouse model of AD was used to study the decline in cognitive abilities in AD.

1.6 Research objectives

Since its first description, AD has gone from a rarely reported disorder to one of the most common disabling diseases among older adults. Although many studies have shown a link between AD pathology and decrease in cognitive abilities but provide inconclusive evidence linking AD and a decline in social interaction and anxiety.

The hypothesis of the study was "AD pathology leads to a decline in cognitive abilities-in particular social interactions, which results in social isolation and anxiety leading to a submissive behavior in AD".

The main objective of the study was to investigate the "effects of intensive treatment of $AlCl_3$ on cognitive abilities in AD in particular to the effect on social interactions, anxiety responses and social dominance".

Chapter 2

LITERATURE REVIEW

On November 25, 1901 a 51 year old female, Auguste D., of Frankfurt Germany presented to Dr. Alzheimer. She had a group of symptoms including impairment of language ability, disorientation, anxiety, unpredictable behavior, delusions, hallucinations and pronounced psychosocial impairment. After the death of the patient, using Bielschowsky silver staining technique, Alzheimer observed the cortex spotted with numerous plaques in the brain tissues of the patient which were nestled among the neurons. He also observed tangled bundle of fibrils, which were present in the the areas of the cortex, where neurons should normally be present (http://hod.kcms.msu.edu).

In 1910, Dr. Emil Kraepelin mentioned “Alzheimer’s disease” for the first time, stating: "The clinical interpretation of this Alzheimer's Disease is still unclear. Although the anatomical findings suggest that we are dealing with a particularly serious form of senile dementia, the fact is that this disease sometimes starts as early as in the late forties".

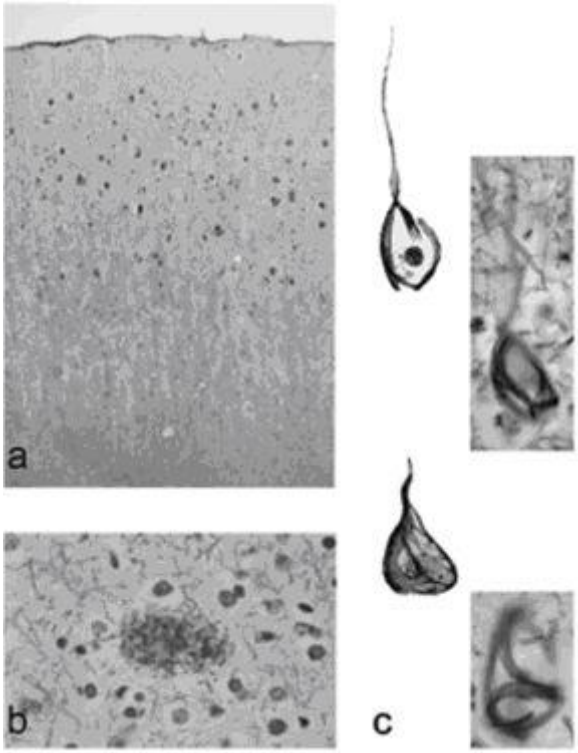

Figure 2.1 Tissue sections from Auguste D.'s brain show amyloid plaques (a,b) and neurofibrillary tangles (c) in the cerebral cortex. The drawings depicted in c were taken from Alzheimer's own work (1911). Primary magnification: x5 (a), x100 (b,c) (www.hod.kcms.msu.edu)

Since the discovery of AD 106 years ago, there have been a significant number of scientific breakthroughs in AD research. In the 1960s, a link between cognitive decline and the presence of plaques and tangles in the brain was discovered. After this discovery, the medical community formally recognized AD as a disease and it was widely accepted that dementia is not a normal part of aging. Before that, AD was little known outside medical textbooks. In the 1970s, AD emerged as a significant area of research interest when scientists made strides in understanding the complete human body. This led to important discoveries and a better understanding of complex nerve cells and the networks in the brains of AD

patients in 1990s. Much of the research was focused on genes involved in AD. FDA approved several drugs to treat the cognitive symptoms of the disease (Alzheimer's Disease Research Summit, 2012).

The onset of AD is characterized by impaired short-term memory, but as the disease progresses, other cognitive skills decline. Later, unpredictable behavior, anxiety, paranoia and control over body functions is lost. The diagnosis of this disease is based on a well-established criterion (McKhann *et al.*, 1984): AD is confirmed at postmortem examination. However the clinical diagnosis of disease is based on a number of factors. A combination of the neurological and mental status examination is done and is reasonably accurate. Unfortunately there are no conclusive diagnostic tests or biological markers of the disease. At death, the most recurrent manifestations in brain include deposits of extracellular β amyloid protein in the form of plaques which contain rudiments of degenerating neurons. Inside the neurons changes include deposits of hyperphosphorylated tau protein which forms neurofibrillary tangles. Loss of neurons and synapses is widespread in case of AD (Dickson, 2001).

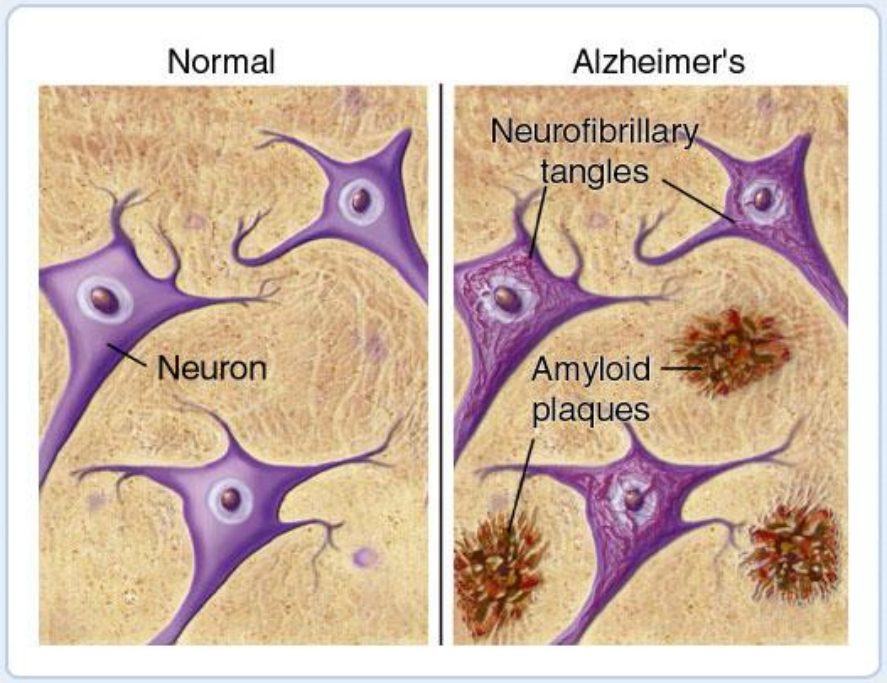

Figure 2.2 Artist's depiction of normal neurons and amyloid plaques outside and neurofibrillary tangles inside the neurons in case of Alzheimer's (American Health Assistance Foundation, 2000-2012).

The incidence of AD increases approximately 1 percent annually among people aged between 65 to 70 years. For people above 85 years of age this increase is approximately 6 to 8 percent (Del Tredici *et al.*, 2002; Di Carlo *et al*., 2002; Fratiglioni *et al*., 2000; Hebert *et al*., 2001). The incidence of AD is slightly higher for women (Miech *et al*., 2002; Ruitenberg *et al*., 2001). The duration of illness varies considerably from 2 to 20 years from person to person affected with AD. Different population-based studies showed that the median survival time for patients with AD was 3 to 4 years (Helmer *et al.*, 2001; Wolfson *et al*., 2001). AD represents the 8th leading cause of death for people over the age of 65 years (Hoyert and Rosenberg 1997).

Most of the research focus in case of AD is on the genes that could be involved in the manifestation of the disease. Mutations in three genes, the amyloid precursor protein (APP) gene located on chromosome 21, the presenilin 1 (PS1) present on chromosome 14, and the presenilin 2 (PS2) on chromosome 1, have been found in families with a history of early-ons*et al*zheimer's disease with onset as early as the third decade of life. PS1 mutations are reported to be the most frequent cause of familial AD, occurring in 11% of patients who were referred for testing (Rogaeva *et al*., 2001). However, only a small proportion of patients with the disease have these genetic mutations. Association of an allelic variant of apolipoprotein-E (APOE) ε 4 has also been seen in case of sporadic and familial AD with onset after age 65 years. Slooter *et al*. (1998) showed that a single APOE-ε 4 allele increases the risk of AD to twofold, whereas if the allele is present in homozygous configuration, it results in a fivefold increase in the risk of acquiring the disease. It is reported that in 20% of the cases, APOE-ε 4 is involved making it the single most important risk factor for AD (Slooter *et al*., 1998). Mutations in the genes that cause early-onset AD elevate levels of amyloid β peptide (Aβ 1-40 and Aβ 1-42), which is formed as a result of proteolytic cleavage of the amyloid precursor protein. Amyloid β consequently aggregates in the brain of the patients in the form of neuritic plaques. Hyslop (2000) showed that the allelic variant APOE may be involved in the clearance or degradation of amyloid β. Thus, a pathway leading to the pathogenesis of AD has been identified by the investigation of families with the disease. Genetic linkage studies have discovered several additional presumed loci for Alzheimer's disease on multiple chromosomes (Bertram *et al*., 2000; Ertekin-Taner *et al*., 2000; Kehoe *et al.*, 1999; Mayeux *et*

al., 2002; Myers *et al*., 2000). Genes that are involved in the metabolism and clearance of amyloid are being investigated, but no specific gene variant has yet been identified.

Apart from genetics, scientists are trying to find possible links of the disease with other factors that play a crucial role in the development of neural connections in the brain of the individuals. A study conducted by Stern *et al.* (1994) showed that poorly educated individuals are at a higher risk of getting AD than well-educated persons. The hypothesis behind this study is that as a result of formal education, a "cognitive reserve" is developed which has a protective effect. The quality and kind of environment during childhood may also play an important role because individuals from supportive and nurturing households and communities had a lower risk of acquiring disease as adults (Hall *et al.*, 2000; Moceri *et al*., 2000). Moreover, research showed that time spent engaged in a physical and mental activity during late life is associated with a lower risk of developing AD. Risk is lowest for individuals having complex activity patterns that include frequent brain activities such as playing puzzles and physical activities (Lindsay *et al.*, 2002; Scarmeas *et al*., 2001). In the Canadian Study of Health and Aging, it was seen vigorous exercise had the strongest effect in lowering the risk of AD (Lindsay *et al.*, 2002). Guo *et al*. (2000) and Plassman *et al*. (2000) showed that head injury increases the risk of Alzheimer's disease. The mechanism by which trauma increases the risk is unknown, but it has been seen that closed head injury in humans and rodents is followed by Amyloid β deposition (Horsburgh *et al.*, 2000; Jellinger *et al.*, 2001; Uryu *et al.*, 2002). However, a relationship

between head injury and genetic variants has not been established in the pathogenesis of AD.

A number of environmental factors have also been investigated as possible causative agents of AD in some people. Among these is Aluminium (Al). There is an indirect evidence that links Al with AD, but no causal relationship has yet been proved. According to Suwalsky *et al*. (1999), Aluminum is a neurotoxic agent but there is little evidence regarding its molecular cytotoxicity. Gomez *et al*. (1997) showed that brain aluminum concentrations were found higher as compared to aluminum levels in other organs. Research showed that Al is highly neurotoxic and has an inhibitory role in prenatal and postnatal development of the brain in humans and experimental animals at high concentrations (Yumoto *et al.*, 2001). A number of neurological manifestations have been attributed to Al neurotoxicity in humans. These include memory impairment, tremor, myoclonic jerks, impaired coordination, slow motor movements, ataxia, and generalized convulsions (Zatta *et al.*, 1991; Crapper and DeBoni, 1980). The neuropathological conditions that are associated with high levels of Al in the brain include Alzheimer-type senile and presenile dementia, Down syndrome with associated AD, Parkinson's with dementia, neurofibrillary degeneration and senile plaques of AD (Zatta *et al.*, 1991; Crapper and DeBoni, 1980).

In AD, there is a global shrinkage of brain tissue. The sulci (plural of sulcus), which are the grooves or furrows in the brain, are noticeably widened. Shrinkage of the gyri (plural of gyrus) which are the well-developed folds of the brain's outer layer is seen. In addition, the chambers within the brain (containing

the cerebrospinal fluid) are evidently enlarged. In the early stages of the disease, short-term memory is impaired when the cells in the hippocampus (part of the limbic system) start degenerating. The ability to perform everyday tasks also declines. As the disease advances, it spreads through the cerebral cortex (the outer layer of the brain). As a result judgment declines, emotional outbursts may occur and language is impaired. As the disease progresses to final stages, more nerve cells die, leading to changes in behavior, such as wandering and agitation. In the final stages of the disease, patients lose the ability to recognize faces and communicate. They are unable to control bodily functions and require constant care (Alzheimer's Disease Research, 2012).

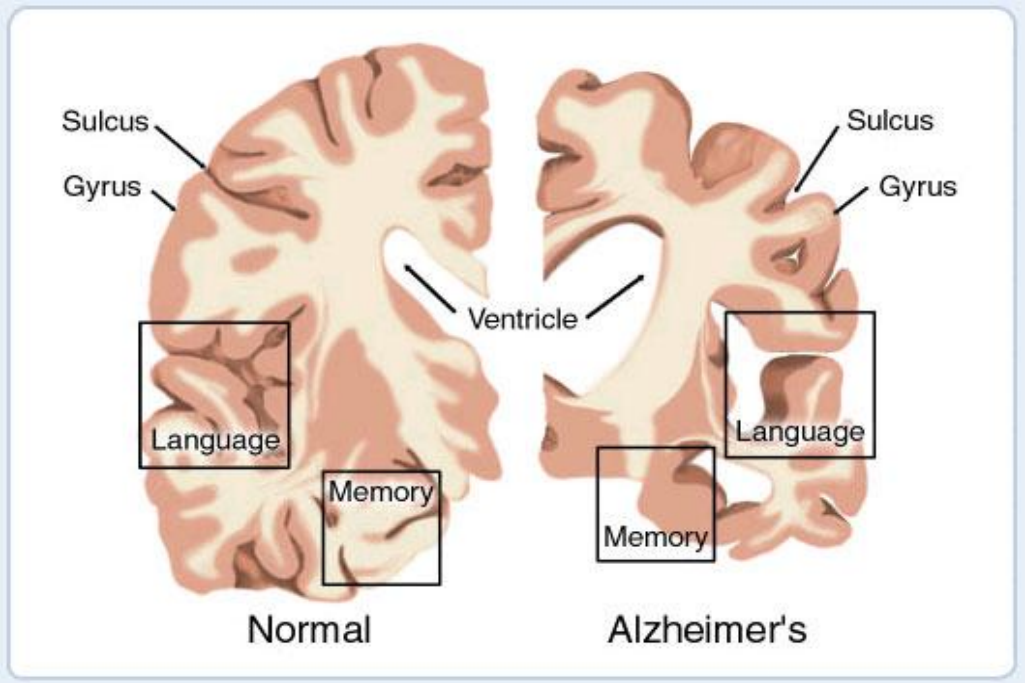

Figure 2.3 A comparison of a normal brain and a brain affected with AD (American Health Assistance Foundation, 2000-2012).

Cognitive decline and Alzheimer's disease are one of the main causes of morbidity and mortality around the world and are significantly burdensome to the patients, their families, and society in general. Research over the past 20 years has provided important information regarding the nature of Alzheimer's disease and its relation to cognitive decline and the combined magnitude of the problem. At present, conclusions cannot be drawn regarding the association of any risk factor with AD or cognitive impairment. Cognition is defined as a combination of skills including attention, social interaction, learning, memory, language, visuo-spatial skills, and executive functions, such as making a decision, setting a goal, planning, and judgment. Cognitive impairment ranges from severe form of dementia as in the case of AD, to mild cognitive impairment (MCI) and age-related decline in cognition. Cognitive impairment can be due to a number of reasons, and MCI does not always lead to dementia. A number of neuropsychological testing is done for the above-mentioned skills and has been a predominant method for the evaluation of any change in cognitive abilities, but functional cognitive decline is only linked with pathologic changes typical of Alzheimer's disease (National Institute of Health, 2010). In case of AD, it has been seen that the patients display impaired awareness of their cognitive deficits (Kotler and Camp, 1995). Correa *et al*. (1996) showed that in most of the cases, AD patients are unaware of the decline in their cognitive abilities, limitations in the performance of everyday activities (Vasterling *et al*., 1995) and mood disturbances (Burke *et al*., 1988). A domain that has surprisingly received very little attention is the effect of Alzheimer's disease on emotional control and social interaction (i.e. the ability to interact with others in society and handle emotions effectively). Although aberrant social behaviors have

been observed among AD patients (Bozolla *et al*., 1992), awareness of these problems has not been put on focus.

Mice have proven to be a very good model to study the progression and pathogenesis of AD and its effect on cognitive behavior. (Rebai and Djebli, 2008; Rui and Yongjian, 2010; Ashe, 2001). After the completion of the human genome project in 2001 and complete sequencing of mouse genome the following year (2002) it was seen that the genes of mice and humans are virtually identical. The obvious differences between humans and mice lie not in the genes themselves but in which part of the body, at which point during the life and how those genes are activated. Therefore it is not wrong to conclude that the physiology and anatomy of mouse and humans are pretty much the same (http://www.usatoday.com).

As a number of cognitive functions such as fear, anxiety, social interaction, social dominance and the establishment of social hierarchy are intrinsic behaviors in all animals thus the behavior of mice can be extrapolated to humans. Cognitive abilities of mice can be quantified using a number of behavior analysis tests. One of the tests that are widely used to study anxiety-like behavior in mice is elevated plus maze (EPM). EPM exploits the conflict between the innate fears of rodents for open areas versus their desire to explore novel environments. Refuge is provided by the closed arms whereas the open arms offer exploratory value. When anxious, the natural tendency of mice is to prefer enclosed dark spaces to opened brightly lit spaces. In this context, anxiety-related behavior is measured by the degree to which the rodent avoids the open arms of the maze (Komada *et al.,* 2008).

Social interactions are a fundamental and adaptive component of the biology of numerous species. Social recognition is one of the most important factors for the structure and stability of the networks and relationships that identify societies. For animals, such as mice, recognition of conspecifics may be important for maintaining social hierarchy and for mate choice (Berry and Bronson, 1992). A variety of neuropsychiatric disorders are characterized by disruptions in social behavior and social recognition, including AD, depression, autism spectrum disorders, bipolar disorders, obsessive-compulsive disorders, and schizophrenia (Robinson *et al.*, 2005).

Standardized behavioral assays that quantify the preference of mice for initiating social interactions with novel conspecifics is of great value for measuring decline in social activity of mouse models of Alzheimer's disease. Crawley's sociability and preference for social novelty protocol has been successfully employed to study social association and social memory in several rodent models (Clapcote *et al.*, 2007; Kaidanovich-Beilin, 2009). The main principle of this test is based on the free choice by a subject mouse to spend time with any of the two containment cages during two experimental sessions that includes indirect contact with one or two mice with which it is unfamiliar. To quantify the social tendencies of the experimental mouse, the main tasks that are measured are a) the time spent with a novel mouse and b) preference for a novel versus a familiar mouse. Thus, the experimental design of this test allows evaluation of two significant but distinguishable aspects of social behavior, that are social attachment and social novelty. "Sociability" in this case is defined as inclination to spend time with another mouse, as compared to time spent alone with an identical but empty cage.

This test provides robust results, which are then carefully analyzed and interpreted (Moy *et al*., 2004).

Aggression is a common component of social interaction and the establishment of dominance hierarchies among animals including primates and has gained considerable attention over the past few decades. The focus of most of the research is on investigating the neural origins of aggression. According to Fischer (2010), in case of dominant male rodents, there is an increased rate of neuron formation compared to subordinate counterparts. The Tube Dominance test assesses cognition in rodent models of brain disorders Alzheimer's for example, particularly social dominance through the measurement of aggression. Subjects are released into opposite ends of a clear, narrow tube. The animals interact in the middle of the tube; the more dominant animal will show greater aggression and force its opponent out of the tube. When one animal has all four paws out of the tube, it is declared as loser, while the animal remaining inside the tube is the winner, ending the match. The number of wins is reported as a percentage of total number of matches (www.sbfnl.stanford.edu).

Chapter 3

MATERIALS AND METHODS

3.1 Chemicals

Aluminium chloride hexahydrate (Product no. 11091) was obtained from Sigma-Aldrich Canada Ltd, 2149 Winston Park Drive Oakville ON L6H 6J8 Canada. It was a crystalline, colorless product. The formula of the compound was $AlCl_3.6H_2O$ and molecular weight was 241.43 g/mol. The compound had ≤ 0.005% total impurities of heavy metals (as Pb). The product container was kept tightly closed and in a well ventilated area. Chemical solutions were made fresh everyday by dissolving the chemical into distilled water.

3.2 Animals

Experiments performed complied with the rulings of the Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA (Guide for the Care and Use of Laboratory Animals: Eighth Edition, 2011). The protocol was approved from the Internal Review Board (IRB), Atta ur Rahman School of Applied Biosciences, National University of Science and Technology (NUST). BALB/c mice (9-11 months of age and weight range of 27-55gm) were purchased from National Institute of Health (NIH), Islamabad. Mice were housed in the animal house of Atta ur Rahman School of Applied Biosciences, NUST, under a controlled environment. The temperature was maintained at a constant of 25±2˚C and the natural light and dark cycle (14 hrs

light and 10 hrs dark) was followed. Animals were given tap water and a standard diet *ad libitum* consisting of 30% crude protein, 9% crude fat, 4% crude fiber and 10% moisture. A total of 30 mice were used in this study and each mouse was given a unique identity. 5 mice were kept in one cage (40x20.5x20.5cm) and all animals had equal access to food and water.

3.3 Intra peritoneal injections

For intra peritoneal (IP) injections BD Ultra-Fine II short needles (30 Gauge x 8 mm) were used. Mice were weighed daily and IP dose of 150 mg/kg body weight was given to the experimental mice everyday between 10 am-11:30 am. The cage lid was removed carefully to avoid excessive disturbance to the animals. The mouse to be injected was restrained smoothly by grasping its tail into forefinger and thumb. The mouse was lifted from the floor of the cage onto the cage lid maintaining a firm grip on the tail. Using the forefinger and the thumb of second hand, loose skin was drawn up form over the shoulders and held securely to restrict movement of the mouse's head. Maintaining the grip on the scruff and the base of the tail, the mouse was lifted and turned over so that the body was supported on the palm of the hand. "Two man" procedure was employed in which one investigator restrained the mouse by the scruff with one hand and slightly extended the body of the animal by holding tail with the other hand. The body was tilted so that the head was facing downwards and the abdomen was exposed. The second investigator inserted the needle into the abdomen at about 30˚ angle to minimize the penetration into the abdominal organs. IP injection was made into the

right or left lower quadrant of the mouse. After the injection, the needle was withdrawn and the mouse was put back into its cage and released.

Table 3.1: Plan to develop a chemically induced mouse model of Alzheimer's disease

S. No.	**Groups**	**No. of animals**	**IP injection**	**No. of days**
1.	Control	10	Vehicle	14
2.	AD mice	10	150 mg/kg	14

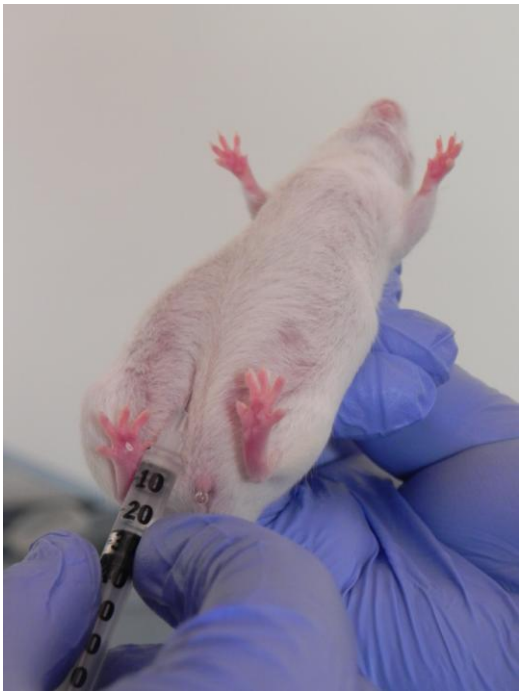

Figure 3.1: Two man method for intraperitoneal injection

3.4 Behavior testing

Behavior testing was performed between 12pm-6pm on the 14th day of the treatment with Aluminium chloride ($AlCl_3$). The mice were transferred to the testing room 30 minutes prior to the beginning of the first trial to let it habituate to the conditions of the testing room. The testing room was well lit and the temperature was maintained at 25±2˚C. There was no human presence in the behavior analysis room during the testing procedure and all the tests were recorded on a video camera.

3.4.1 Elevated plus maze

The testing procedure was the same as described by Munekazu Komada *et al*. (2008) with slight modifications. The experimental apparatus used for elevated plus maze test was in the configuration of a + comprising of two open arms (50.5x10cm) across from each other and perpendicular to two closed arms (49.5x10cm) with a center platform (10x10cm). The walls of closed arms were 49.5cm high. The entire apparatus was elevated above the floor at a height of 75.5cm. The apparatus was made of iron alloy and the walls were opaque.

The test mouse (experimental/control) was placed in the centre platform of the maze with the head pointing towards the closed arm. Test mouse was allowed to explore freely about the maze. Animal was considered into an arm when all four of its paws were in the respective arm. The following parameters were noted:

i. Percentage of time spent in open or closed arms.

ii. Number of entries into each arm.

iii. Production of fecal boli.

The duration of the test was 10 minutes.

After each trial all the arms and the center area were cleaned with 70% ethanol to prevent a bias based on olfactory cues.

Table 3.2: Matrix for behavior analysis using elevated plus maze

S.No.	**%Time spent in open arms**	**%Time spent in closed arms**	**No. of entries into open arms**
1. AD mice (10)			
2. Control (10)			

Figure 3.2: Elevated plus maze apparatus consisting of two open arms and two closed arms

3.4.2 Social novelty test

The testing procedure was the same as described by Beilin *et al.* 2011 with some modifications as per requirement of our study. The apparatus for preference for social novelty test comprised of a square box (40x40x40cm) and was made from steel. Two identical hollow spherical cages made of plastic were placed in the box and were large enough to hold a single mouse (stranger). Each container had holes on its surface that allowed the exchange of air between the interior and exterior of the cage but small enough to prevent direct physical interactions between the subject and the stranger.

Two classes of mouse were used in this test. One that acted as a control, naïve or unfamiliar animal known as the "stranger" and the other was the test subject. Mice of the same strain, age and weight were used as controls, without any

prior contact (littermates) with the subject. Two control mice were required per experiment and in some cases same control mice were used between trials.

Two empty cages were placed diagonally into the social activity box so that the subject had access to all sides of the cages. The subject mouse was placed at center of the box for adaptation and was allowed to explore freely for 10 minutes.

3.4.2.1 Social affiliation aspect of the test (Session I)

One of the control mice (Stranger 1-opposite gender) was placed into one of the cage that was located on one side of the box. The placement of the stranger 1 was systematically altered between trials. The subject mouse had free access to explore around the containers.

Immediately following parameters were monitored and recorded:

i. Duration and

ii. Number of direct (active) contacts between the subject and the cage housing or not housing the Stranger 1, for each sphere individually.

iii. Duration and

iv. Number of other behaviors by the subject mouse around each cage including walking, self grooming, lack of body movement for more than 5 seconds (freezing), as well as any unusual or repetitive behavior such as jumping etc.

The duration of session I was 5 minutes.

3.4.2.2 Social novelty/preference session of the test (Session II)

The second control mouse (Stranger 2-opposite gender) was placed inside the cage that was positioned diagonally to the cage in which Stranger 1 was present (that had been empty during session I). Same parameters were monitored and recorded as described in session I, differentiating the behaviors of the subject mouse in the presence of stranger 1 as compared with Stranger 2.

The duration of session II was 5 minutes. After the completion of the sessions the complete apparatus including the cages was cleaned with 70% ethanol to avoid bias based on olfactory cues.

Table 3.3: Matrix of behavior analysis using social novelty test

Social affiliation aspect of the test (Session I)			
S. no.	Time spent with stranger I (sec)	Time spent with empty container (sec)	Total time (sec)
1.			
Social novelty/preference session of the test (Session II)			
S. no.	Time spent with stranger I (sec)	Time spent with stranger II (sec)	Total time (sec)
2.			

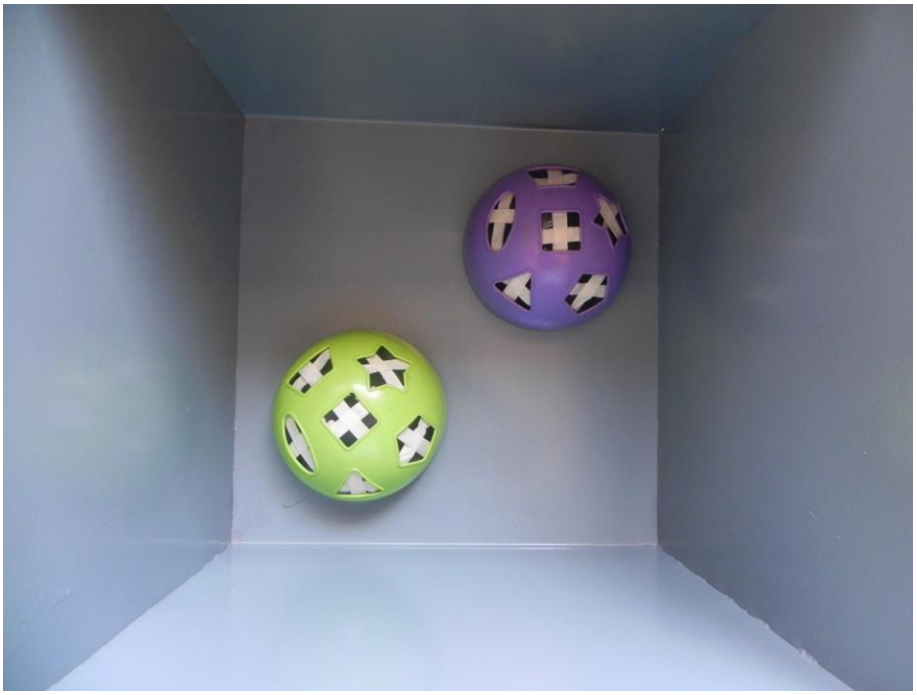

Figure 3.3: Social novelty test apparatus

Figure 3.4: Subject exploring one of the containment spheres

3.4.3 Social dominance/Tube dominance test

The testing procedure was the same as described by Rodriguiz *et al.* (2004). The apparatus consisted of clear narrow plastic tubing (diameter of 3.5cm and length of 30cm). The apparatus was anchored to a solid wooden base. Subjects of the same gender and approximately of the same weight were placed on the opposite ends of the tube facing each other and released. The subjects interacted in the middle of the tube. Social behaviors such as agonistic behavior (biting, clawing, tail rattling, forcing the opponent to retreat) and pushing were continuously monitored for each animal until one of the subjects withdrew from the tube or 3 minutes elapsed. The following parameters were defined:

i. Each subject was given three rounds of matches and the number of wins was reported as the percentage of total number of matches.

ii. The submissive mouse was identified as the animal that first withdrew from the tube and had its all four paws out of the tube.

iii. If none of the subjects withdrew the tube before the time elapsed, the match was coded as a "tie" between the subjects.

After each match the apparatus was cleaned with 70% ethanol to prevent bias based on olfactory cues.

Figure 3.5: Mice at the starting positions of the tube in tube dominance test

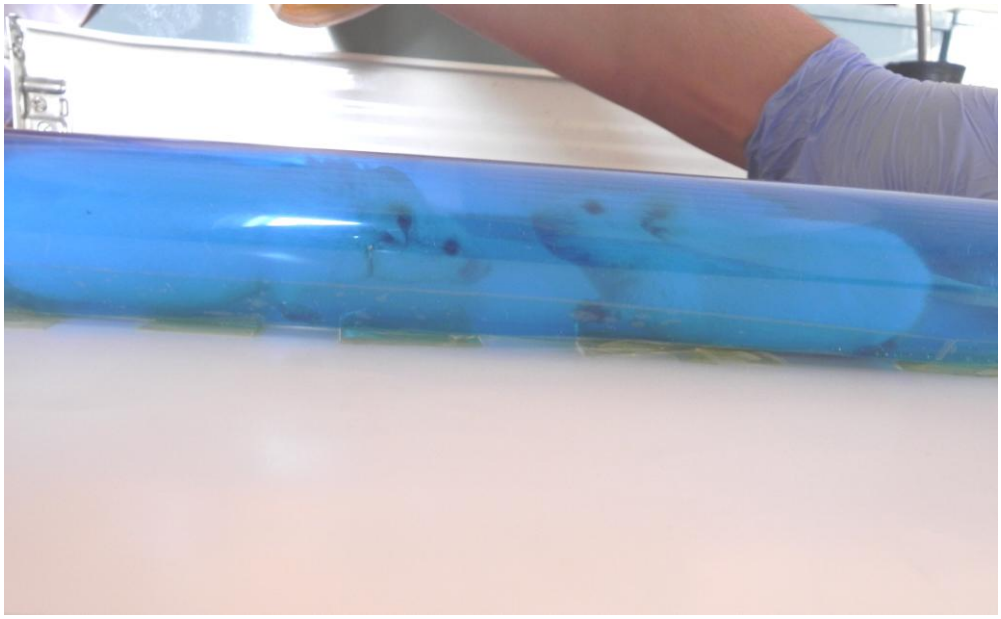

Figure 3.6: Mice in the middle of the tube, interacting with each other

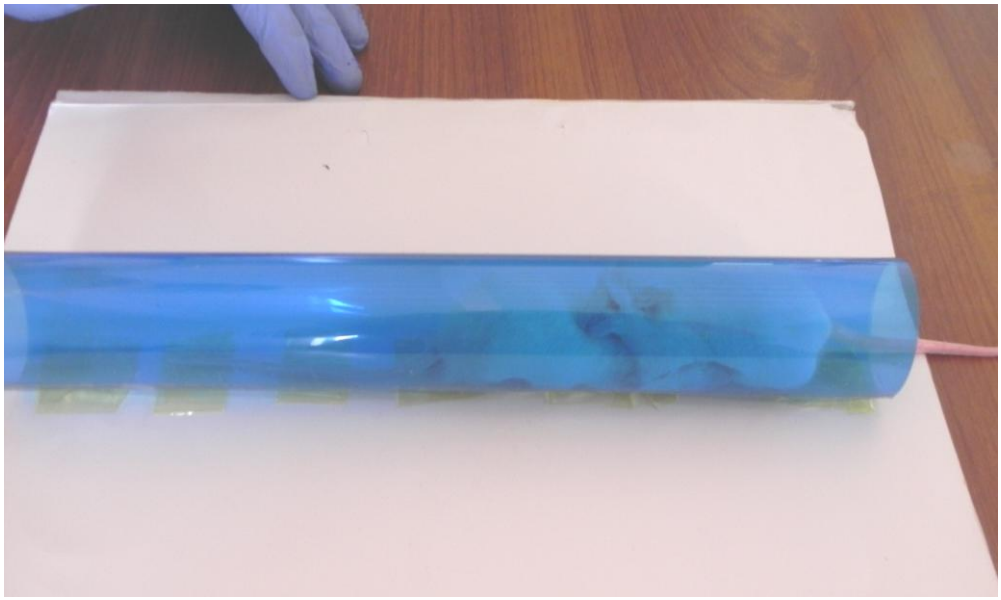

Figure 3.7: One of the subjects forcing the other out of the tube

Table 3.4: Matrix for behavior analysis using tube dominance test

No. of matches	**%Win**	**%Loose**	**%Tie**
1. AD mice vs. control			
2. AD mice vs. control			
3. AD mice vs. control			

3.5 Statistical analysis

Statistical analysis was done using Graphpad prism version 2.0. Two tailed t test was applied to the data and p value less than 0.05 was considered significant. The data was represented as mean ± SEM up to two significant figures with a 95% confidence interval.

Chapter 3

RESULTS

4.1 Assessment of anxiety and exploratory behavior in $AlCl_3$ treated mice

Elevated plus maze test was used to assess the exploratory behavior and anxiety responses in $AlCl_3$ induced AD mice models. The assessment of anxiety was done by calculating the percentage of time spent in closed arms relative to the percentage of time spent in open arms. Time spent in closed arms by AD mice was significantly higher (7.73±1.99, p=0.001) as compared to control mice. Exploratory behavior of AD mice was assessed by the number of entries into open/closed arms. The number of entries into open arms was significantly (8.60±2.05, p=0.0005) lower in AD mice.

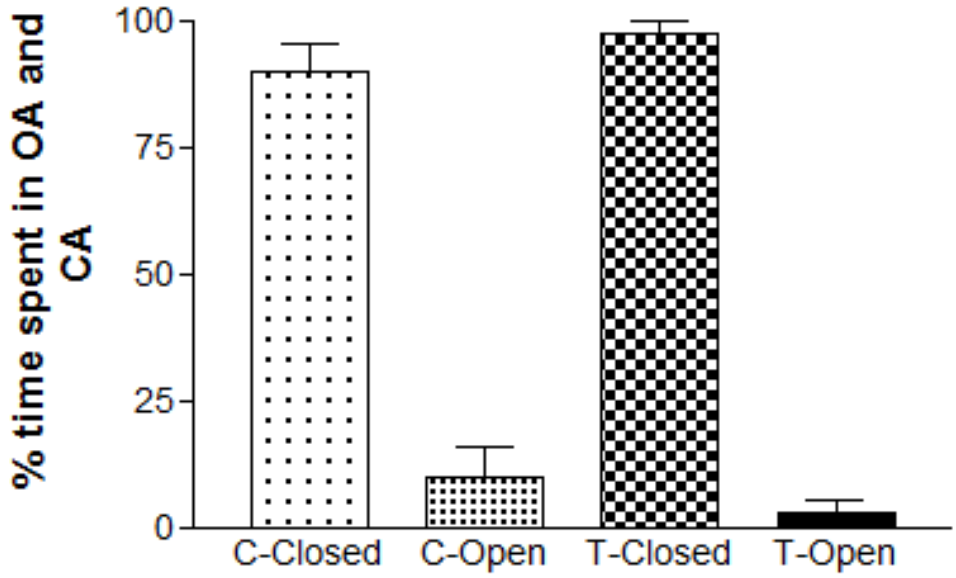

Figure 4.1: A comparison of percentage of time spent in open arms (OA) and closed arms (CA) of elevated plus maze by control (C) and AD (T) mice (n=10). The mean percentage of time spent by AD mice in closed arms was 97.53%. Mean percentage time spent by control mice in closed arms was 89.80%. The difference in the mean percentage time spent by AD and control mice was statistically significant (p=0.001). Similarly the mean percentage time spent by AD mice in open arms of the maze was 3%. The mean percentage time spent by controls in open arms was 10.20% and the difference in the mean percentage time of AD and control mice was statistically significant (p=0.002). (Two-tailed t test).

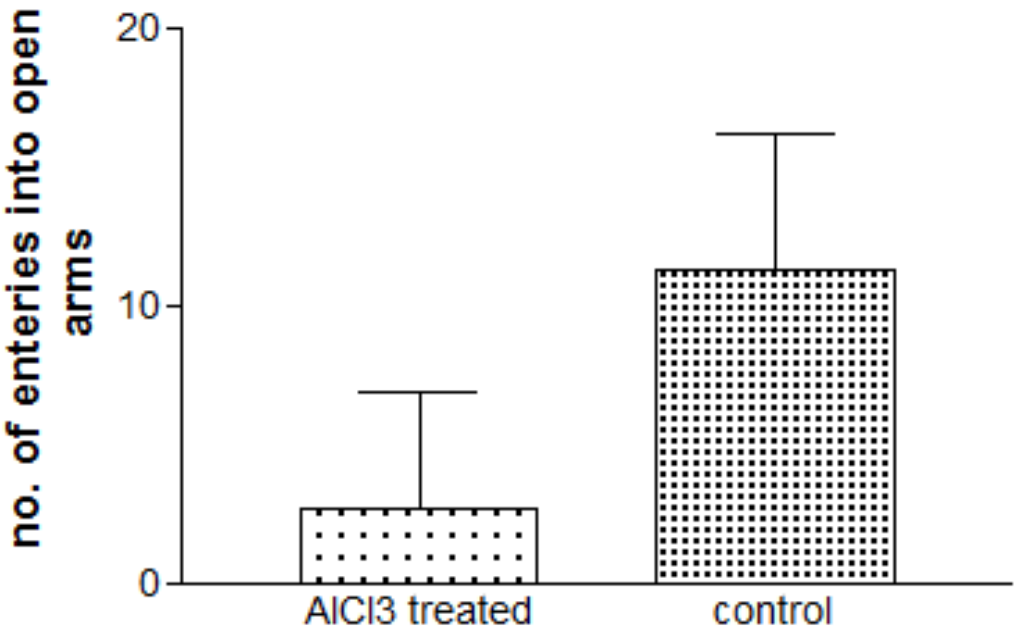

Figure 4.2: A comparison of no. of entries into open arms by AD and control mice (n=10). The average number of entries in open arms by AD mice was 2.7 and the average number of entries by control mice was 11.3. A significant difference in the number of entries is shown (p=0.0005). (Two-tailed t test).

4.2 Assessment of sociability and interest in social novelty in $AlCl_3$ treated mice

Social novelty test was performed to assess the effect of $AlCl_3$ treatment on sociability and preference for social novelty in AD mice models. In session I of the test, the time spent with stranger 1 by AD mice was significantly (106.9±29.36, p=0.0019) less as compared to control. During session II of the test, the mouse was presented with a choice between the stranger 1 (now-familiar) and stranger 2 (novel). A significant deficit in sociability was observed in AD mice (138.2±30.98, p=0.0003).

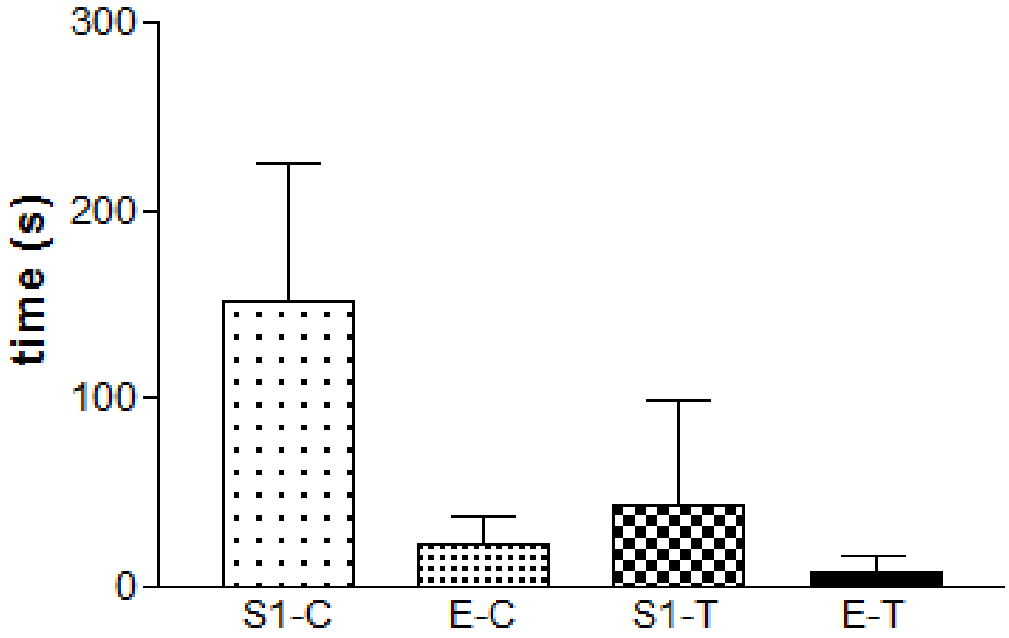

Figure 4.3: Social affiliation session (session I) of the test. A comparison of the time spent by control (C) and AD (T) mice (n=10) with stranger 1 and empty cage. Both AD and control mice showed preference to stranger 1 as compared to empty cage but AD mice spent significantly less time with stranger 1 as compared to control (p=0.0019). The difference in the time spent with stranger 1 and empty cage by AD mice was not statistically significant (p=0.0585). The difference in the time spent by control mice with stranger 1 and empty cage was significant (p=0.0001). (Two-tailed t test).

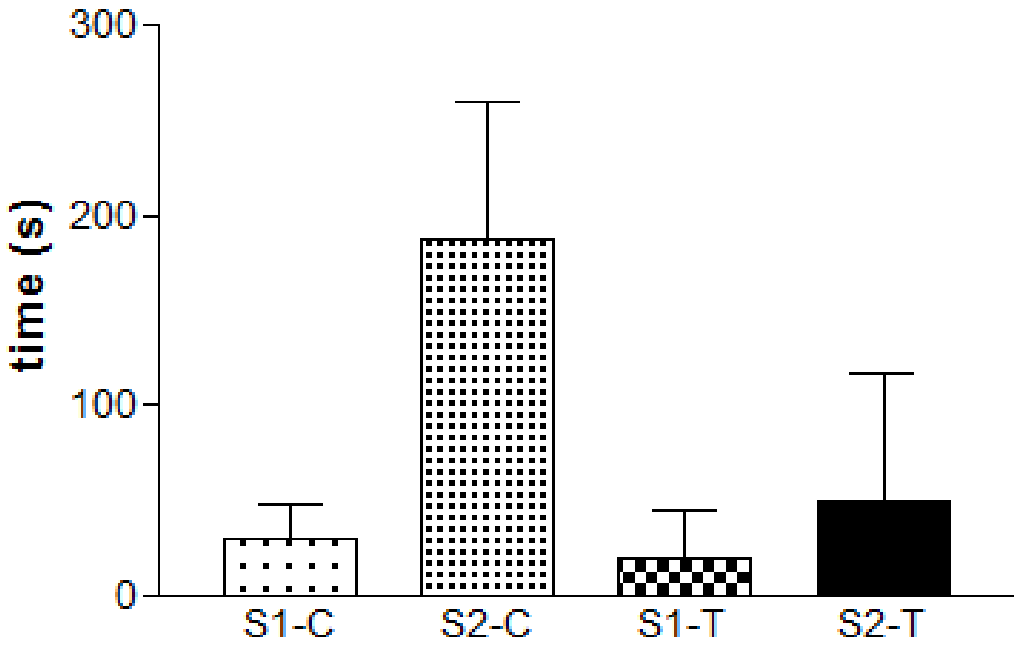

Figure 4.4: Social novelty/preference session (session II) of the test. A comparison of the time spent by control (C) and AD (T) mice (n=10) with stranger 1 and stranger 2. Both AD and control mice showed preference to stranger 2 as compared to stranger 1 but AD mice spent significantly less time with stranger 2 as compared to control (p=0.0003). The difference in the time spent with stranger 1 and stranger 2 by AD mice was not statistically significant (p=0.2005). The difference in the time spent by control mice with stranger 1 and stranger 2 was highly significant (p<0.0001). (Two-tailed t test).

4.3 Assessment of dominance and aggressive tendencies in $AlCl_3$ treated mice

In order to measure aggressive behavior in AD mice models, tube dominance test was performed. Each mouse was given three rounds of matches and then the percentage of wins was calculated and compared with control mice. The percentage of wins was slightly higher in AD mice showing aggressive tendencies however the difference was not statistically significant (13.40 ± 15.09, $p > 0.05$).

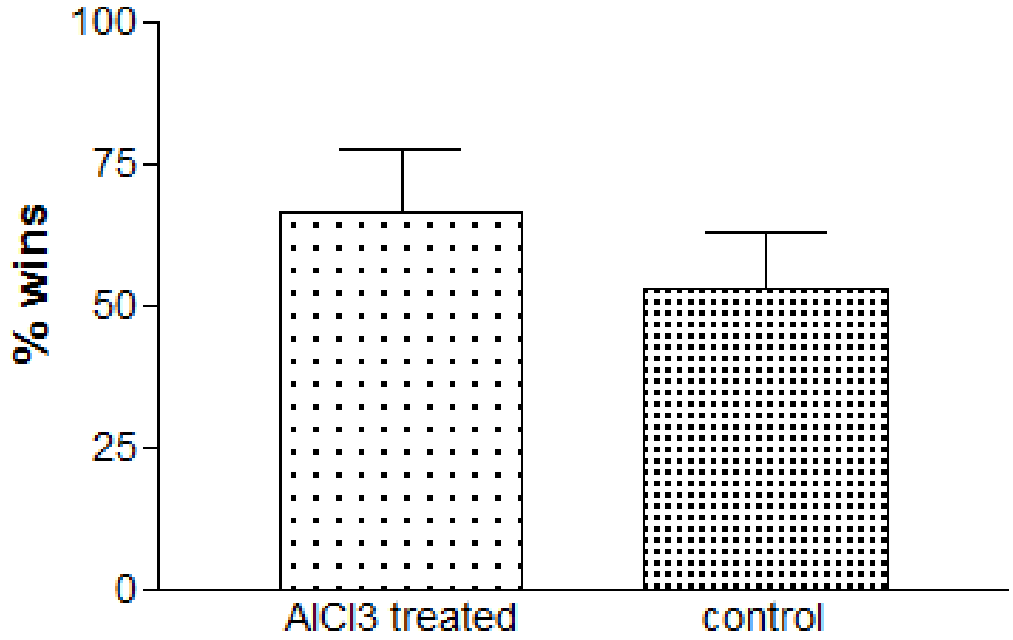

Figure 4.5: Percentage of wins in AD mice and control mice (n=10). The mean percentage of wins during the three rounds of matches in AD mice was 66.40±11.13. Mean percentage of wins in control mice was 53±10.18. The difference in the mean percentage of wins was higher in AD mice as compared to control but the difference was not statistically significant (p=0.386). (Two-tailed t test).

Chapter 5

DISCUSSION

In this study chemically induced AD mouse model was developed by treating 11 months old BALB/c mice with $AlCl_3$ intraperitoneally for a period of 14 days. $AlCl_3$ has been used in a number of studies to induce dementia and AD in mouse models (Shati *et al*., 2011; Ding and Yang, 2010; Ouafa and Nour, 2008). The dose selected was higher (150 mg/kg body weight) than the reported doses (40 mg/kg body weight) for inducing AD in mice but lower than the lethal dose of $AlCl_3$ (940 mg/kg body weight) (Shati *et al*., 2011; Ouafa and Nour, 2008; Pharmacology and Toxicology Vol. 60). The main objective of the study was to investigate the effects of intensive treatment of $AlCl_3$ on cognitive abilities in AD in particular to understand the cognitive impairment at initial stages of AD.

For the assessment of exploratory behavior and anxiety responses, elevated plus maze (EPM) was used. The results indicated an increase in anxiety responses of experimental mice as evident from the percentage of time spent in closed arms and production of fecal boli. Experimental mice spent more time in closed arms and had little exploratory activity as compared to controls. These results were consistent with the fact that emotional symptoms like anxiety and neo phobia contribute significantly to the clinical profile of early stages of AD (Ferretti *et al.*, 2001; Hwang *et al.*, 2004). According to Raber, (2004) in 70% of the patients suffering from AD, increased anxiety may occur during the course of the disease. However the findings were contradictory to the reported results of EPM in

transgenic mice models of AD (Schindowsky *et al.,* 2006; Arendash *et al.,* 2001; Harrison *et al.,* 2003). In these studies, when the transgenic mice were subjected to EPM test, they showed an increase in exploratory activity in open arms of the maze as compared to controls. A number of factors contribute to these contradictory results. The stain of mice (C57BL6) used in these studies were different as compared to BALB/c mice used in our study. Level of anxiety in different strains of mice is genotype dependent (Griebel *et al.,* 2000). Another important aspect could be that these in transgenic mice, all of the pathological features of AD were not taken into account. Schindowsky *et al.* (2006) study was focused on investigating the mechanisms underlying cognitive deficits during pathogenic tau aggregation. Similarly work by Arendash *et al.* (2001) was on spatial memory deficiencies in APP+PS1 transgenic models of AD. Harrison *et al.* (2003) worked on hBACE1 knockout transgenic mice which had increased exploratory behavior. BACE1 knockout mice were however more timid and spent less time in open arms. The timid and bold phenotypes of knockout BACE1 and transgenic hBACE1 suggested the role of BACE1 activity in anxiety.

Another factor that is of due importance is the stage of the disease. In early stages of the disease, amygdala; the emotional center of the disease is affected which results in elevated anxiety response (Braak and Braak, 1991; Hyman *et al.,* 1990). However in moderately severe AD, repetitive behavior is one of the hallmarks as shown in several studies using transgenic mice models of AD and in patients suffering from moderately severe AD (Schindowsky *et al.,* 2006; Arendash *et al.,* 2001; Harrison *et al.,* 2003; Janssen Pharmaceutica, 2012).

The social novelty test was used to assess sociability and interest in social novelty. In sociability session (session I) of the test, both AD mice and control mice preferred to spend more time with the cage containing stranger 1 as compared to empty cage. However, the time spent with stranger 1 by $AlCl_3$ treated mice was less as compared to controls indicating deficit in social interaction. During preference for social novelty session (session II) of the test, control mice spent more time exploring and sniffing the cage containing stranger 2 as compared to stranger 1 but $AlCl_3$ treated mice did not demonstrate such preference. Comparing the results with controls revealed a significant decrease in the preference of stranger 2 as compared to stranger 1 in $AlCl_3$ treated mice. Our results were consistent with the reported results in Thy1-hAPP$^{Lond/Swe+}$ mouse models of AD (Faizi *et al.*, 2012). $AlCl_3$ treated mice displayed a decrease in social interaction that closely resembles the deficit in sociability in AD patients.

AD is presented with a spectrum of behavioral changes. Agitation, irritability and aggressive behavior are also in the range of behavioral disturbances associated with AD (Micheal *et al*., 1996). In order to assess the aggressive tendencies and dominant/submissive behavior in $AlCl_3$ treated mice, tube dominance test was performed. When compared with controls, a significant difference in aggression was not observed. These results were consistent with the fact that in AD not all the patients are prone towards aggression (alzheimers.aplaceformom.com). A number of factors play a role in aggressive behavior in AD and still this factor is under debate in the scientific community that why certain AD patients show aggression and other not. One factor that can be considered is genetics of the individual. Harrison *et al.* (2003) showed that in

BACE1 knockout mice, the aggressive tendencies were reduced concluding that BACE1 had an important role in behavioral phenotype in AD. Moreover it was not known whether the $AlCl_3$ treated mice were dominant or submissive animals. A study by Lewejohann *et al.* (2009) on transgenic mice models of AD showed that APP transgene does not affect the dominant/submissive behavior of mice.

AD is a multifaceted disease that involves a number of genetic and environmental factors. The major clinical hallmark of AD is a progressive cognitive impairment that leads to memory loss, deficit in sociability, anxiety, aggressive behavior and delusions to name a few. Despite considerable progress, the complete molecular pathology of the disease has not been elucidated. A number of animal models have been used in different AD studies but different models emphasize on different aspects of the disease. In this regard, there is a need for an AD animal model that depicts and develops some or all aspects of this unique human disease in a reproducible manner. This is crucial for the development and testing of potential treatments. A valid AD animal model should show progressive AD-like neuropathology and cognitive impairment. Chemically induced mouse model for AD was developed in this study. After intensive treatment with $AlCl_3$, the treated group showed significant cognitive decline. Elevated anxiety response, decrease in exploratory activity and deficits in social interaction were seen in $AlCl_3$ treated mice as compared to controls. Our results were consistent with the reported results in transgenic mouse models of AD. However one advantage that our AD mouse model had over the transgenic models is that it can be used to study the progression of the disease. The spectrum of behavioral deficits that we investigated in our study is related to early and mid

stages of the disease (http://www.psychiatry24x7.com). Prolonged treatment of $AlCl_3$ can be used to induce late or severe stage of the disease and further decline in cognitive functions can be evaluated. $AlCl_3$ induced mouse model of AD can be used to investigate the pathogenesis of the disease as well as the clinical symptoms of AD. Further investigation is required to quantify APP isoforms and tau proteins and their role in establishment of AD in $AlCl_3$ induced mouse models. Moreover the progression of the disease, its associated pathogenesis and cognitive impairment can also be investigated to complete the disease profile of this model.

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